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Marchand A[1], Ericsson M[1], Audran M[1], Fenaille F[2], Capdeville P[1], Martin L[1]

EPO Biosimilars with unusual profiles: characterization and detection

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Abstract

Biosimilars of recombinant erythropoietin (rEPO) can present some small structural differences compared to the reference product. Three biosimilars, with apparent lighter molecular weight than the genuine rEPO (Eprex®), were selected for further investigation: Jimaixin™ authorized in China, and Hemax ® and Epotin™ authorized in Algeria. The aims of this research were: i) to study the electrophoretic EPO profiles obtained after a spike of the three biosimilars in urine and plasma by using improved IEF-PAGE and SDS-PAGE methods and to evaluate their identification using reevaluated criteria ii) to test complementary strategies to improve their detection: a two-dimensional electrophoresis approach (SDS separation following IEF) or a neuraminidase treatment of the sample (proposed to increase the separation between recombinant and endogenous EPO by SDS-PAGE), iii) to compare using MALDI-TOF the specific N-glycosylation pattern of each biosimilar with the original rEPO Eprex®.
Experiments with spiked samples indicated that the biosimilars were more difficult to detect at low doses than Eprex®, in particular, Epotin™ and Jimaixin™ by IEF-PAGE in urine and Hemax ® by SDS-PAGE in plasma. IEF-PAGE for plasma samples and SDS-PAGE for urine samples had the highest identification rates considering the three biosimilars. Two-dimensional electrophoresis experiments did not improve the detection. Samples pre-treatment with neuraminidase gave more promising results for low doses as it slightly increased the detection.
Compared to Eprex® N-glycosylations, all three biosimilars presented a decrease in the most complex forms (loss of sugar antennae and sialic acids) while bi and tri antennae forms were enriched.

Introduction

Since expiration of the patents for the first generation of recombinant erythropoietin (rEPO) drugs, pharmaceutical companies world-wide have begun to produce rEPOs copies. All licensed EPO biosimilars shall demonstrate the same safety and efficacy for therapeutic use than the original drug but they can present some small structural differences compared to the reference product due to some variations in the production process [1]. Consequently the electrophoretic profile of a biosimilar can be slightly different from a conventional rEPO one and its detection in a doping control sample might be challenging.
After analyzing various EPO biosimilars, three drugs were selected for further characterization: Jimaixin™ (Jintan Ltd, China), Hemax® (Biosidus, Argentina) and Epotin™ (Gulf Pharmaceutical Industries, United Arab Emirates). They presented slight differences compared to the original epoetin alpha Eprex® using SDS-PAGE (lower molecular weight) and IEF-PAGE (more isoforms in the basic area).

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The aims of this research were:
i) to study EPO electrophoretic profiles obtained by IEF-PAGE and SDS-PAGE for the 3 biosimilars after a spike in urine and plasma and to evaluate their detection following WADA's criteria [2],
ii) to test potential complementary strategies to improve detection: a two-dimensional electrophoresis approach or a neuraminidase treatment of the sample (cleavage of sialic acids) before electrophoretic separation,
iii) to evaluate the specific N-glycosylation pattern of each biosimilar (Jimaixin™, Hemax® and Epotin™) and compare it with Eprex® to identify differences with the original epoetin alpha drug.

Experimental

Detection of rEPOs by IEF and SDS-PAGE analysis

Spikes of Jimaixin™, Hemax®, Epotin™ and Eprex® were performed in 6 different urine samples (starting volume 9 mL) and 3 different plasma samples (starting volume 500 µL). After an estimation of endogenous EPO content in each sample (using human EPO Quantikine ELISA kit from R&D Systems), addition of rEPO was performed to obtain mixes of rEPO/endogenous EPO with approximate abundances of 35/65, 50/50 and 65/35 respectively. EPO analyses were performed as in Martin et al. [4] using optimized protocols to improve sensitivity of detection and, for IEF-PAGE method, criteria slightly modified compared to WADA's TDEPO to recruit more suspicious samples. All urine and plasma samples were immuno-purified using an anti-EPO coated well from a human EPO ELISA detection kit (STEMCell Technologies) for urine and magnetic beads coated with anti-human EPO antibody 9C21D11 (R&D systems) for plasma. Eluates were analyzed. A single-blot was performed after IEF-PAGE and SDS-PAGE, and EPO immuno-detection was conducted using a biotinylated anti-EPO antibody (clone AE7A5, R&D Systems).

Complementary tests

Neuraminidase treatment: Biosimilars and Dynepo® standards as well as retentates resulting from the ultrafiltration of urine samples (spiked with the biosimilars (50/50 mix) or not) were incubated in wells of a Stemcell EPO ELISA plate for 2 hours at 37°C. After binding of EPO, desialylation was performed by addition of a 5 mU neuraminidase solution and incubation 1 h at room temperature as previously described [3]. After elution, SDS-PAGE analysis was performed as usual and IEF-PAGE using a 2-10 pH gradient instead of 2-6.
2D-gel: Standards and eluates from urine samples with or without spike of biosimilars (50/50 mix) and with or without neuraminidase treatment were analyzed as in Desharnais et al. [3]. For the IEF part, samples without neuraminidase were separated on a 2-6 pH gradient gel and samples treated with neuraminidase on a 3-11 pH gradient gel.

Biosimilar glycan characterization by MALDI-TOF/TOF

rEPO standards (Eprex® and the three biosimilars) N-glycosylations were characterized by mass spectrometry using a previously published protocol [5,6]. Briefly, after protein denaturation, 4 µg of each rEPO were treated with 2 units of N-Glycosidase F (Roche) overnight at 37°C to release N-glycans. After acidification, proteins were precipitated using ice-cold ethanol and resulting N-linked glycans were then

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purified on a porous graphitic carbon SPE cartridge using a mixture of 40% acetonitrile, 0.1% trifluoro-acetic acid for elution. After a permethylation step, N-glycans were finally purified on C-18 spin columns (Pierce) and eluted in 80% acetonitrile. After evaporation, final reconstitution was made in 50% methanol. 0.5 µL were mixed with 0.5 µL DHB matrix (10 mg/mL 2,5 dihydroxybenzoic acid (DHB) in methanol/H2O (50/50, v/v) containing 1 mM sodium acetate). Analysis were performed using a MALDI-TOF/TOF instrument UltrafleXtreme instrument (Bruker Daltonics). Resulting spectra were then analyzed using flexAnalysis software for identification of N-glycan specific rEPO structures. Three separated experiments with 2 or 3 technical replicates each time were performed and the mean of these replicates was considered representative of the glycosylations of each rEPO. The relative abundance of each glycan structure was estimated.

Results and Discussion

Standards characterization

The formulation composition indicated the presence of human albumin in all three biosimilars contrary to Eprex®. This was corroborated by Coomassie blue staining after SDS-PAGE analysis. Strong signals around 60 kDa attributable to albumin and other bands probably due to other serum proteins were detected (Figure 1A). After immuno-extraction (data not shown), almost all additional protein signals were eliminated. After immuno-detection of EPO, only a clear rEPO signal was found. Compared to Eprex®, Jimaixin™ and Epotin™ had broader bands by SDS-PAGE with half of the signal below the Dynepo® reference corresponding to the endogenous EPO zone. Hemax® signal showed a more compact band with an apex close to the Dynepo® reference. By IEF-PAGE (Figure 1B) all three biosimilars had a basic profile clearly indicative of a recombinant EPO. Epotin™ and Jimaixin™ presented more isoforms than Eprex® in the basic area, in particular an intense fifth band, and Jimaixin™ and Hemax® had respectively two and one isoforms more acidic than Eprex® and located in the endogenous area of the gel. Two batches of each biosimilar were tested and showed similar profiles but batch-to-batch variability might exist.

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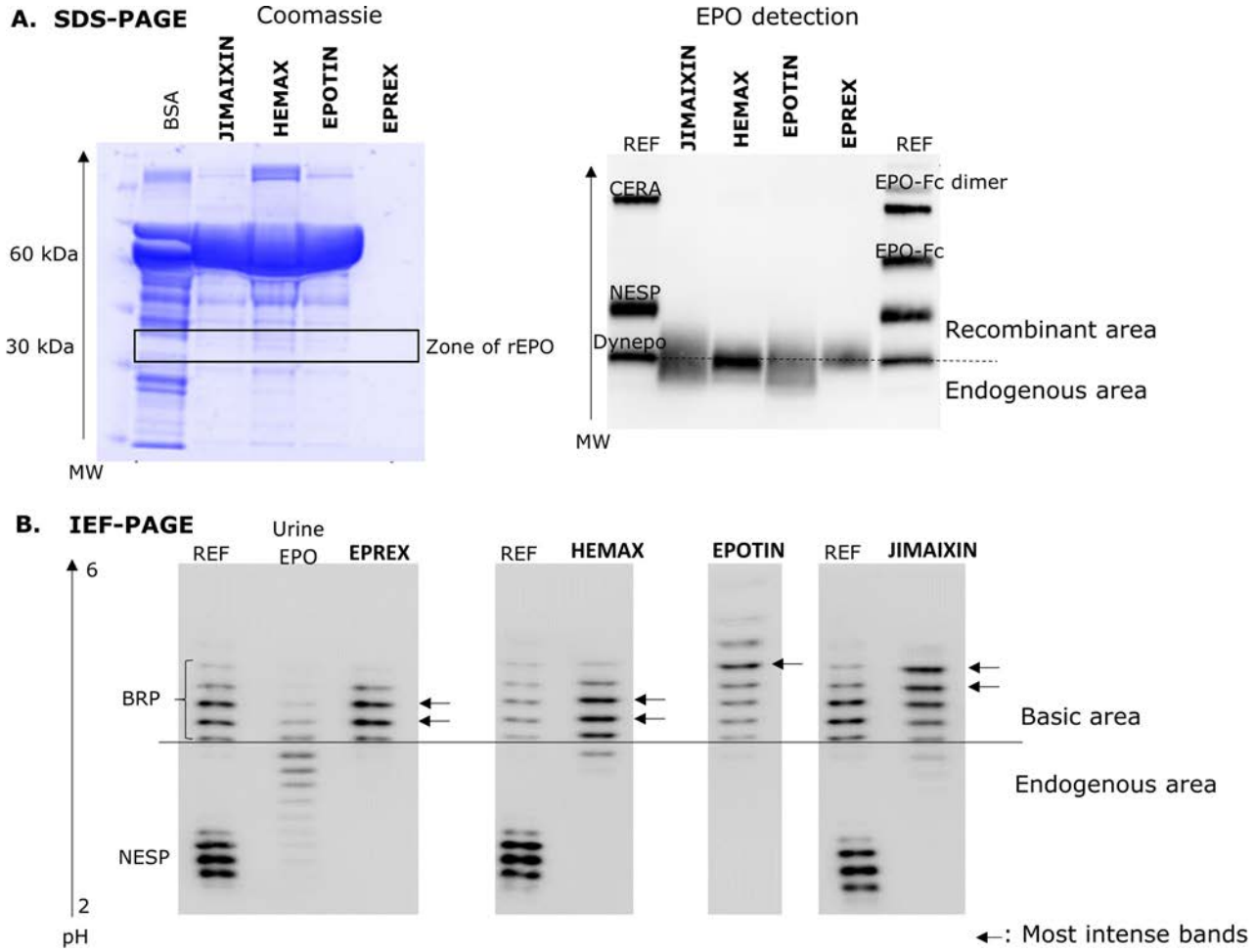

Figure 1. Analysis of the three biosimilars by SDS-PAGE and IEF-PAGE. A. SDS-PAGE Left part: Coomassie staining- Right part: EPO immuno-detection after Western-Blot. Bovine serum albumin (BSA), Eprex® and urinary EPO (uEPO) were added for comparison. **B.** IEF-PAGE. The most intense bands in each profile are indicated by black arrows. EPO references (REF): BRP and NESP as well as Eprex® and urine (endogenous EPO) were added for comparison.

Detection in urine and plasma after spiking

rEPOs were identified according to the following criteria. For SDS-PAGE, the presence of a smear above endogenous EPO and Dynepo® apex position was indicative of the presence of rEPO. For IEF-PAGE, a ratio between the second most intense band in the basic area of the gel and the most intense band in the endogenous area (2B/N) was calculated. The criteria for a suspicious sample was 2B/N > 0.85 for urine, and the criteria used for plasma samples was 2B/N ≥ 1.30. These criteria were slightly modified compared to WADA's TDEPO criteria after a reevaluation study in order to recruit more suspicious samples without increasing the number of false suspicions [4]. For urines using this new criteria 2B/N > 0.85 instead of TDEPO's criteria (summarized as 2B/N > 1) led to 11 more samples classified as suspicious on 72 spiked samples analysed (+15%). Similarly for plasma samples using 2B/N ≥ 1.30 instead of TDEPO's criteria (summarized as 2B/N ≥ 2) led to 10 more samples classified as suspicious on 36 spiked samples analysed (+28%). All non spiked samples were negative with the new criterias applied. Increasing concentrations of biosimilar were reflected in the increasing 2B/N ratios by IEF-PAGE and increasing smear intensity above endogenous EPO signal by SDS-PAGE (Figure 2 and 3).

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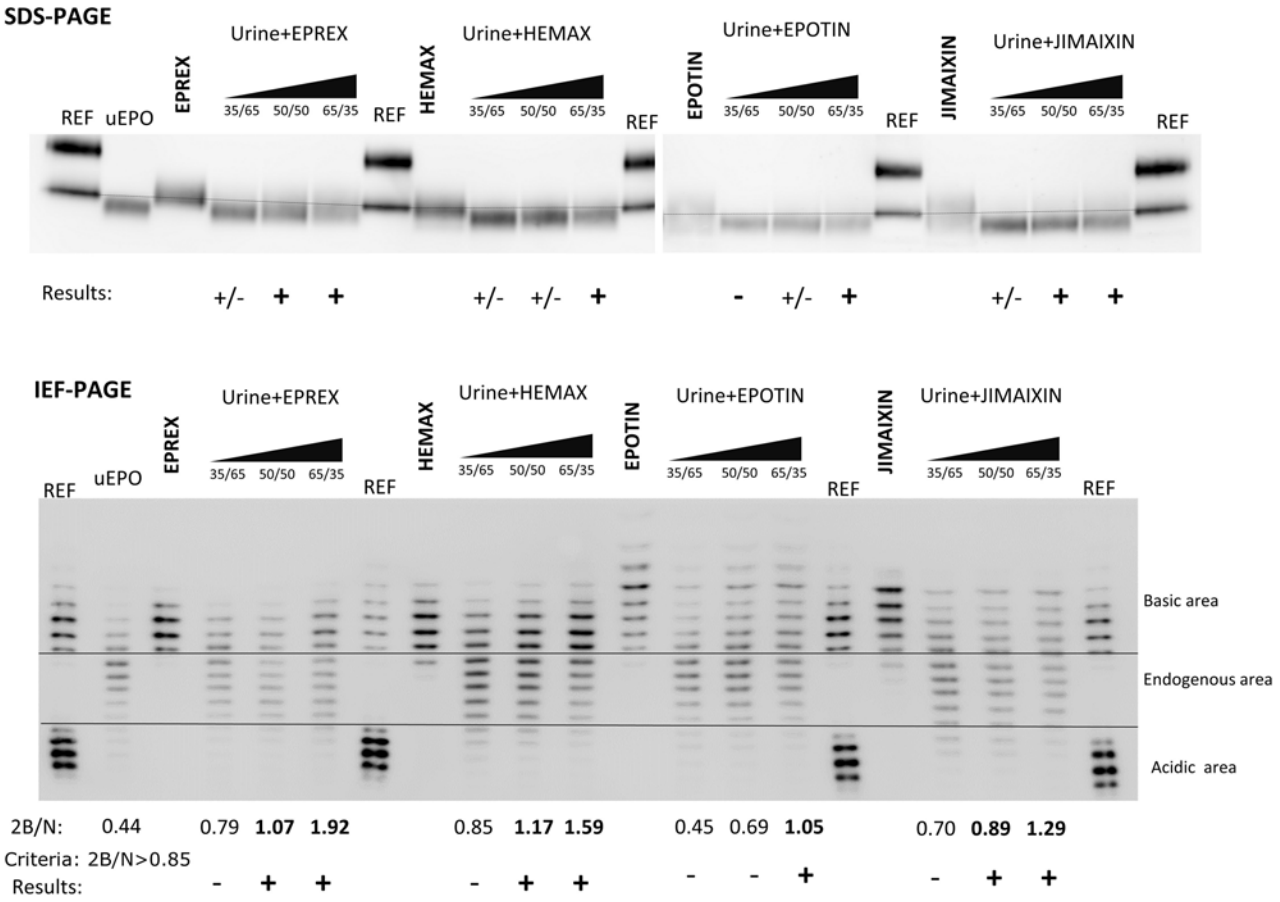

Figure 2. rEPO analysis of one urine spiked with increasing concentrations of each Biosimilar and Eprex®. The endogenous EPO in the urine without spike (uEPO) and a standard of each biosimilar were also analyzed. **A**. SDS-PAGE method: REF corresponded to Dynepo® (lower band) and NESP (upper band). The results of identification are indicated (– = negative results, +/– = suspicious, + = positively identified). **B.** IEF-PAGE method: REF corresponded to BRP (upper bands) and NESP (lower bands). The results of the 2B/N ratio (> 0.85 = suspicious) and results of identification are indicated.

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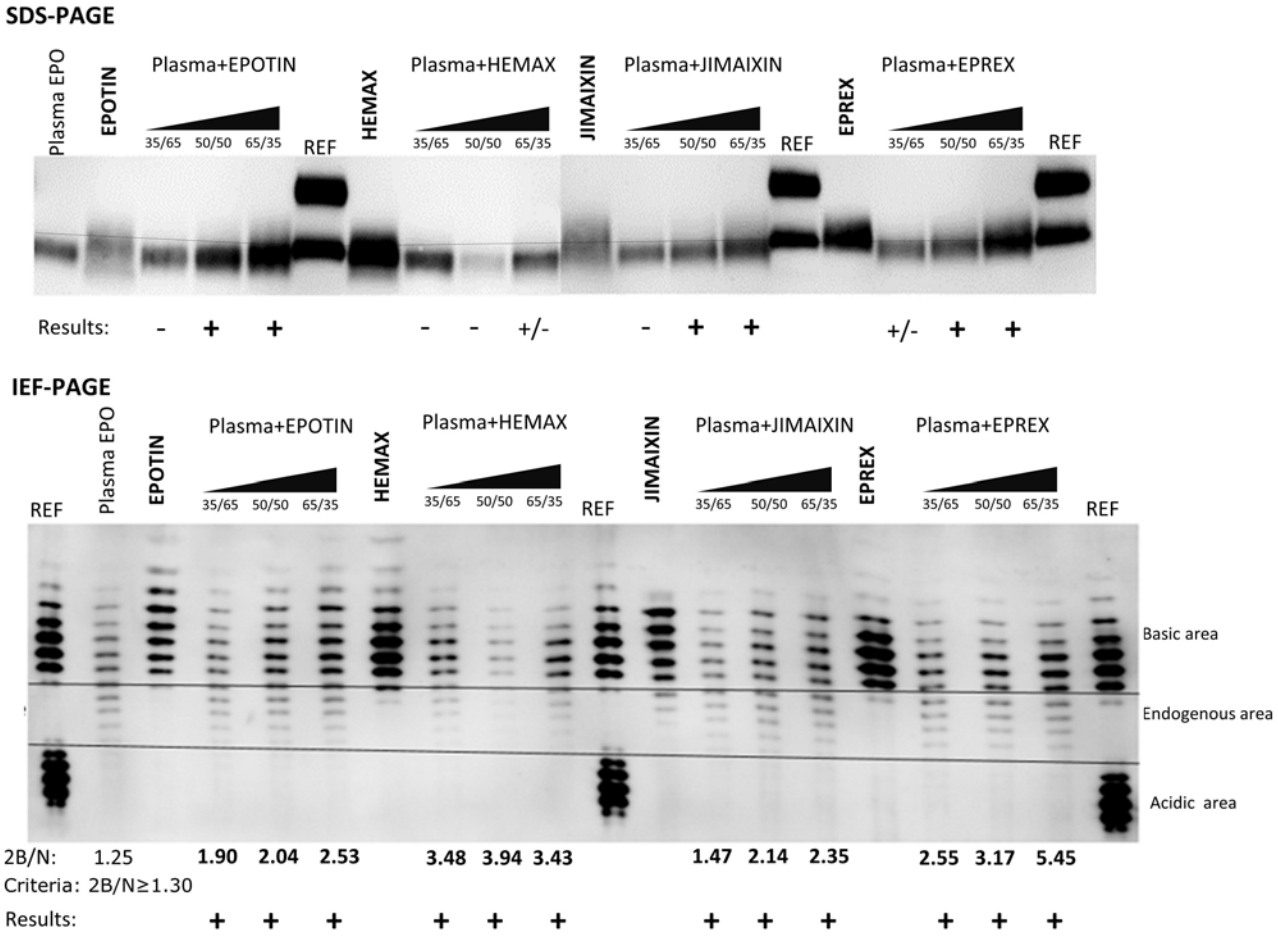

Figure 3. rEPO analysis of one plasma spiked with increasing concentrations of each biosimilar and Eprex®. The endogenous EPO in the plasma without spike (plasma EPO) and a standard of each biosimilar were also analyzed. **A.** SDS-PAGE method: REF corresponded to Dynepo® (lower band) and NESP (upper band). The results of identification are indicated (– = negative results, +/– = suspicious, + = positively identified). **B.** IEF-PAGE method: REF corresponded to BRP (upper bands) and NESP (lower bands). The results of the 2B/N ratio (≥ 1.30 = suspicious) and results of identification are indicated.

The results (summarized in Table 1) demonstrated that while Eprex ® was efficiently identified by both IEF-PAGE and SDS-PAGE methods, the identification of the biosimilars was more challenging in particular for the lowest dose (35:65 mixes of rEPO:endogenous EPO). The two different methods of separation were complementary because, depending on the biosimilar, one or the other was more efficient. Jimaixin™ and Epotin™ were more easily identified by SDS-PAGE while Hemax® was better identified by IEF-PAGE. SDS-PAGE method applied on urine samples or IEF-PAGE on plasma samples appeared the best options to obtain the greatest number of suspicions.

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Suspicious		EPOTIN	HEMAX	JIMAIXIN	EPREX
URINE	IEF (% with new criteria - % with TDEPO criteria)	33% - 22%	**83% - 72%**	44% - 28%	72% - 50%
	SDS	**78%**	67%	**83%**	**89%**
PLASMA	IEF (%with new criteria - % with TDEPO criteria)	**67%** - 33%	**89% - 67%**	**78%** - 44%	**89%** - 67%
	SDS	**67%**	33%	67%	**89%**

Positive Identification		EPOTIN	HEMAX	JIMAIXIN	EPREX
URINE	SDS	56%	33%	67%	67%
PLASMA	SDS	56%	11%	67%	56%

Table 1. Summary of the results obtained for the REPO detection in spiked urine and plasma using either IEF-PAGE or SDS-Page methods. Six urines and 3 plasma samples were spiked at three different ratios of recombinant EPO to endogenous EPO (35/65, 50/50 and 65/35). Percentage of suspicious samples identified by each method are indicated (upper table). For IEF-PAGE the percentages obtained when using the new criteria for suspicions and those obtained when using TDEPO's criteria are indicated. In addition percentage of samples with clear positive identification in case of confirmation by SDS-PAGE are also indicated (lower table). The best conditions are shown in bold.

Complementary tests to increase identification

Neuraminidase treatment: Neuraminidase cleaves the sialic acids at the end of the N-glycan chains and this was suggested as a way to increase the distance of migration between recombinant EPO and endogenous EPO (which contains more sialic acids)[3].

- SDS-PAGE results (Figure 4A and 4B):

All EPO profiles after treatment by neuraminidase had lower molecular weight and wider signal. Dynepo® treated by neuraminidase was evaluated: the Dynepo®+neuraminidase band was just above the endogenous EPO+neuraminidase signal and presented a sharp band: it could be used as a marker to separate the endogenous zone from the exogenous zone. With this marker, urines spiked with biosimilars were slightly more suspicious than before neuraminidase treatment in particular for Hemax®. However a loss of intensity could sometimes be observed after neuraminidase.

- IEF-PAGE results (Figure 4C):

Due to the cleavage of sialic acids, neuraminidase treatment induced a shift of the EPO profiles towards the basic part of the gel and a 2-10 pH gel gradient was necessary. After spike, for all three biosimilars, three small bands could be observed interspaced between the three most basic isoforms of EPO even at low amounts. This specific pattern was a distinctive sign and increased the sensitivity of detection compared to the conventional IEF-PAGE method.

Samples analyzed by both IEF-PAGE and SDS-PAGE before and after treatment by neuraminidase indicated that the detection of biosimilars by IEF-PAGE method (intact EPO) was less performant than the IEF-PAGE protocol with neuraminidase-treated samples. IEF-PAGE on neuraminidase treated samples had similar sensitivity than the SDS-PAGE method on untreated samples. Finally SDS-PAGE method on neuraminidase treated samples was a bit more performant to identify rEPO biosimilars at low doses and could be used in case of a suspicious sample.

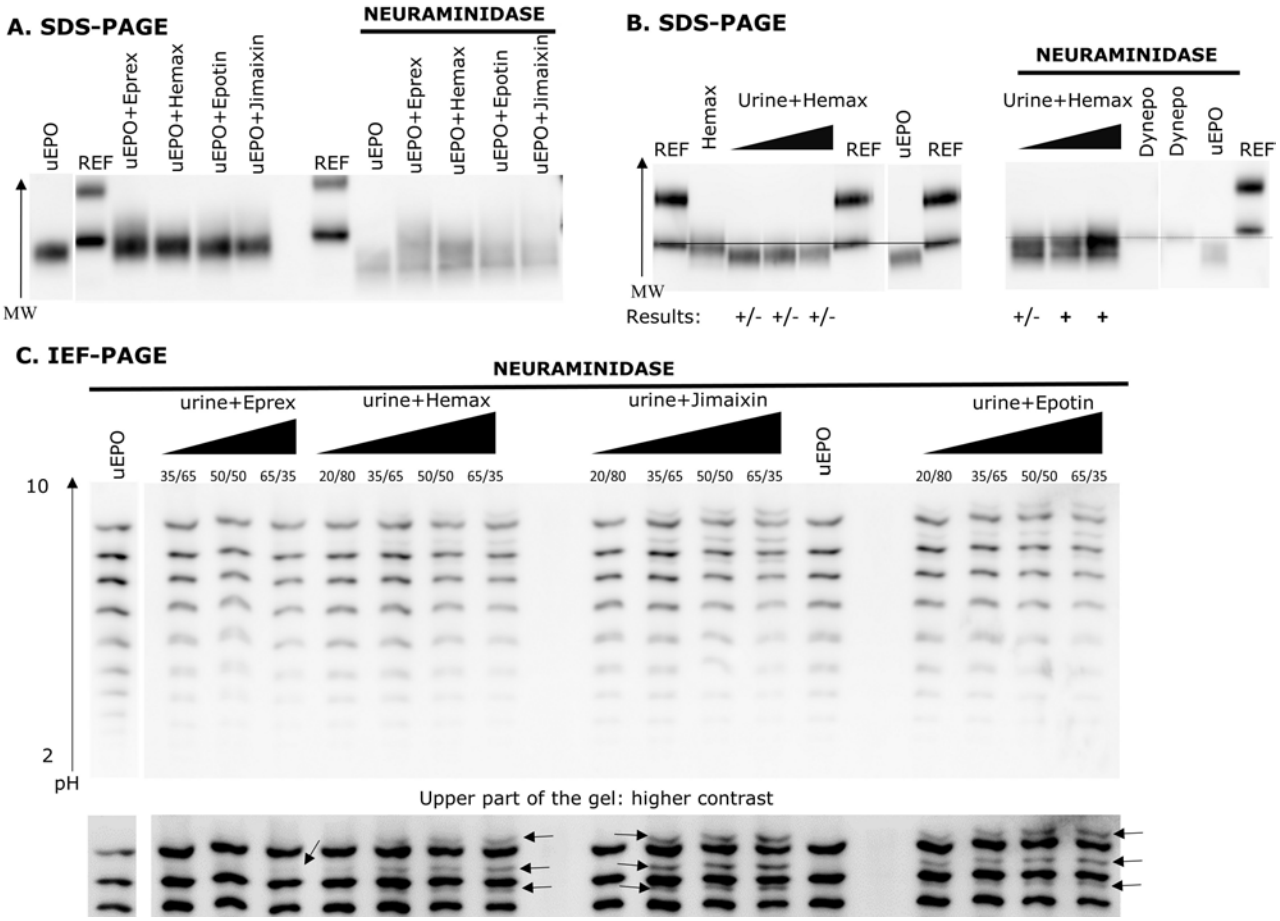

Figure 4. Effect of neuraminidase on EPO profiles of endogenous EPO (urine), Eprex® and biosimilars. **A.** SDS-PAGE. Urine EPO without spike (uEPO) or spiked with each rEPO at 50:50 with or without neuraminidase treatment were analyzed. **B.** SDS-PAGE: uEPO or urine spiked with increasing amount of Hemax® as well as Dynepo® without and and with neuraminidase treatment were analyzed. Dynepo® was used to delimitate endogenous and exogenous zone for EPO and results of identification are shown. **C**. IEF-PAGE. uEPO standard or urine spiked with increasing amount of each biosimilar treated by neuraminidase were analyzed on a 2-10 pH gradient gel. Black arrows indicate newly identified bands specific of rEPOs and well expressed in biosimilars.

Two dimension gel electrophoresis : The first attempts confirmed the previously published observations with Eprex® [3], but the 2D-separation did not improve biosimilars identification compared to IEF-PAGE or SDS-PAGE (data not shown). This method is also technically complex and no clear criteria were identified for biosimilar detection.

Analysis of N-Glycosylations

As shown in Figure 5, all profiles were qualitatively similar between Eprex® and the 3 biosimilars: they all exhibited the same N-glycan forms. This indicates that EPO was correctly produced. However, there were significant differences in relative abundance for some specific hyposialylated N-glycan forms. In particular we identified a significant enrichment in bi-antennary and tri-antennary forms compared to Eprex® and a decrease of the tetra-antennae tetrasialylated form (the main N-glycan form of Eprex®) towards an increase of the tetra-antennae N-acetyl-lactosamine (LacNAc) bisialylated form for Epotin™ and Jimaixin™. This might happen due to the conditions of culture or during the purification process [7-9]. Epotin™ and Jimaixin™ presented the most pronounced differences while Hemax® had a

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glycosylation profile closer to Eprex®. This was in good agreement with the IEF-PAGE profiles of all 3 biosimilars.

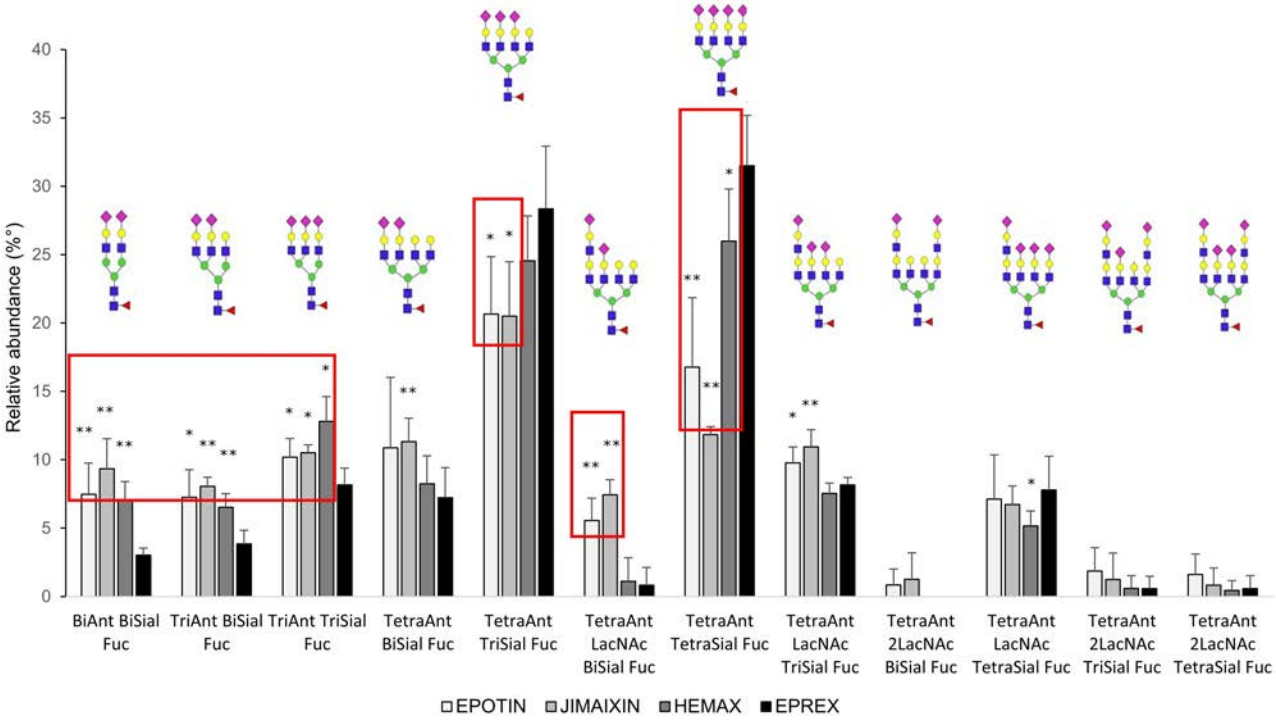

Figure 5. Graphical representation of the relative abundance of each N-glycan EPO structures for Eprex® and the three biosimilars. Mean+/-SD of all replicates obtained for each immuno-purified biosimilar (n=5 for Epotin™, n=6 for Hemax® and Jimaixin™) and for Eprex® (n=6) are presented. Major differences with Eprex® are shown in red squares. Statistical comparison with Eprex® for each N-glycan structure were performed: *: $p < 0.05$, **: $p < 0.01$. Green circles, mannoses; yellow circles, galactoses; blue squares, N-acetyl-glucosamines; red triangles, fucoses; purple diamonds, sialic acids.

Conclusions

In this study, 3 biosimilars of rEPO (Epotin™, Hemax® and Jimaixin™) were characterized and presented unusual profiles by IEF-PAGE and/or SDS-PAGE. After a spike in urine and plasma, the detection was slightly more difficult than conventional rEPO Eprex® in particular with low doses. SDS-PAGE analysis and urine matrix were the best option for the detection of all biosimilars. In addition, this method avoid the risk of false suspicions due to active/effort urines that might happen with IEF-PAGE. Using a 2D-gel analysis approach was not useful but a treatment with neuraminidase slightly improved the detection. Comparison of N-glycosylations between the biosimilars and Eprex® indicated an increase in less acidic and less complex glycan forms the three biosimilars in line with their different electrophoretic profiles. These findings needs to be confirmed by an appropriate administration study in healthy men.

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Heiland CE, Masquelier M

Sepharose gel beads: an effective technique for recombinant erythropoietin immunopurification from blood

Doping Control Laboratory Karolinska University Hospital, Stockholm, Sweden

Abstract

We have previously shown that the performance of the immunopurification step used to concentrate erythropoietin (EPO) and its recombinants before electrophoretic analysis has an impact on the detection of EPO. Although urine is the most common doping control matrix, blood seems to be a better alternative for abuse detection as highlighted by WADA's published statistics for EPO between 2015 and 2018. In this study, an EPO Purification Gel Kit for Blood (EPGK for Blood; MAIIA Diagnostics) containing anti-EPO antibody-linked sepharose gel beads was used to immunopurify blood samples fortified with four erythropoiesis stimulating agents (BRP, NESP, EPO-Fc and CERA) and the results were validated, with special attention to detection limit and eluate stability determination. Lately, dried blood spots (DBS), an alternative collection method and matrix, has been attractive for the anti-doping scene. Therefore, the same immunopurification technique was also applied to extract ESA from DBS to evaluate the impact of low blood volume of the spots on the detection limit and if multiple spots can compensate and increase the method sensitivity.

Introduction

Between 2015 and 2018, erythropoiesis-stimulating agent (ESA) adverse analytical findings (AAFs) in blood represented over 20% of the total number of ESA AAFs, although blood tests made up only about 8% of the total ESA tests taken [1], showing the importance of blood as a matrix for recombinant erythropoietin (rEPO) detection. This could be attributed to blood stabilizing effects from high protein concentration and to the cold transport requirement reducing erythropoietin (EPO)-degrading bacteria in blood. Consequently, more blood analysis should result in better detection of rEPO. Choosing an effective immunopurification method is also crucial, as shown previously [2]. The EPO Purification Kit (EPK; MAIIA Diagnostics), commonly used in anti-doping laboratories, has caused filtration issues in some urine, therefore MAIIA developed a gel kit (EPGK) using sepharose gel beads coupled to anti-EPO antibodies, aiming to simplify purification, increase sample throughput, and reduce sample handling, while keeping a good EPO detection both in urine [3] and in blood. The possibility of using DBS for EPO detection after ELISA immunopurification has been described by Reverter-Branchat et al. [4], thus initiating interest in the EPGK's capabilities for this matrix. Therefore, in this study, serum and DBS were spiked and immunopurified with the EPGK to evaluate and validate the kit for EPO blood analysis. The validation evaluated the detection limit and immunoextract stability, the application of this technique to DBS, and the impact of DBS sample volume on EPO detection.

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Experimental

Materials

Reference standards used for spiking were: Biological Reference Protein (BRP) (European Directorate for the Quality of Medicines and Healthcare; Strasbourg, France); NESP (Aranesp; Amgen, Thousand Oaks, CA, USA); CERA (Mircera; Roche, Mannheim, Germany); and EPO-Fc (Prospec, Ness-Ziona, Israel). EPO Purification Gel Kit (EPGK) for Blood was provided by MAIIA Diagnostics (Uppsala, Sweden). Whatman 903 protein saver cards were from GE Healthcare (Uppsala, Sweden). Trayster Digital (IKA; Staufen, Germany) was used for EPO extraction. Biotinylated AE7A5 monoclonal antibody from R&D Systems (MN, USA) was used as the detection antibody. Streptavidin-HRP was from Calbiochem (CA, USA).

Method

Sample collection: Serum samples were collected from volunteers, anonymized, and pooled. Samples were either kept blank or fortified with four ESAs. Whole blood was collected from volunteers, anonymized, spiked and pipetted onto Whatman 903 protein saver cards (50 µL/spot).

Immuno-extraction and -purification: The method was performed according to the manufacturer's instructions [5]. Briefly, 0.5 mL of serum or 1 whole blood spot (50 µL) and Sample Buffer were placed in a column attached to a funnel, which was then inserted into a Falcon tube, and incubated end-over-end. The sample was filtered with vacuum pressure, the gel beads were washed, and the EPO was eluted with Elution Buffer (35 µL for serum and 50 µL for DBS).

SAR-PAGE and Western blot: A single blot analysis was done as previously described [3], with a few changes to the sample volume used for analysis and the detection antibody. 8 µL (serum) or 16 µL (DBS) of sample were loaded onto the electrophoretic gel. Immunodetection was performed as follows: biotinylated antibody (0.5 µg/mL) overnight, wash, streptavidin-HRP (0.5 µg/mL) for one hour, and a wash.

Serum stability: The stability of spiked serum eluates was investigated at -20°C, 4°C, and room temperature for 0, 1, 7, and 30 days after immunopurification. Serum samples were spiked with BRP (150 pg/mL serum), NESP (30 pg/mL serum), CERA (1000 pg/mL serum), and EPO-Fc (500 pg/mL serum).

Limit of detection for serum: Five decreasing concentrations of the four ESAs were analyzed in 1 mL of serum by spiking the samples as follows: 2000 – 18.8 pg CERA; 1000 – 1.8 pg EPO-Fc; 60 – 3.8 pg NESP; 300 – 18.8 pg BRP.

Limit of detection for DBS: Six decreasing concentrations of the 4 ESAs were analyzed in 50-µL DBS by spiking whole venous blood as follows: 200 – 6.3 pg CERA; 50 – 1.6 pg EPO-Fc; 5 – 0.2 pg NESP; 25 – 0.8 pg BRP.

DBS sample volume: Venous blood was spiked with 4 ESAs (126 pg BRP, 50 pg NESP, 1000 pg CERA, and 250 pg EPO-Fc per mL whole blood) and pipetted onto a filter paper support and four 50-µL spots were immunopurified together and compared with immunopurification of one spot and 100 µL of serum spiked with the same ESA concentrations. Purification of four 50-µL DBS discs is equivalent to approximately 100 µL of serum. The volumes of immunopurified sample were adjusted for loading on the electrophoretic gel.

Results and Discussion

The stability of spiked serum eluates is presented in Figure 1. All ESAs spiked in serum samples were quite stable at all temperatures for at least 30 days, with a decrease mainly for CERA and EPO-Fc at room temperature. Therefore, the immunopurified samples should preferably be kept at 4°C or -20°C.

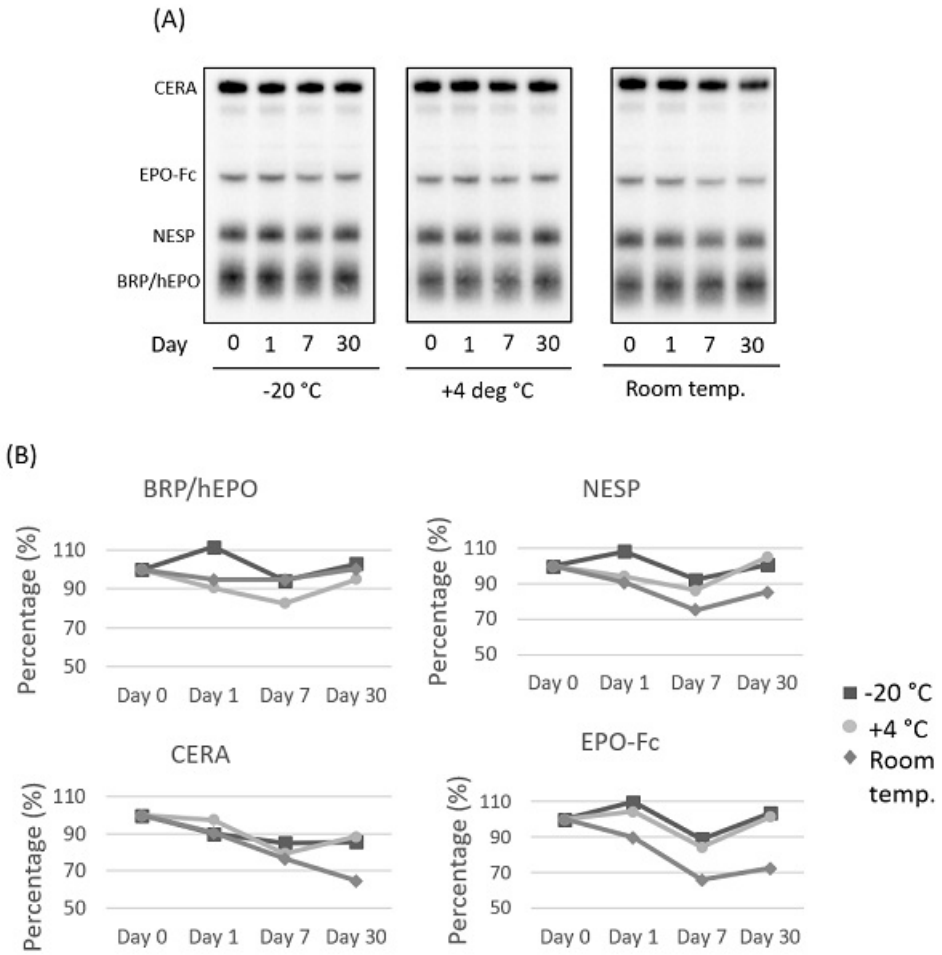

Figure 1. Stability experiment of eluates from serum samples spiked with 4 ESAs and immunopurified with EPGK. Samples were stored at -20 °C, 4 °C, and room temperature for 1, 7, and 30 days and compared to freshly purified sample (Day 0). (A) Gel image of the eluate stability experiment. (B) Graphs of the variation in percentage of band intensity of stored samples compared to freshly purified sample.

For serum spiked and immunopurified by EPGK, LOD was determined to be approximately 31.2 pg for CERA, 15.6 pg for EPO-Fc, 3.8 pg for NESP, and 18.8 pg for BRP per mL serum. However, this is calculated assuming a recovery range of 100% and for application of only 8 µL of immunopurified eluate on the gel. The LOD obtained for DBS was determined to be approximately 125 pg for CERA, 62.5 pg for EPO-Fc, 12.5 pg for NESP, and 62.5 pg for BRP per mL whole blood. The detection limits determined by Reverter-Branchat et al. (1200 pg CERA, 60 pg for NESP, and 60 pg for BRP per mL whole blood) and obtained using 25-µL spots of modeled-blood [4] were similar for BRP but higher for NESP and CERA

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compared to our results. Indeed, for DBS our LOD was comparable or better, but it was still 3 to 4 times higher than the LOD we obtained for serum immunopurification, as could be expected, which was confirmed in Figure 2. When comparing band intensities of these two matrices, spiked alike, it is apparent that the ESA detection is comparable to each other (Figure 2A and B). Therefore, it seems that multiple DBS are an effective approach in order to reach the same sensitivity as in serum. The importance of sample volume for EPO analysis was further demonstrated when 4 DBS were compared to only 1 DBS (Figure 2C). There is a noticeable improvement in EPO detection as the number of discs increases.

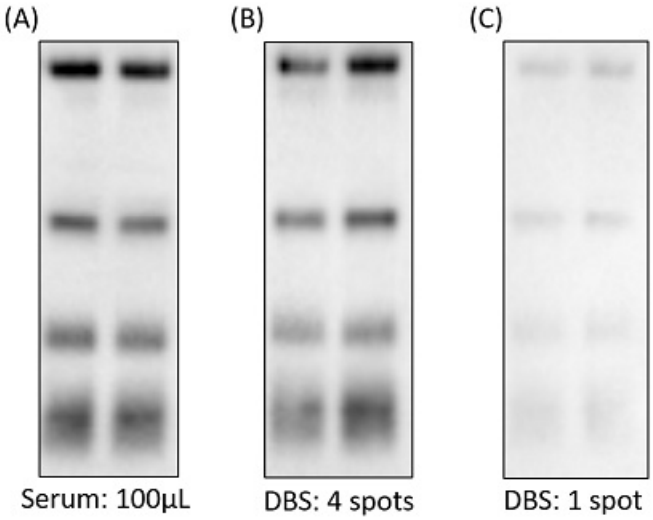

Figure 2. Sample volume comparison for serum and DBS. (A) Serum samples (two replicates of 100 µL each) spiked and immunopurified with the EPGK. (B) DBS samples (two replicates of four 50-µL spots each) spiked and immunopurified with the EPGK. (C) DBS samples (two replicates of one 50-µL spot each) spiked and immunopurified with the EPGK. Concentrations (pg/mL whole blood): BRP: 125 pg, NESP: 50 pg, CERA: 1000 pg, EPO-Fc: 250 pg. Volumes loaded onto electrophoretic gel: 11.2 µL for serum and 16 µL for DBS.

Since sample volume is a limitation for optimal EPO detection in DBS, there is a need for sufficient sample volumes and sensitive methods when DBS are added to the anti-doping sample matrix repertoire. Therefore, future experiments will include efforts to improve sensitivity by concentrating the DBS eluate during immunopurification and loading the entire volume onto the electrophoretic gel in order to compensate for the small initial volume of whole blood. This will hopefully optimize ESA detection in DBS. Other possible solutions might be to optimize transfer during the blotting step by performing a discontinuous semi-dry transfer as described [6], instead of using the typical continuous transfer system.
While the possibility of detecting EPO in patient samples has been demonstrated by Reverter-Branchat et al. [4], it would be interesting to investigate lower doses of rEPO that an athlete might potentially use, and this investigation is currently underway.

Conclusions

The EPO Purification Gel Kit for Blood can easily be used to immunopurify serum spiked with ESAs, giving

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stable eluates and detection comparable to the previous method. Moreover, it could be successfully applied to extract EPO from dried blood spots. More investigations are needed to further evaluate the detection limits, such as a large-scale study where DBS samples would be collected from participants administered with rEPO.

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Sigmund G, Geyer H, Goergens C, Thevis M

Prevalence of the imidazole derivatives naphazoline, oxymetazoline and xylometazoline in doping control samples

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Abstract

In 2017 an AAF for the imidazole derivative naphazoline was reported the first time in the WADA testing figures since their implementation in 2003. This reporting of an AAF was particularly noteworthy as the Cologne laboratory frequently detects imidazole derivatives in in-competition samples, but these findings are not reported as AAFs due to the remark in the prohibited list that imidazole derivatives are permitted for topical/ophthalmic use.

To assess the prevalence of imidazole derivatives among German and international elite athletes, the Digital Matrix (full scan data of GC/MS analyses) of 5500 in-competition (IC) doping control samples from different sports, collected from January till June 2017, were evaluated for the imidazole derivatives naphazoline, oxymetazoline and xylometazoline. Out of the 5500 evaluated samples, 199 samples contained imidazole derivatives (3.6 %). Out of the 199 samples, xylometazoline was detected in 178 samples (89.4%), oxymetazoline in 11 samples (5.5 %) and naphazoline in 10 samples (5.0 %). The majority of the target analytes (45.2 %) was detected at concentrations between 101-500 ng/mL. Twelve samples (6.0 %) showed concentrations above 1000 ng/mL.

Introduction

According to the WADA Prohibited List, S6 stimulants, imidazole derivatives are prohibited except for dermatological, nasal or ophthalmic use [1]. In 2017 an AAF for the imidazole derivative naphazoline was reported the first time in the WADA testing figures since their implementation in 2003 [2]. Details about the reporting of the naphazoline (e.g. extraordinary high concentrations, missing declaration in DCF, missing TUE etc.) were not available. This reporting of an AAF was particularly noteworthy as the Cologne laboratory frequently detects imidazole derivatives in in-competition samples, but these findings are not reported as AAFs due to the remark in the prohibited list that imidazole derivatives are permitted for topical/ophthalmic use.

The time interval from January till June was chosen to cover different seasons of the year. The DCFs were not evaluated for the declaration of imidazol derivatives.

Experimental

The Digital Matrix, i.e. full scan data of GC/MS analyses, of 5500 in-competition (IC) doping control samples from different sports were evaluated for the presence of the imidazole derivatives naphazoline, oxymetazoline and xylometazoline. Additionally, the concentrations of these derivatives were estimated.

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In order to cover different seasons, the samples analyzed came from the period from January to June 2017. The DCFs were not evaluated for the declaration of imidazol derivatives.
Samples were analyzed according to the method described by Thevis et al. in 2007 [3]. The used analytical method in brief: LLE of 5 mL urine at pH 14 with TBME, injection of 5 µL of organic layer into a GC/MS/NPD system. The mass spectrometer was operated with EI and full scan analysis *m/z* 40-400, 4 scans/s.

Results and Discussion

Out of the 5500 evaluated samples, 199 samples contained imidazole derivatives (3.6 %). 10 samples contained 2 different imidazole derivatives. Out of the 199 samples, xylometazoline was detected in 178 samples (89.4%), oxymetazoline in 11 samples (5.5 %) and naphazoline in 10 samples (5.0 %). The majority of the target analytes (45.2 %) was detected at concentrations between 101-500 ng/mL (see Figure 1). Twelve samples (6.0 %) showed concentrations above 1000 ng/mL. Corresponding sample details are presented in Table 1.
The imidazole derivatives were detected in 46 different sports. Most findings were detected in football (Table 2).

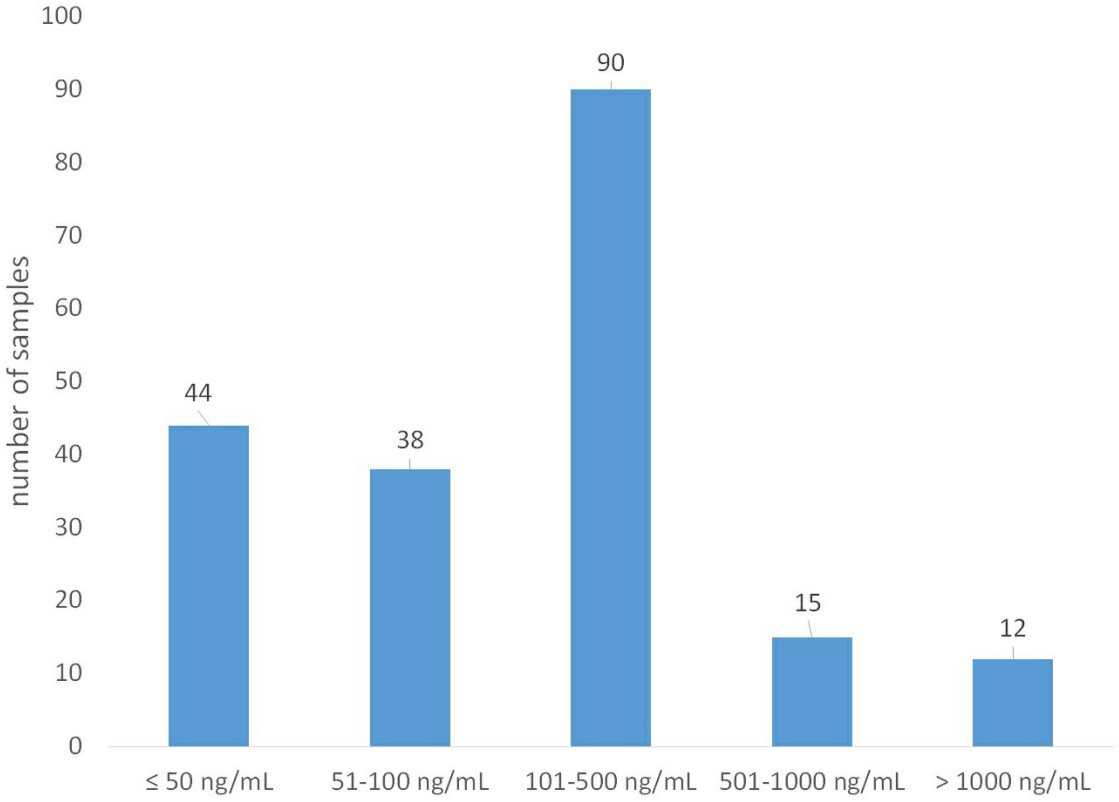

Figure 1. Estimated concentrations of the imidazole derivatives in the 199 findings

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sports	sex	conc. [ng/mL]	imidazole derivatives
Powerlifting	m	5260	naphazoline
Weightlifting	f	4500	xylometazoline
Taolu	f	4100	naphazoline
Powerlifting	m	3150	xylometazoline
Boxing	m	1900	xylometazoline
Wrestling	m	1700	xylometazoline
Football	m	1500	xylometazoline
Football	m	1500	xylometazoline
Ice Hockey	m	1350	xylometazoline
Ice Hockey	m	1300	xylometazoline
Artistic	m	1150	xylometazoline
Judo	f	1050	xylometazoline

Table 1: Samples with concentrations of imidazole derivatives > 1000 ng/mL

sports	number
Football	40
Weightlifting	16
Basketball	12
Ice Hockey	12
Boxing	9
Powerlifting	8
Judo	7
Cycling	7
Swimming	7
Speed Skating	6
Field Hockey	5
Wrestling	5

Table 2: Sports with most frequent findings of imidazole derivatives

Conclusions

This is the first time that the frequency of the use of imidazole derivatives in elite athletes has been evaluated. More than 3% of all tested IC samples showed the presence of imidazole derivatives. Xylometazoline was the most frequently detected imidazole derivative.

These data should be presented to the WADA Prohibited List expert group to facilitate the decision on whether or not a reporting limit should be implemented for these substances (e.g. > 1000 ng/mL). If it is agreed to implement such a reporting level, as next step excretion studies with imidazole derivatives

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have to be conducted, to evaluate whether the detected concentrations are consistent with a permitted topical/ophthalmic use or prohibited routes of administration.

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Acknowledgements

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Wicka M, Kaliszewski P, Grucza K, Stanczyk D, Drapala A, Konarski P, Zalewska Z, Siek P, Michalak D, Kwiatkowska D

Elimination profile of triamcinolone acetonide and its metabolite in human urine after multiple transdermal administration

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Abstract

Triamcinolone acetonide (TA) is prohibited in competition and only for oral, intravenous, intramuscular or rectal administration. In order to distinguish whether TA was administered by one of the prohibited routes or not, a specific reporting limit for urinary concentrations of the parent compound and its metabolite was established at 30 ng/mL. Triamcinolone acetonide is a synthetic corticosteroid applied topically to treat various skin conditions, intra-articularly to treat joint injuries, intramuscularly for the control of severe allergic states. In nasal spray form, it is used to treat allergic rhinitis. The aim of the present work was to study the metabolic profile of Triamcinolone acetonide in urine following transdermal administration. Four healthy male volunteers applied 4 g of Pevisone (econazole nitrate 10 mg/g + Triamcinolone acetonide 1.1 mg/g) once a day and twice a day throughout three consecutive days. Four spontaneous urine samples were collected per day i.e. in the morning, midday, afternoon and evening. Urine samples were analyzed by liquid chromatography combined with mass spectrometry to measure TA and 6β-OH triamcinolone acetonide (6β-OH TA), the main TA metabolite. After administration, the concentration of TA was significantly lower than of 6β-OH TA. For 6β-OH TA, a greater concentration was obtained: 0.10 - 4.36 ng/mL. However, concentrations of TA and 6β-OH TA were lower than 30 ng/mL for all volunteers.

Introduction

Triamcinolone acetonide is asynthetic corticosteroid used as anti-inflammatory drug to treat various diseases. It is applied topically to treat various skin conditions (e.g., severe erythema multiforme and pemphigus), intra-articularly to treat joint injuries (e.g., gout, psoriatic arthritis and systemic lupus erythematosus), intramuscularly for the control of severe allergic states. In nasal spray form, it is used to treat allergic rhinitis. It is used to treat many different conditions such as gastrointestinal diseases (e.g., ulcerative colitis)[1]. Triamcinolone acetonide is widely used by athletes to treat articular pain, injures and sprains [2]. According to the WADA Prohibited List [3], glucocorticosteroids are prohibited in competition and only when administered by oral, intravenous, intramuscular or rectal routes. In order to distinguish whether glucocorticosteroids were administered by one of the prohibited routes or not, a specific reporting limit for urinary concentrations of parent compounds and their metabolites was established at 30 ng/mL [4].

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Experimental

Four male healthy subjects, aged 28, 29, 41 and 60 years, height and weight respectively 179 cm/84 kg, 175 cm/67 kg, 178 cm/83 kg, 178 cm/90 kg (subject 1M, 2M, 3M and 4M), applied 4 g of Pevisone (econazole nitrate 10 mg/g + Triamcinolone acetonide 1.1 mg/g) once a day for three days and then twice a day for three consecutive days – transdermal administration. One dose of the preparation that was applied to the skin weighed 4 grams and provided a one-time dose of 4.4 mg of the active substance acetonide triamcinolone. All volunteers applied the preparation to the sacrolumbar part of the spine. Four spontaneous urine samples were collected per day, i.e. in the morning, midday, afternoon and evening. Urine samples were collected for 5 days after the final administration (Figure 1).

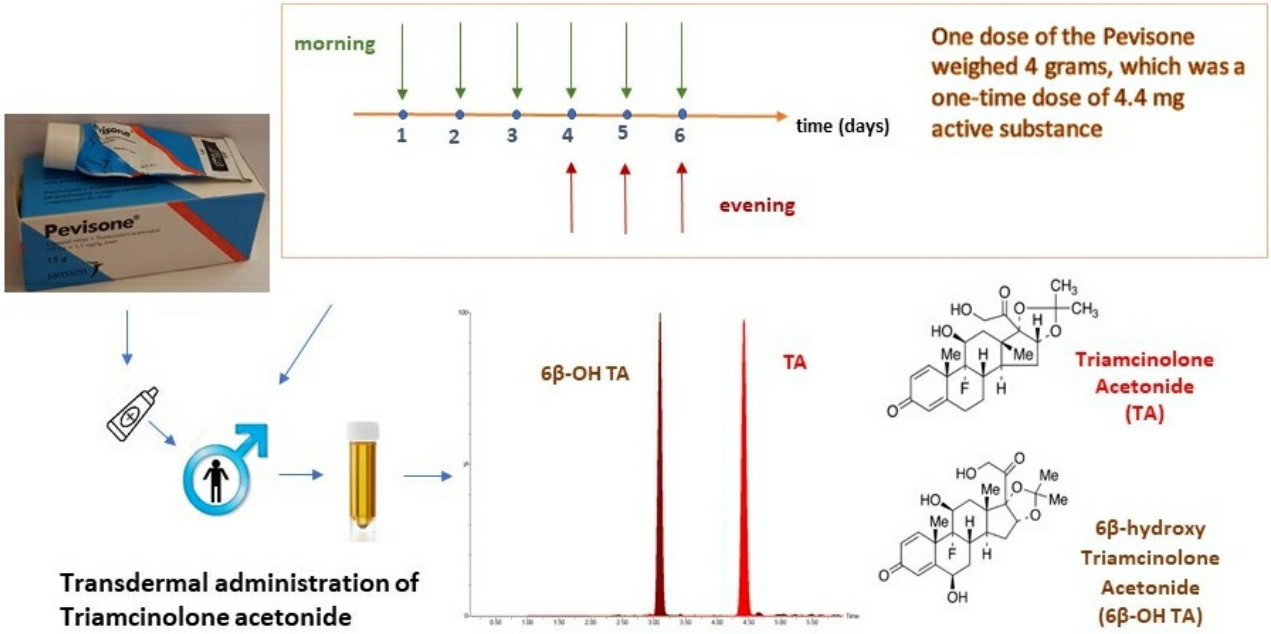

Figure 1. Scheme of dosage of Pevisone and administration

The sample preparation is a two-step procedure involving enzymatic deconjugation of glucuronides and then liquid-liquid extraction with methyl tert-butyl ether. Chromatographic separation was conducted using a Waters Acquity UPLC System liquid chromatograph with BEH C18 (1.7 µm, 100 mm x 2.1 mm) from Waters. The mobile phase consisted of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B), and LC gradient was employed at the constant flow rate of 300 µL/min at 45°C. Triamcinolone acetonide (TA) retention time was 4.43 min, while that of 6β-OH triamcinolone acetonide (6β-OH TA) was 3.10 min. MRMs of the studied substances were traced with a Micromass Quattro Premier XE mass spectrometer equipped with an ESI source. All analytes were investigated in the ESI^+ mode. Desolvation gas flow was set at 643 L/h at 450°C with ion source temperature at 120°C. Capillary voltage was 3.0 kV.

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Results and Discussion

Characteristics of the calibration curves are shown in Table 1.

	Linearity			
Analyte	Range [ng/ml]	Equation of linear regression[b]	(r)[c]	LOD [ng/ml]
TA	0.5 – 30.0	y = 0.03x + 0.01	0.997	0.05
6β-OH TA	0.5 – 30.0	y = 0.02x + 0.01	0.998	0.05

[a]) n=6, [b]) mean value, [c]) coefficient of correlation

Table 1. TA and 6β-OHa curve parameters

The LOD for TA and 6β-OH TA were estimated at 0.05 ng/mL. The linear regression curve ranged from 0.5 to 30 ng/mL and had a satisfactory coefficient of correlation. Typical MRM chromatograms generated from a urine sample spiked with 30 g/mL of Triamcinolone acetonide and the metabolite 6β-OH Triamcinolone acetonide are shown in Figure 2.

Blank urine control samples which were analyzed in each series of the validtion procedure (n=6) were free of compounds wich might be eluted in the retention times of TA and 6β-OH TA. As shown in Figure 2, TA and 6β-OH TA could be identified in human urine after transdermal administration at low concentrations.

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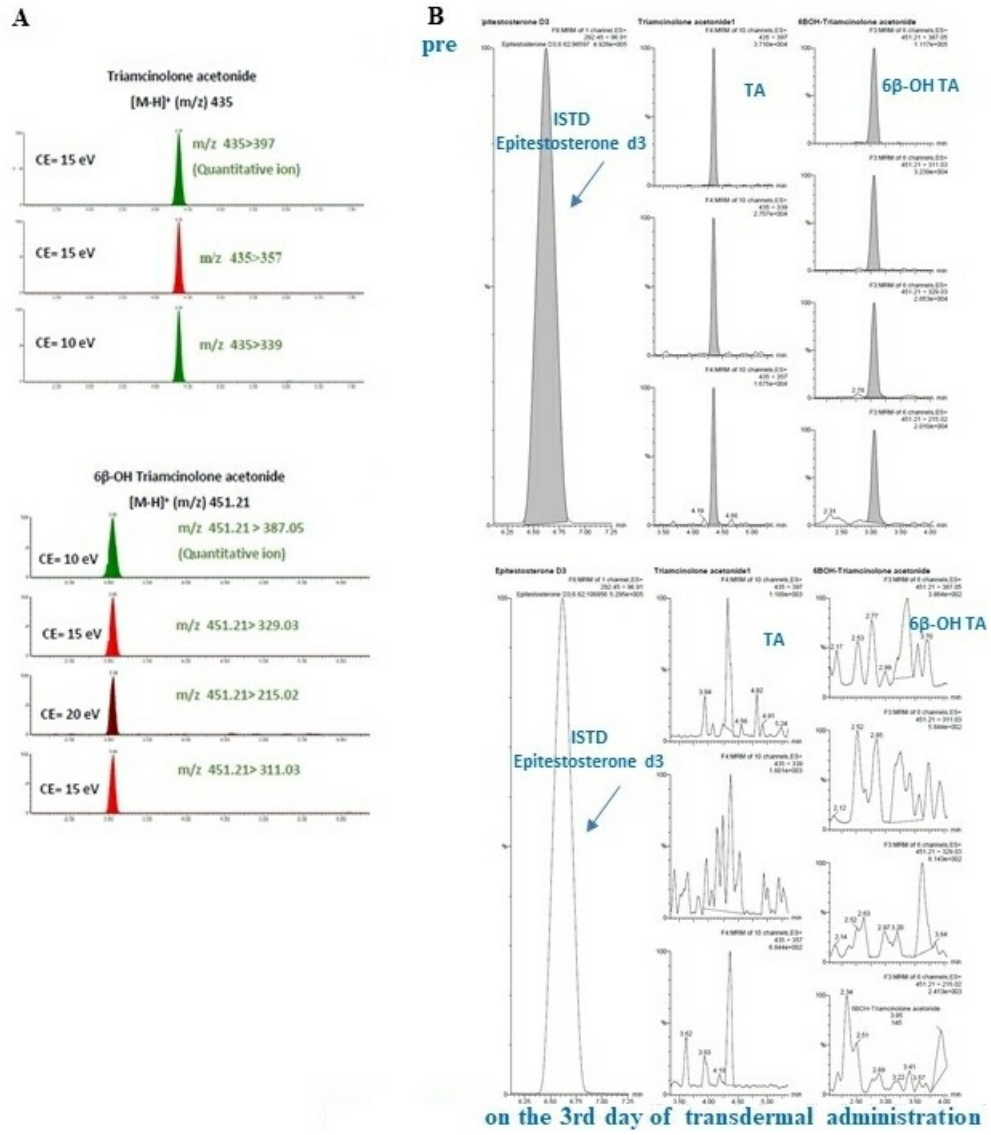

Figure 2. Typical MRM chromatograms: (A) spiked urine 30 ng/mL; (B) urine sample pre and 3rd day after transdermal administration

In our study, 4 g per day for three days, and 8 g per day of TA cream for three consecutive days (total 36 mg of cream, equivalent 39.6 mg of the active substance acetonide triamcinolone) were administered to the volunteers. The topical dose was administered for six consecutive days, to determine if repeated doses lead to accumulation of TA in urine. Figure 3 shows the changes in the concentrations of TA and 6β-OH TA after administration. The maximum concentration of TA were in the range of 0.7 – 0.8 ng/mL. However, the maximum concentation of 6β-OH TA was in the range of 1.93 – 4.36 ng/mL.The low concentration values obtained for TA, as in case [1], were resulted from a low absorption of TA by the skin. There was also a visible lack of TA accumulation after multiple administrations. The high inter-individual variability in concentrations between volunteers was probably depending on the age and the metabolism associated with it.

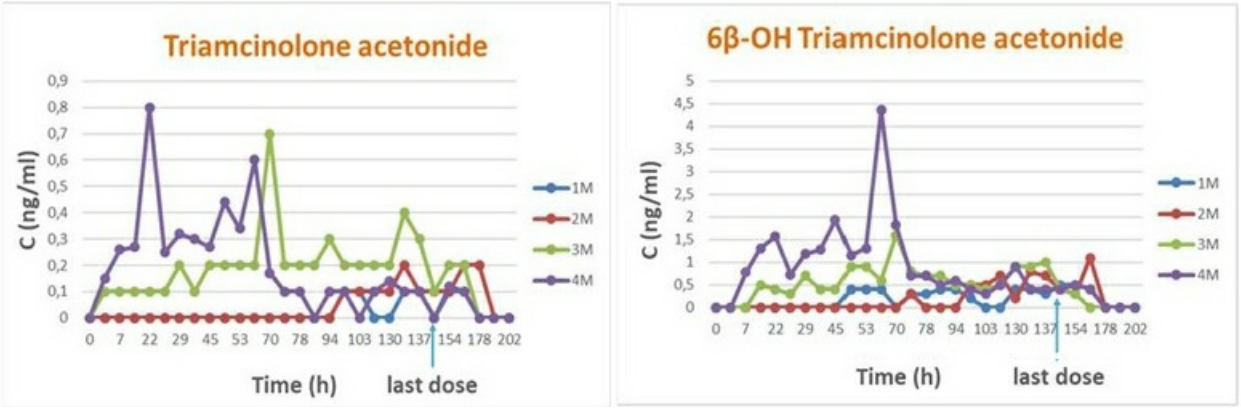

Figure 3. Changes in the concentrations of TA and 6β-OH TA in urine after administration

Conclusions

1. During monitoring of the profile excretion of Triamcinolone acetonide, the concentration values ranged from 0.10 ng/mL to 0.80 ng/ml. While for the metabolite of Triamcinolone acetonide (6β-OH Triamcinolone acetonide), the concentration values ranged from 0.10 ng/mL to 4.36 ng/mL.

2. After the end of the last dose of Pevisone, the presence of both substances was observed for an average of 2 days.

3. Based on the data collected, the Triamcinolone acetonide and 6β-OH-Triamcinolone acetonide were not detected above the reporting limit.

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Trend analysis and decision making in the Havana Anti-Doping Laboratory

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Abstract

Trend analysis is a technique widely used in the fields of finance, marketing, and decision making in general. The Havana Anti-Doping Laboratory, accredited uninterruptedly since 2003, possesses and applies internal procedures to internally calibrate the measurement instruments. The objective of the present study was to evaluate the trends of nine calibration parameters of three Agilent Technologies 5973N Simple Quadrupole, in order to provide data for a feasible decision making.
Three tests were applied to the data (nine parameters evaluated annually from 2003 to 2019) in order to check the presence of trends: Mann-Kendall trend test, Spearman test and Kendall tau test. Then, a prediction was estimated by the slope, intercept and, the residues values. The study was carried out for gas chromatograph instruments and for the calibration parameters as injector and detector linearity, flow and injector accuracy and uncertainty.
The results allowed to obtain the approximate date that the instruments could reach a maximum value of the calibration acceptance criteria. This criterion could (should) be used by the Laboratory Manager in order to plan their replacement and to plan the use of the instrument. One of the results was that quantification assays should be suspended for the instrument coded as MSD4 with the aim of maintaining the reliability of the results reported by the Laboratory.

Introduction

The decision process has characteristics that make it continuous, interrelated and the result of a statistical process. According to Fundamental and General Metrology Terms, “calibration” is the set of operations with which, under specified conditions, the correspondence between the values indicated in the instrument, equipment or measurement system and the values are established represented by a reference material or by known values corresponding to a measurement or standard. The equipment and patterns responses, vary over time due to different causes, i.e. aging, deterioration, improper cleaning and chemical reactions [1,2].
The main objective was to evaluate trends based on the data obtained in the calibration processes of three GC/MS in the Havana Antidoping Laboratory during sixteen years.

Experimental

Data and methods applied

Three Agilent Technologies 5973N Simple Quadrupole coded as MSD2, MSD3 and MSD4 were studied through nine parameters that define the instrument performance. The verification of these parameters is

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carried out every year and the present study included the results obtained from 2003 to 2019. Table 1 describes the parameters and the acceptance criteria applied in the evaluation of the instruments.

Statistics. Data obtained in the lapse from 2003 and 2019 was statistically evaluated through three tests in order to check the presence of trends: Mann-Kendall trend test; Spearman test and Kendall tau test with 95% confidence interval [3,4].

After to applied the tests to the data, it was selected the parameters that show trends in their behaves between 2003 and 2019 and finally; a long term prediction by the application of the "automatic forecasting" (by the slope, intercept and, the residues values) was made and, it was estimated the year in which the maximum value of the Calibration Acceptance Criteria should be achieve.

Software used were STATGRAPHICS centurion XV, version 15.1.02, StatPoint Inc, 2008 and; PAST 3.26 (Hammer, Ø, Harper, DAT Ryan PD 2001. PAST: paleontological statistics software package for education and data analysis Paleontological Electronica 4 (1): 9pp.

Parameters evaluated during Calibration Process		
ID	**Parameters**	**Acceptance criteria**
EF*	Flow inaccuracy	RSD < 10%
LI	Injector Linearity	$R^2 > 0.98$
PI	Injector precision	RSD < 4 %
PF	Injector Flow precision	RSD < 0.5 %
LD	Detector Linearity	R^2 mayor a 0.98
AI*	Detector carryover	RSD < 0.1 %
RD	Detector noise level	< 25 000 units
DD	Detector drift	< 40 000 units
uc	Combined uncertainty	uc < 20 %
(*) values close to zero from 2003 to 2019		

Table 1. Parameters evaluated during the calibration process of three single quadrupole gas chromatograph-mass spectrometer (GC/MS) and the acceptance criteria in the Havana Anti-Doping Laboratory

Results and Discussion

Annually, the verification of the calibration status for each instrument is perform using the "accepted" or "no accepted" criteria. This does not allow to evaluate trends in the data. After 17 working years, out of the nine parameters verified, the flow (in)accuracy and detector carryover showed values permanently close to zero and for that, they remained out of the analysis.

The Injector Precision (PI) evaluates the precision of the injector by calculating the coefficient of variation of the chromatographic peaks areas of the reference material obtained after to inject consecutively 5 replicates of the 1 µL injection volume. This parameter showed a marked trend for all three instruments (Spearman rho, Kendall tau and Mann-Kendall trend, $\alpha = 0.05$, Table 2). Because it is a significatively contributor to the uncertainty value, also this parameter showed a significant trend for all three instruments [5]. Additionally to injector precision (**PI**) and uncertainty (**uc**), the instrument MSD4 showed a significant trend for the parameter Injector Linearity (**LI**).

Instrument	ID	Spearman rho	Kendall tau	Mann-Kendall trend
MSD2	LI	-0,323	-0,155	-
	PI	**0,610**	**0,515**	**S=70; *p*=0,0045**
	PF	-0,495	-0,347	-
	LD	-0,407	-0,279	-
	RD	0,338	0,235	-
	DD	0,513	0,376	-
	uc	**0,818**	**0,666**	**S=44; *p*=0,012**
MSD3	LI	0,120	0,118	-
	PI	**0,478**	**0,367**	**S=50; *p*=0,044**
	PF	-0,049	0,007	-
	LD	-0,209	-0,125	-
	RD	0,079	0,037	-
	DD	0,026	0,052	-
	uc	**0,763**	**0,636**	**S=35; *p*=0,0081**
MSD4	**LI**	**-0,742**	**-0,574**	**S=-78; *p*=0,0015**
	PI	**0,539**	**0,471**	**S=64; *p*=0,0094**
	PF	0,032	-0,029	-
	LD	-0,039	-0,015	-
	RD	-0,061	-0,015	-
	DD	0,424	0,323	-
	uc	**0,745**	**0,636**	**S=35; *p*=0,0081**

Table 2. Statistical evaluation of the GC/MS calibration data during 17 years. The bold and underlined values represent a statistically significant trend (α = 0.05).

Table 3 shows the results obtained for the prediction analysis. As expected, the uncertainty was the "warning parameter" because its maximum accepted value should be reached firstly that others. Being the Injector the main instrument part linked to the obtained results, the Manager may consider the possibility to replace only this part and no the all instrument.

The instruments included in this trend analysis are GC/MS single quadrupole that are used today to quantified substances with a threshold at high concentrations values (per example Salbutamol or Tetrahydrocannabinol carboxylic acid) and also, they are used for research tasks. After these results, the laboratory routine should be re-organized in order to avoid the use of the instruments at least for quantitation purposes.

	Prediction for MSD2			Prediction for MSD3		Prediction for MSD4		
	IP	DD	uc	IP	uc	IP	IL	uc
Slope	0.119	1195	1.25	0.067	0.735	0.108	-0.00092	0.869
Error	0.0353	356	0.291	0.0306	0.186	0.0454	0.000182	0.248
Intercept	-237.6	-2392700	-2512	-132	-1468	-215	2.84	-1736
Error	70.99	716330	585	61.6	376	91.3	0.365	500
r	0.658	0.655	0.806	0.492	0.795	0.524	-0.795	0.759
p	0.00407	0.00434	0.00154	0.0447	0.00345	0.0307	0.000137	0.00674
Prediction	**2022**	**2036**	**2021**	**2030**	**2025**	**2028**	**2026**	**2022**

Legend: IP: injector Precision; DD: detector drift, U: uncertainty; IL: injector linearity

Table 3. Description of the prediction line obtained for each instrument and the year when the instrument should reach the maximum acceptance criteria in the calibration process

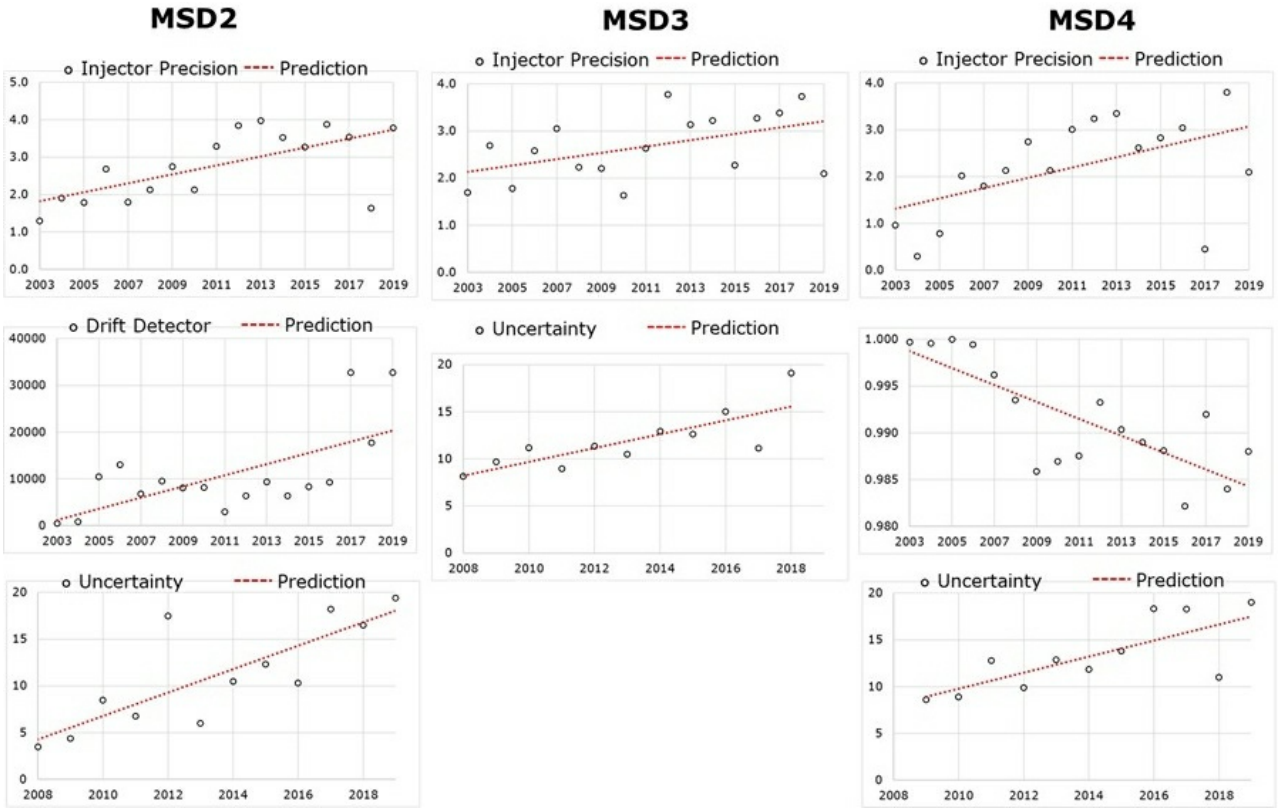

Figure 1. Prediction line obtained for the parameters that showed a statistical trend from 2003 to 2019

Conclusions

The analysis of trends using the data obtained from the calibration process showed results that could (should) be used by the Laboratory Management in order to plan and execute the budget of the laboratory. It is important to take into account that the prediction proposed here are valid as long as the same level of labor and frequency of maintenance operations are maintained. These predictions allow an adequate and prioritized budget enforcement as well as the re-organization of the tasks performed in each instrument.

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Rodriguez Fernandez A, Laffita D, López Castillo D, Terrero O, Montes de Oca R

Increase in the incidence of THC in urine samples of athletes in 2019

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Abstract

According to regulations of the World Anti-Doping Agency (WADA), the use of cannabinoids is forbidden in competition [1]. In doping controls, the detection of cannabinoid misuse is based on the analysis of the pharmacologically inactive metabolite 11-nor-Δ^9-carboxy-tetrahydrocannabinol-9-carboxylic acid (THC-COOH), and urinary concentrations greater 150 ng/mL constitute as an adverse analytical finding (AAF) [2,3]. This threshold level was only 15 ng/mL until 2013.

The objective of this study is to describe the presence of THC-COOH in samples of athletes received in the Havana Anti-doping Laboratory between 2017 and 2019. Samples were classified by sports, gender, and country, as well as by means of the collection mode In Competition (IC) and Out of Competition (OOC). In 2019, 40.0% of the collected IC samples showed concentrations between 15 and 150 ng/mL. The sports Judo, Football, Sambo and Para Table Tennis were showing concentrations higher than 150 ng/mL. Three of these samples were reported as Adverse Analytical Finding after adjusting the decision limits by specific gravity. In the same year (2019), OOC samples collected from eight different sports showed THC-COOH levels above 150 ng/mL. 18.9% of these samples came from Chile. This data show the increase of THC-COOH consumption during 2019 compared to 2017 and 2018.

Introduction

Tetrahydrocannabinol (THC), also known as Δ^9-tetrahydrocannabinol, is the main psychoactive constituent of cannabis [3,4]. THC is one of the substances of the S8 cannabinoid group that is prohibited in competition; the threshold level of the relvant analyte THC-COOH is 150 ng/mL [1,2]. Years ago this level was only 15 ng/mL. However, in May 2013, the WADA Executive Committee decided to increase the threshold level after considering the numerous presentations received from interested parties during the Code review process. Cannabis data from all sources has been analyzed for the potential benefits and hazards for athletes related to the abuse of cannabinoids for the purpose of doping in sports [3].

The objective of this study was to describe the presence of THC-COOH in samples of athletes between 2017 and 2019 and the increase in consumption in the last year.

Experimental

Data: A total of 13328 urine samples were processed between 2017 and 2019. Samples were collected according to the Initial Testing Procedure which is applied to all doping control samples in our laboratory in Havana.

Sample preparation was done as described by Martínez-Brito [5]. Briefly, 2.5 mL of urine were hydrolyzed with β-glucuronidase, after previous addition of ISTD and pH correction to 7. Afterwards,

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liquid-liquid extraction using *tert*-butylmethyl-ether was done. Then, TMS derivatives were analyzed using an Agilent 7890B gas chromatograph coupled to a mass spectrometer triple quadrupole Agilent 7000. One quality control was added in each analysis batch.

Reagents: THC-COOH was supplied by Cerilliant. All reagents and solvents (sodium bicarbonate, potassium carbonate, sodium phosphate, sodium hydrogen phosphate) were of analytical grade. *Tert*-butylmethyl-ether was HPLC grade. β-glucuronidase (*Escherichia coli* K12) from Roche Diagnostic (Mannheim, Germany). Mercaptoethanol, Ammonium Iodide and MSTFA were purchase by Sigma-Aldrich.

Results and Discussion

From all processed samples between 2017 and 2019, 1.9% showed the presence of THC-COOH in 2017, 1.8% in 2018 and 2.8% in 2019, showing an increase during the last year. The higher incidence of this substance was observed in males with 81.2%. These findings results from the screening and identification was done using a positive THC-COOH control. If the THC-COOH concentration, estimated with the positive control, is greater than 75 ng/mL (50% of the threshold), THC-COOH was quantified with three new aliquots of the sample.

If we compare the presence of THC-COOH among sports in 2019, an increase was seen in some of them (Figure 1). Weight lifting, Judo, Athletics, and Tae-Kwon-Do were those with the highest levels.

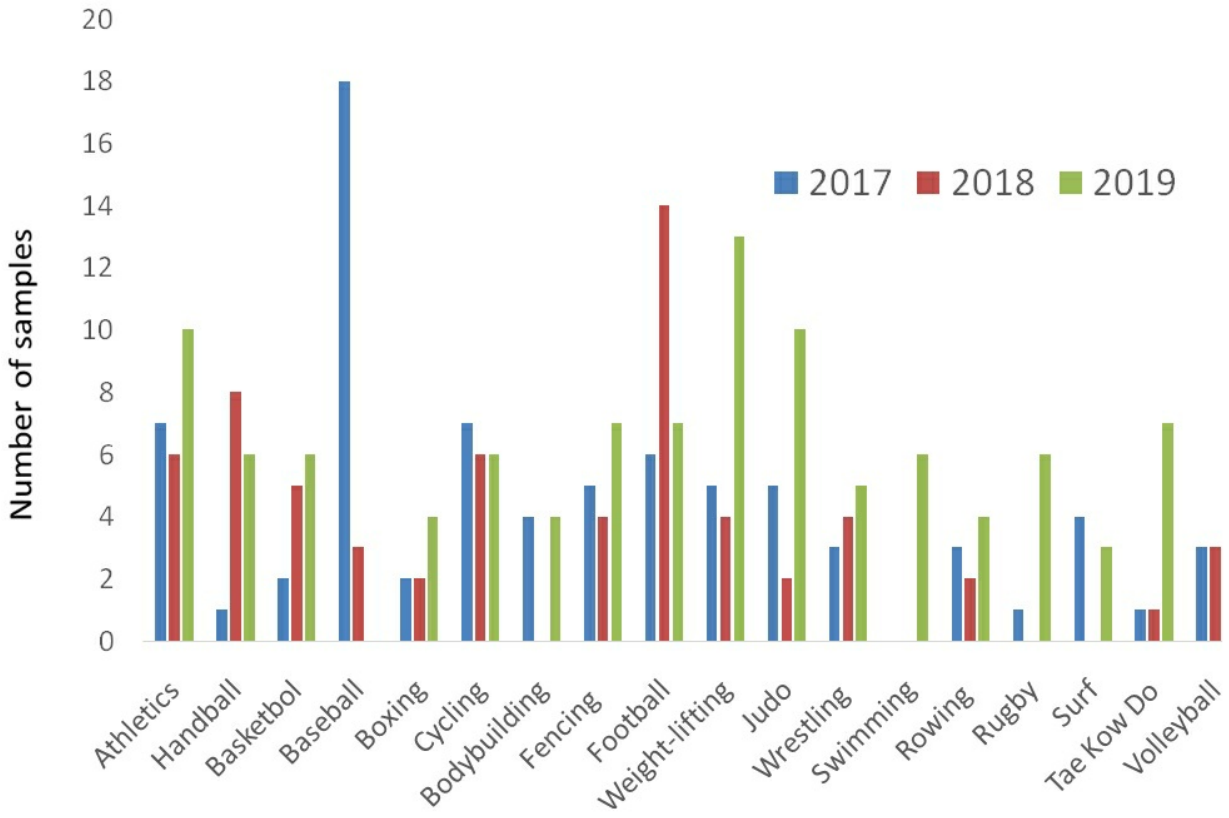

Figure 1. Sports with a greater number of samples where the presence of THC-COOH was detected (2017-2019)

In 2019, there was an increase of IC and OOC samples with THC-COOH concentration levels above 15 ng/mL (Figure 2). From the IC samples showing THC-COOH, 55.7% had a concentration higher than 15 ng/mL. This value was only 39.1% in 2018 and 26.8% in 2017. Out of 61 IC samples with presence of THC-COOH in 2019, 29 showed concentration levels between 15 and 150 ng/mL, representing 47.5%. In this study, concentration ranges of 15 ng/mL, 15-150 ng/mL and more than 150 ng/mL were determined, since the quantification thresholds set by WADA before and after 2013 were 15 ng/mL and 150 ng/mL.

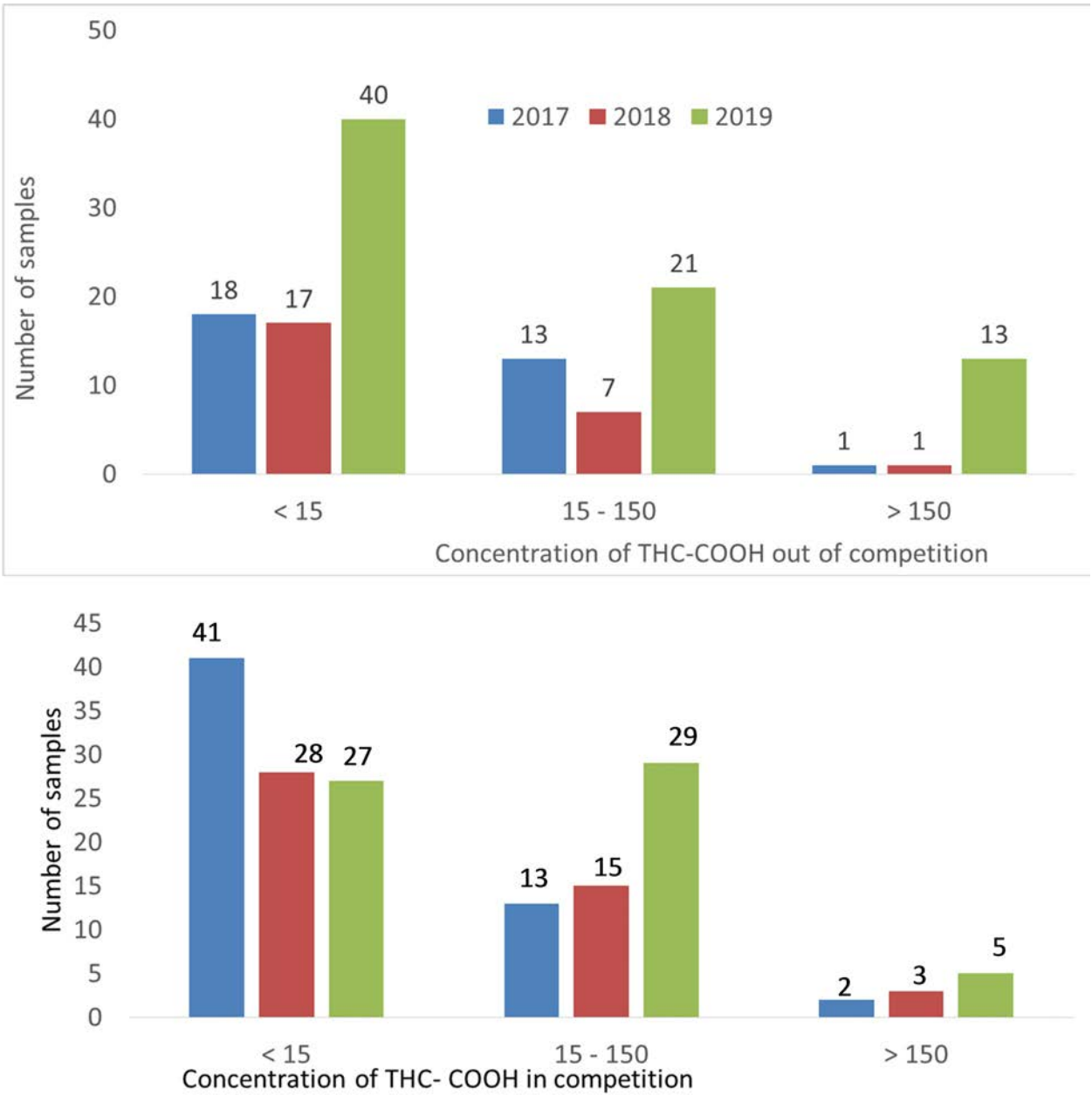

Figure 2. Representation for years of the concentration of THC-COOH detected by number of samples

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In 2019, Athletics, Basketball, Fencing, Weight lifting and Volleyball had more OOC samples with concentration levels of THC-COOH above 15 ng/mL. On the other hand, Athletics, Football, Judo and Weight lifting were the sports with the highest incidence of this indicator for IC testing controls (Figure 3). After the adjustment of the decision limits for the specific gravity, the Adverse Analytical Findings reported in 2019 correspond to Sambo (219.8 ng/mL), Football (790.0 ng/mL) and Para Table tennis (1456.0 ng/mL).

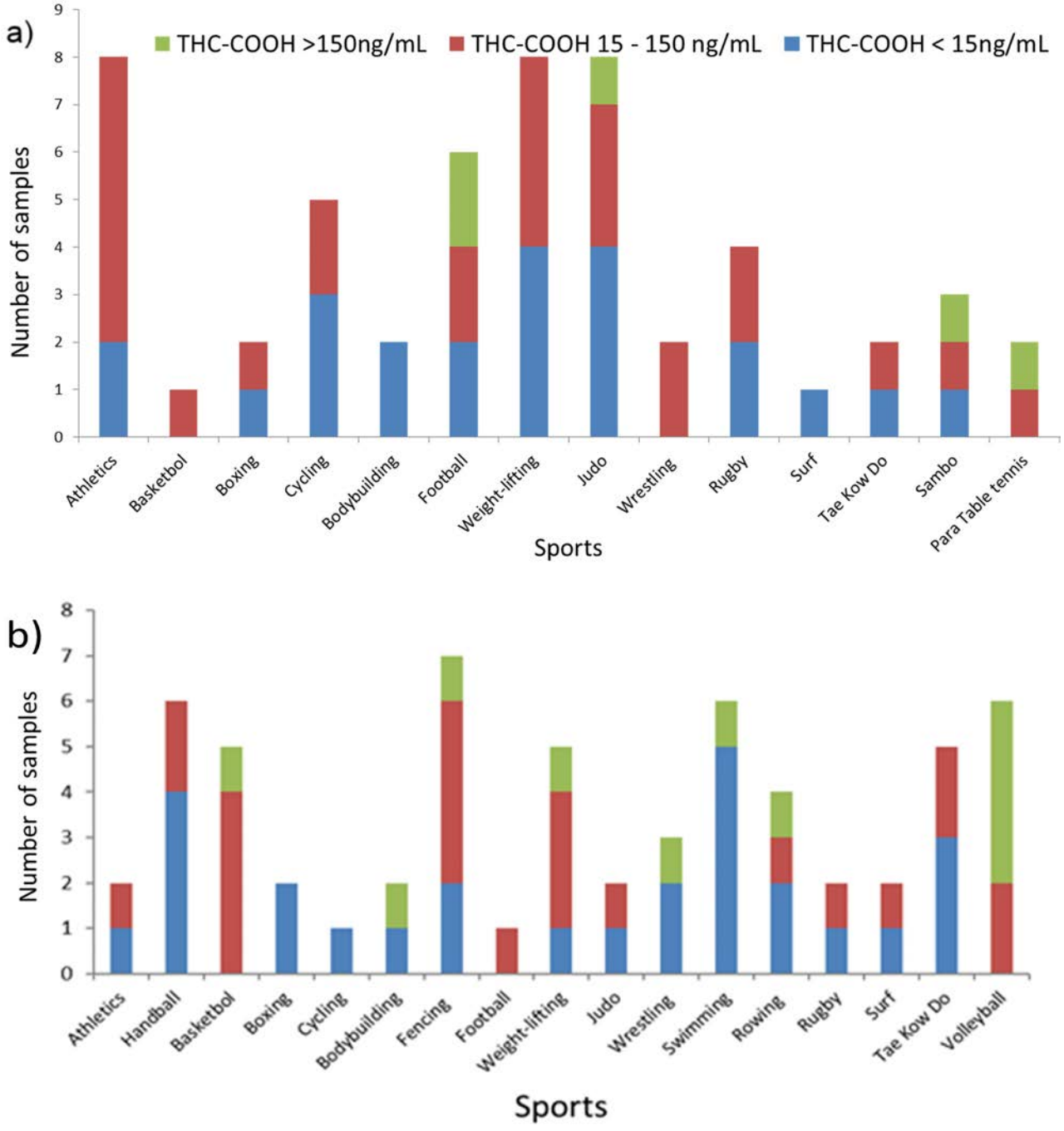

Figure 3. Sports with the highest incidence of THC-COOH in samples collected IC (a) and OOC (b) in 2019

In 2019, the countries with the highest presence of THC-COOH in the OOC controls were Chile (26 out of 137 analyzed), Mexico (23 out of 1019 analyzed) and Cuba (8 out of 593 analyzed) (Figure 4).

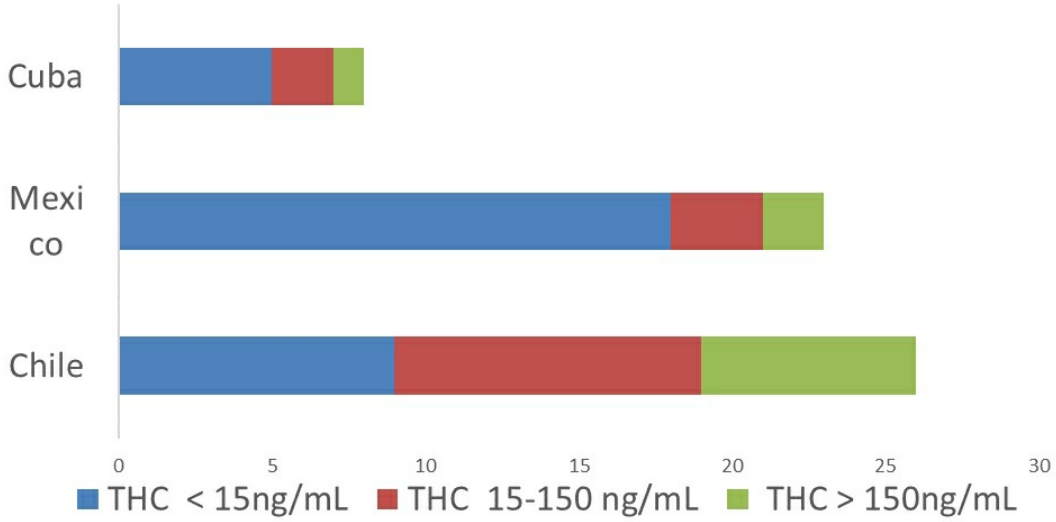

Figure 4. Countries with the highest presence of THC-COOH in the controls OOC in 2019

Conclusions

In 2019, the presence of THC-COOH increased in the total samples received. Compared to previous years, 70% of sports showed an increase in THC-COOH detection. In 2019, IC samples from Judo, Football, Sambo and Para Table Tennis athletes were analyzed with THC-COOH levels higher than 150 ng/mL. Three of them were reported as Adverse Analytical Finding. During the same year, OOC samples were positive for THC-COOH levels higher than 150 ng/mL in 8 sports. Chile shows the highest presence of THC-COOH in OOC samples collected in 2019, representing 18.9 % of the samples received.

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Biasini GM, Donati F, Rutigliano L, de La Torre X, Botrè F

Proteomic analysis of biomarkers of red blood cells storage: towards the development of direct detection of autologous blood transfusion

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Abstract

In recent years one of the greatest challenges for antidoping in sports has been to develop a direct method to detect the misuse of autologous blood transfusions (ABT) that can be tempted by cheating athletes to increase oxygen delivery to muscles so enhancing sport performance. Despite being prohibited by the World Anti-Doping Agency (WADA), there is currently no direct method to tackle ABT doping strategies. Storage of erythrocytes, under standard blood banking conditions, for transfusion purpose, induces biochemical and physiological changes that affect red blood cells (RBCs) shape, proteome, metabolism as well as affecting oxidative stress. At the protein level, alterations of the morphological structure of erythrocytes involve Band 3, the most abundant RBC transmembrane protein that plays a key role in membrane integrity and cytoskeletal organization. Moreover, loss of the oxidant-antioxidant balance mostly depends on Peroxiredoxin-2 (PRDX2), one of the primary RBC antioxidant enzymes whose effect is to limit hydrogen peroxide (H_2O_2)-mediated toxicity. The aim of the present project is to evaluate alterations occurring in RBC proteome during storage, comparing their proteome before and after different periods of time, under standard blood banking conditions. In this study, it has been demonstrated that levels of Band 3 decrease over storage time, highlighting its role in the structural and morphological organization of RBCs; moreover, levels of PRDX2 are shown to considerably increase over storage time with the consequence of the increased oxidative stress in erythrocytes.

Introduction

ABT is a banned method by the WADA [1] and consists of the withdrawal of a sufficient volume of blood from an individual, its storage, and its reinfusion. During storage, under standard blood banking conditions, erythrocytes undergo several modifications: loss of shape and membrane integrity [2,3], increased oxidative stress [4] and changes in metabolome and proteome [5]. Phenomena of aggregation and degradation occur to proteins over time, representing a "signature" of RBCs storage. We selected two new biomarkers: Band 3, a transmembrane protein of RBCs involved in cytoskeleton organization [6,7], and PRDX2, the main erythrocytes peroxidase [8,9], to evaluate their levels before and after different periods of storage, to assess the suitability of these biomarkers for the development of a direct method for ABT detection.

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Experimental

Preparation of samples

Whole blood samples were analyzed fresh at T0 and divided into two aliquots. Aliquoted samples were stored at 4°C in K_2EDTA tubes and stained for flow cytometry (FC) and western blotting (WB) applications after 20 (T1) and 40 days (T2). For the WB assay, blood samples (n=2) were washed twice with phosphate buffer (PB). Pellet was resuspended in N-ethylmaleimide (NEM) 0.1 M in PB solution and incubated for 15 minutes at RT. After incubation, 0.1 M of NEM in PB/2% cOmplete Protease Inhibitor Cocktail (Roche, Switzerland) solution was added to samples, and cells were lysed mechanically through a glass homogenizer. Samples were washed twice, and the pellet was resuspended in phosphate-buffered saline (PBS). Protein concentration was estimated through Bradford assay. Samples were denaturated at 70°C for 10 minutes.

SDS-PAGE and Western Blotting

Denatured protein samples (n=2) were analyzed through SDS-PAGE. 5 µg or 300 ng of each sample, respectively for PRDX2 and Band 3 analysis, were loaded in the gel (NuPAGE 4-12% Bis-Tris Gel, Novex-life technologies). Band 3 and PRDX2 standards were loaded as controls. The WB analyses were performed on PVDF membranes against PRDX2 (Anti-h/m/r Peroxiredoxin2 at 1/1600), Band 3 (SLC4A1 Monoclonal Antibody-BIII136 at 1/100000). The membrane was incubated with the secondary antibody (Gt anti-MS IgG (H+L) cross-adsorbed secondary antibody, HRP-conjugate) for 90 minutes. Images were acquired through the ImageQuant LAS4000. Bands of interest were quantified by densitometry by means of the GASepo software.

Flow Cytofluorimeter assay

Whole blood samples (n=5) were washed twice with saline. Band 3 antibody (CD233, Band 3 extra-cellular domain) was added to samples and incubated for 45 minutes. Samples were washed twice to remove the excess of antibody. Glycophorin A (GYPA) antibody as control was added and incubated for 30 minutes. Samples were rewashed to allow FC acquisition (FC500 Beckman Coulter).

Results and Discussion

Our results demonstrated a trend towards the increase of PRDX2 protein amount during storage, while β-actin, the normalizing control, remains stable over storage conditions. Levels of PRDX2 increased progressively after 20 days ($p=0.09$) and after 40 days ($p=0.07$) of storage compared to freshly collected blood samples.

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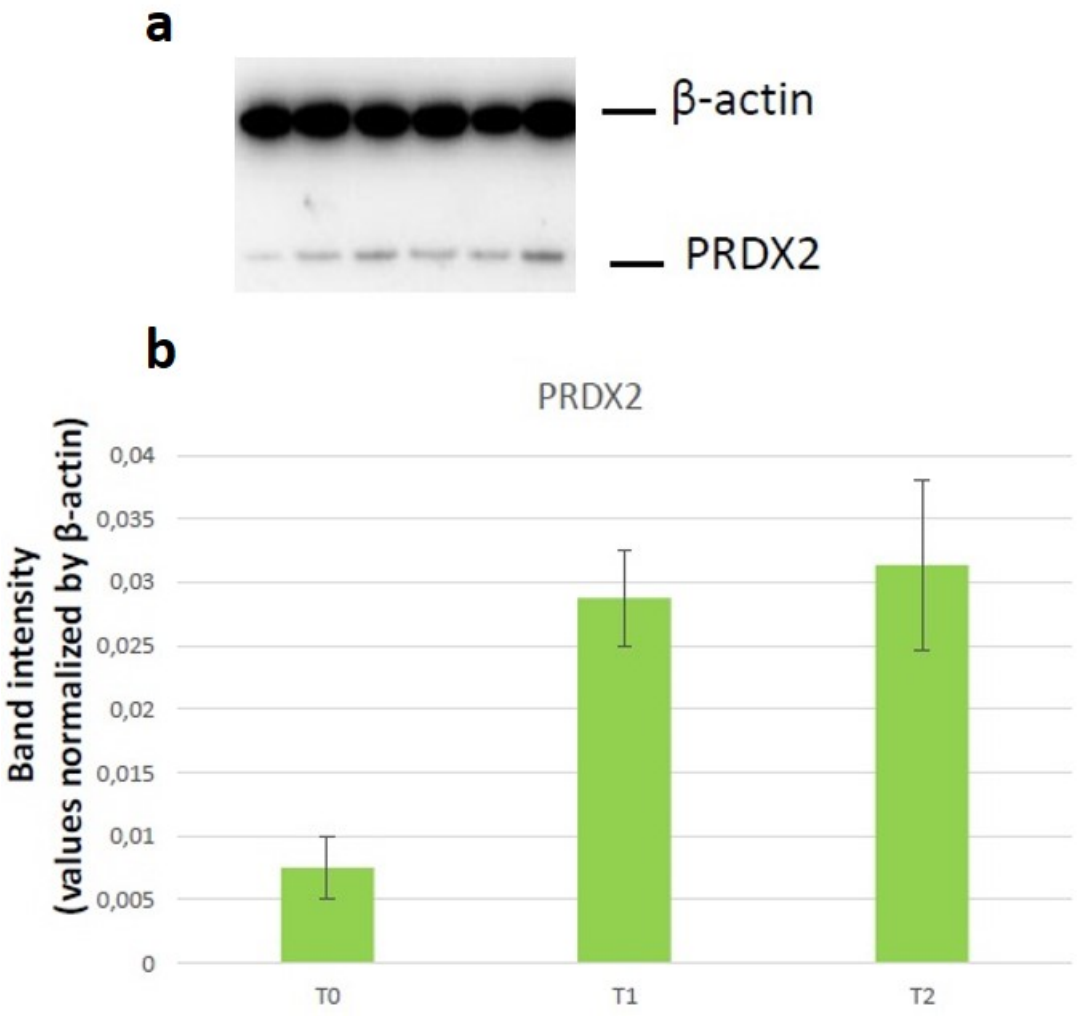

Figure 1a and b. The amount of PRDX2, present in erythrocytes, changes during storage. (**a**) Representative western immunoblots analysis of blood samples against PRDX2 and β-actin (**b**) Different levels of PRDX2 after different periods of storage (T1=20 days, T2=40 days) normalized with levels of β-actin, the loading control. PRDX2 levels progressively increase after 20 days (p=0.09) and after 40 days (p=0.07).

The storage causes damages to the structure of PRDX2 that aggregates in high molecular weight complexes migrating from the cytosol to the membrane of the erythrocytes and, consequently, it becomes more detectable in RBCs membranes [9]. In parallel, there is a trend towards the decrease of Band 3 levels over storage time. The total amount of Band 3 decreases progressively after 20 days (p=0.99) and after 40 days (p=0.62) of storage compared to freshly collected blood samples.

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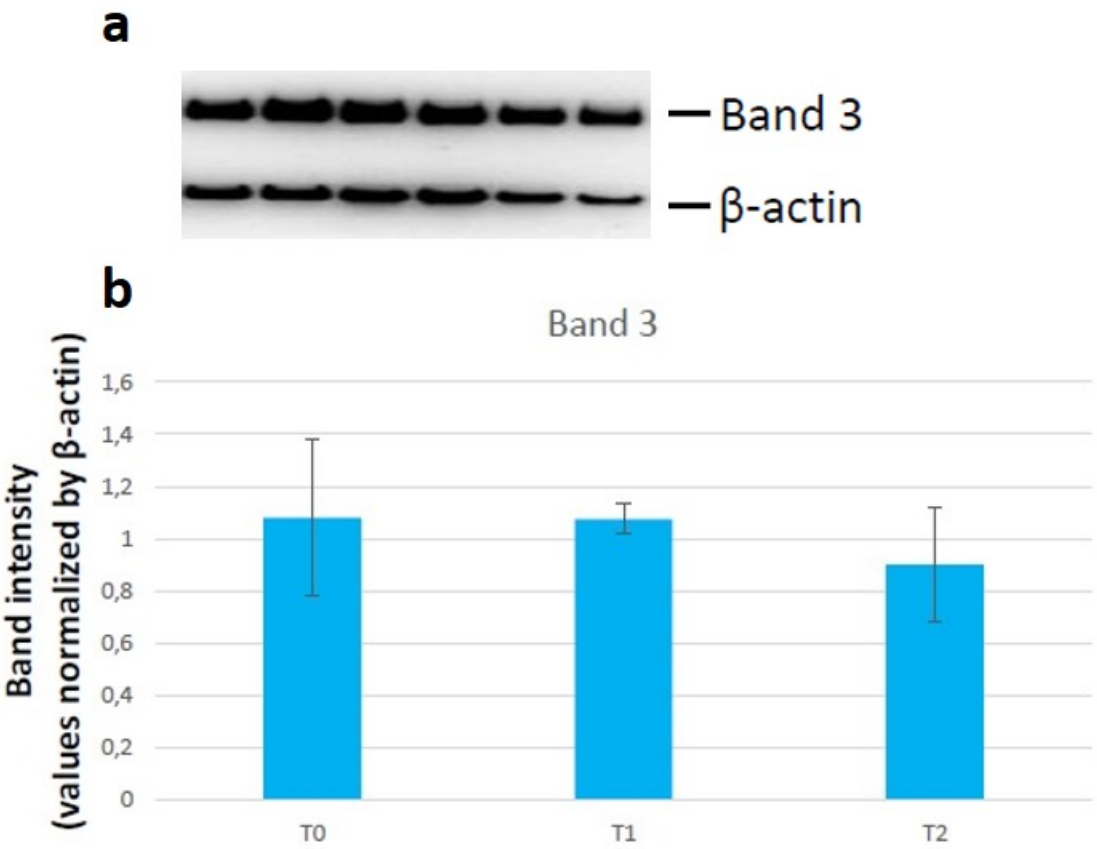

Figure 2a and b. The amount of Band 3, the major integral RBCs protein, changes during storage. (**a**) Representative western immunoblots analysis of blood samples against Band 3 and β-actin. (**b**) Different levels of Band 3 after different periods of storage (T1=20 days, T2=40 days) normalized with levels of β-actin, the loading control. Band 3 levels progressively decrease after 20 days (p=0.99) and after 40 days (p=0.62).

To better assess the alterations that occur to Band 3 due to the storage of RBCs, we also conducted a dual-color staining FC assay. Figure 3a, b, and c shows the progressive decrease in both Band 3, GYPA levels, and their ratio during storage. As we can see, the levels of Band 3 decrease over storage time with a percentage of 19% after 20 days ($p=0.06$) and 39% after 40 days ($p<0.01$) compared to fresh blood samples. However, GYPA levels also decreased due to storage compared to non-stored samples ($p<0.001$), probably due to the reduced size of erythrocytes during storage. The promising result is the progressive decrease of their ratio: Band 3/GYPA levels decreased by 32% ($p=0.02$) and 37% ($p=0.01$) of their value compared to freshly collected blood samples, after 20 and 40 days, respectively. Our results demonstrate that the altered morphology of stored erythrocytes can depend on the altered expression of Band 3, responsible for both membrane and cytoskeletal organization.

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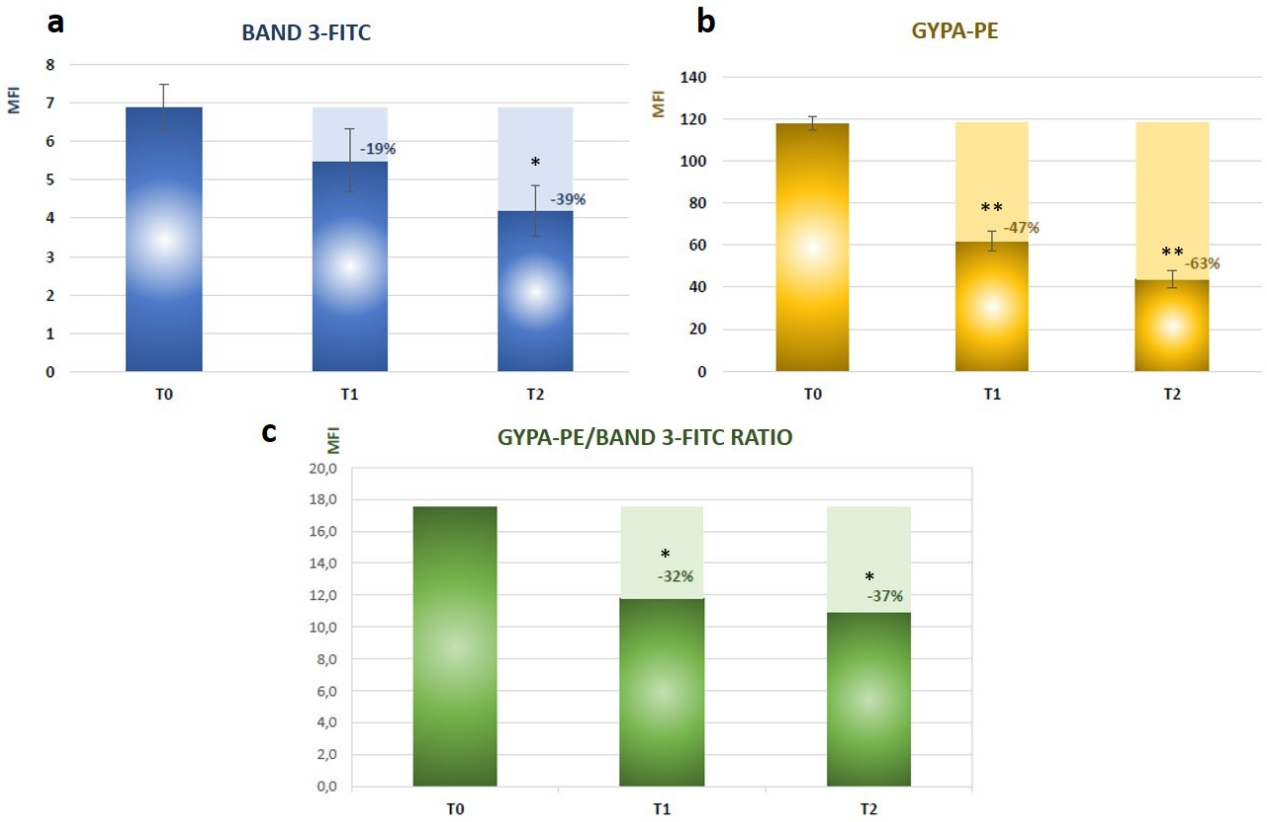

Figure 3a, b and c. The amount of Band 3 and GYPA changes during storage. (**a**) Levels of Band 3 decrease by 19% after 20 days of storage (p=0,06) and by 39% (p<0,01) after 40 days. (**b**) The total amount of GYPA decreases by 47% after 20 days (p<0,001) and by 63% after 40 days (p<0,001) of storage. (**c**) GYPA/Band 3 ratio decreases by 32% after 20 days (p=0,02) and by 37% after 40 days (p=0,01) of storage.

Conclusions

The results obtained in this study allowed to clarify many changes in the proteome of RBCs during blood storage in blood bank simulated conditions that can be interesting to develop a direct method for the detection of ABT. From the data we obtained the following conclusions can be drawn:

1. Band 3 levels progressively decrease during storage due to degradation processes [7]
2. PRDX2 levels increase during storage due to the aggregation of the protein in macrocomplexes [9]
3. Band 3 and PRDX2 can be considered two promising biomarkers for storage to develop a direct method for ABT detection
4. The pool of biomarkers of storage needs to be implemented with more proteins to further improve the results

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Donati F, Botrè F, de La Torre X, Sian V, Biasini GM, Concetti L, Rutigliano L

Evaluation of the impact of genetic polymorphisms on basal values of human serum and urinary biomarkers: implications for anti-doping analyses

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Abstract

The aim of this work is to assess whether and to what extent human genetic polymorphisms can affect the results of both direct and indirect doping analyzes, with particular reference to those markers that require the quantitative determination in order to evaluate whether they exceed the population or individual threshold. In anti-doping research field, the presence of polymorphisms involves a series of questions regarding the genetic influence on the susceptibility to both screening and confirmation analyzes of prohibited substances. Single Nucleotide Polymorphisms (SNP) and Copy Number Variation (CNV) of genes of particular interest in anti-doping context were selected for the scope and DNA was extracted both from urinary or blood matrix. The results of the individual genotyping have been utilized in order to determine the correlation with the basal values of biomarkers used for the anti-doping methods.

Introduction

The individual response variability, due to genetic polymorphisms, can significantly affect the determination of the value of specific biomarkers, and the extent of the variation could lead to errors in the interpretation of the experimental data, with the risk of both "false-negative" and "false-positive" results [2]. Among the most common and impacting categories of genetic polymorphisms there are single nucleotide polymorphisms (SNP) and variations in the number of gene copies (Copy Number Variation, CNV). Both types of polymorphisms can determine a reduction or an increase in the expression of a structural protein or a certain enzyme, causing a specific cellular activity to be increased, decreased or silenced [3-4]. Improving the knowledge about the impact of the presence or absence of a specific polymorphism on biochemical pathways involved in drug and/or biomarker metabolism is an essential step that helps to better understand the results of doping tests in sport. Several polymorphisms having an impact on the interpretation on doping test results have already been identified and are reviewed in Kuuranne et al [1]. Here we aim to verify these results as well as investigating some SNPs possibly influencing the tests used to detect doping with human growth hormone and IGF-1. Moreover, we have investigated genetic variants of biomarkers (specifically BDNF and proBDNF) of a new practice feared to be abused by athletes - namely transcranial direct current stimulation (tDCS)[5].

Experimental

SNP and CNV polymorphisms selected for the aim of this work are described in Figure 1 as well as the

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number of athlete samples analyzed for each genetic system. All the samples analyzed belong to healthy athletes subjected to routine anti-doping controls who have expressed their consent that their biological sample can be used anonymously at the end of the routine analyzes. Urinary samples were used for UGT2B15, UGT2B17 and SULT2A1 comparisons, while serum samples were used for the association with GHR, IGF1 and BDNF polymorphism genes. The study concerning the association of blood values with GH and IGF1 polymorphisms was performed exclusively on male samples to minimize the baseline variability due to intersex differences.

SNP ID	Gene	SNP	Type	Target	Consequence	Sample N.	Wildtype %	Heterozygous %	Full Polymorphic %	Literature Allele fr.
rs1902023	UGT2B15	G>T	Transversion	Phase II metabolism glucuronidation	Missense variant	71	27	52	21	T=0.45 G=0.55
rs7703713	GHR	G>A	Transition	Sensitivity to Growth factor	Intron variant	35	49	48	3	A=0.32 G=0.68
rs6873545	GHR	T>C	Transition	Sensitivity to Growth factor	Linked to GHR exon 3 deletion	40	8	35	57	C=0.31 T=0.69
rs1520220	IGF1	C>G	Transversion	Cell growth	Intron variant	45	58	40	2	G=0.32 C=0.68
rs11030099	BDNF-AS	C>A	Transversion	Neurons growth	3'UTR miR26a target site	91	9	25	66	A=0.23 C=0.77
rs11030100	BDNF-AS	G>T	Transversion	Neurons growth	3'UTR miR26b target site	91	66	25	9	T=0.23 G=0.77
rs6265	BDNF-AS	G>A	Transversion	Neurons growth	Missense variation proBDNF sequence	91	66	25	9	A=0.20 G=0.80

CNv ID	Gene	Target	Sample N.	CNV 0 copies fr.	CNV 1 copy fr.	CNV 2 copies fr.
Hs03185327	UGT2B17	Phase II metabolism glucuronidation	48	10.4	43.8	45.8
Hs03013147	SULT2A1	Phase II metabolism sulfation	48	6.3	33.3	60.4

Figure 1. SNP and CNV genetic polymorphisms selected for the aim of this work and their allele frequencies. Global reference allele frequencies were obtained from "1000 genome human variation catalogue" www.internationalgenome.org

Genomic DNA (gDNA) was extracted from urine/whole blood samples by using a PrepFiler DNA extraction kit (Thermo Fisher, Waltham MA, US). Absolute quantification of gDNA is made on a standard reference curve by using the **"**DNA Quantifiler" kit and real time qPCR 7500 fast instrument (Thermo Fisher, Waltham MA, US). Determination of Copy Number Variation (CNV) was performed by a sensitive specific TaqMan assay (Thermo Fisher, Waltham MA, US) on a real timePCR 7500 fast by the relative quantification method using RNAase P as endogenous reference assay and standard reference DNA as calibrators. CNV genotyping is determined by Copy Caller software (ThermoFisher, Waltham MA, US).

SNP polymorphisms were detected by a sensitive TaqMan SNP genotyping assay (VIC/FAM probes) on a real time qPCR 7500 fast (Thermo Fisher, Waltham MA, US). Each genotyping analysis was carried out using control DNAs with known genotype (one for each of the three types of possible polymorphisms: homozygous wildtype, heterozygous, homozygous full polymorphic). Reference standard DNA was purchased from the Coriell institute for medical research, Camden, NJ, USA)

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Although DNA extracted from urine is generally of lower quality than DNA extracted from blood matrix (mainly due to degradation phenomena), no lower quality typing results were recorded than those performed from DNA obtained from blood matrix since both the dna extracted from urine and the dna extracted from blood gave similar and reproducible results in terms of fluorescence emitted by the taqman probes. Urinary steroidal markers are detected and quantified as glucurono-conjugates or sulfate conjugates after enzymatic hydrolisis with β-glucuronidase from E.Coli and chemical hidrolysis with a mixture of etylacetate/methanol and sulphuric acid respectively and LL extraction on a Agilent 7890A/7000 triple quadrupole MS. Recombinant and Pituitary GH were determined by Wada approved luminescent assay (CMZ) on a Berthold lLB953 luminometer. Serum IGF1 were determined IRMA radioimmunoassay (Immunotech) on a Wizard2 Gamma Counter (Perkin Elmer) Serum BDNF and ProBDNF were analyzed by conventional «sandwich» Elisa kit (Aviscera Biosciences) on a Victor3 plate reader (Perkin Elmer). Statistical differences among groups were determined by conventional independent t-test.

Results and Discussion

Effect of polymorphisms on Phase II glucuronidation and sulfation of steroids

Figure 1 reports the genotyping results fron this study. The T/E glucuronide ratio resulted mainly affected by the UGT2B17 CNV polymorphism. This result is in line to what was observed in previous studies [2]. On the other hand, SNP of the UGT2B15 gene impacted on Andro/Etio glucuronide ratio principally (A/Etio ratio was 1.7, 1.4 and 1.3 for wildtype, heterozygous and full polymorphic samples respectively with statistical significance of $P<0.05$). The reduction of the Andro/Etio ratio due to the full polymorphic condition is not of such a scale to suspect the presence of the inhibitor(s) of 5α-reductase. The lowest values of the ratio Andro/Etio are observed in those subjects who are full polymorphic for both UGT2B17 and UGT2B15 genes (A/Etio = 0.9 in the full polymorphic vs. 1.8 of the wild types with $P<0.05$). Subjects carrying UGT2B17 del/del genotype may result negative for doping compared to the subjects carrying the UGT2B17 ins/ins genotype when an equal dose of exogenous testosterone is administered. Excretion of sulfate conjugated substances seems not so clearly correlated with the CNV's genetic polymorphism SULT2A1 except for testosterone sulfate. This difference is observable in the ratio of testosterone/ epitestosterone sulfate excretion. Individuals carrying 2 copies of SULT2A1 do not appear to have a greater excretion compared to subjects having one copy of the gene. However, to carry 0 copies may, in some cases, be responsible for a lesser excretion: that is the case of testosterone, but also of 5-androstene-3β,17β-diol and the T/E ratio. In previous literature studies, urinary excretion of steroids has always been studied in relation to genetic polymorphism of a single gene and multiple genetic polymorphisms. Particularly, in this study, individuals with complete deletion of the SULT2A1 gene were reported and no individuals carrying 3 copies of the gene were found, a circumstance that is different with what is known from previous literature studies [5].

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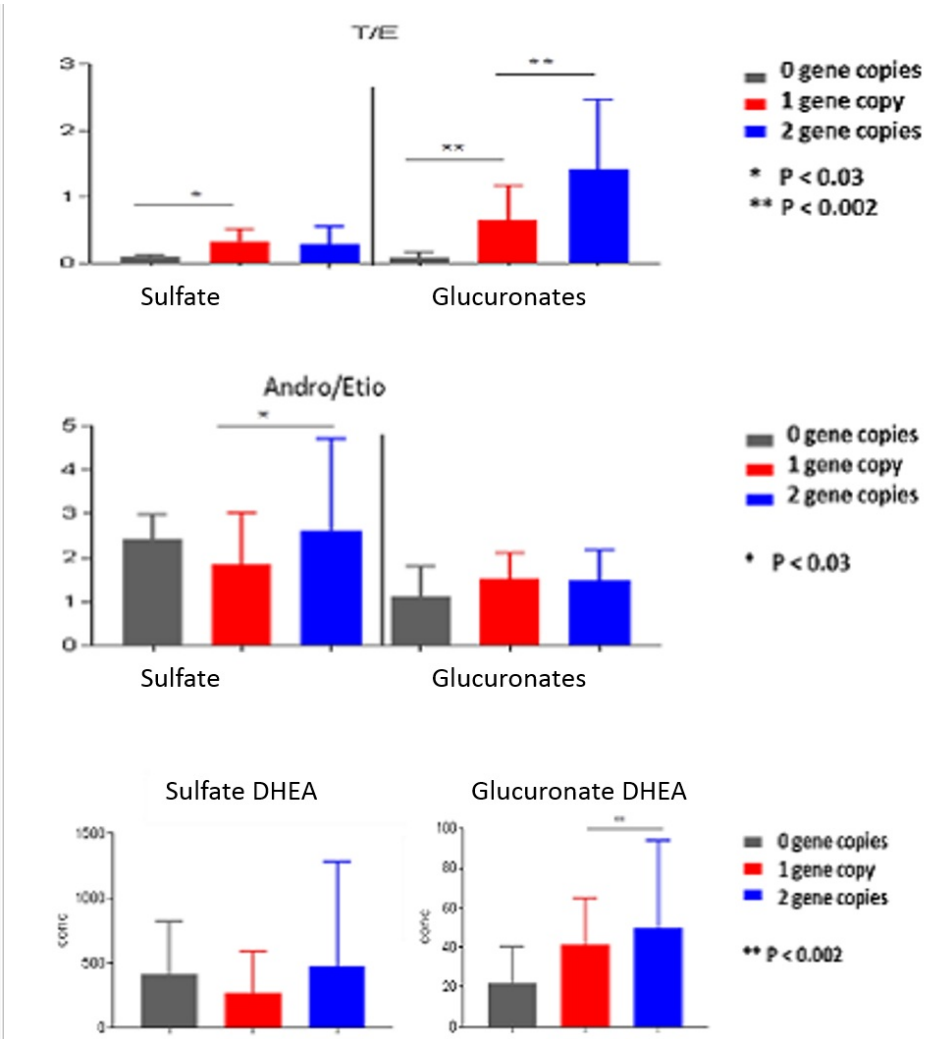

Figure 2. Urinary T/E, Andro/Etio and Sulfate/Glucuronate DHEA in reference to CNV UGT2B17 and SULT2A1genetic polymorphisms in 48 healthy athletes (14 males, 34 females, SULT2A1 N=3, 16 and 29 for 0, 1 and 2 gene copies respectively, UGT2B17 N=5, 21 and 22 for 0, 1 and 2 gene copies respectively)

Serum BDNF and proBDNF

Brain Derived Neurotrophic Factor (BDNF) and its precursor proBDNF are endogenous biomarkers directly related to Transcranial direct current stimulation (tDCS)[6]. tDCS is a brand new practice that is feared to be abused by athletes to improve sports performances by its proved effects on enhancing muscular strength, reducing fatigue and increasing endurance time. Variables affecting the basal serum values of BDNF and proBDNF (such as gender, age, ethnicity, nutrition, genetic polymorphisms) must to be taken into account in the attempt to properly set a threshold for tDCS abuse. The three major SNP polymorphisms here investigated (rs6265, rs11030099, rs11030100) resulted linked together as showed by their relative frequencies and despite their exert effects on different site of the BDNF precursor. SNP rs11030099 and rs11030100 may determine the presence of a microRNA cleavage site on the BDNF mRNA thus affecting its translatability and therefore the final resulting BDNF concentration in serum. Genetic haplotype analysis revealed that serum proBDNF resulted lower when miR26a or miR26b can target the recognition site on 3'-UTR of precursor depending of the presence of a polymorphism on rs11030099 or rs11030100 but not contemporary (that would completely cancel the synergic action of

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miRs). Curiously, not the same pattern has been observed for BDNF so suggesting that proBDNF and BDNF follow two different dynamics when they are in the systemic circulation (Figure 3).

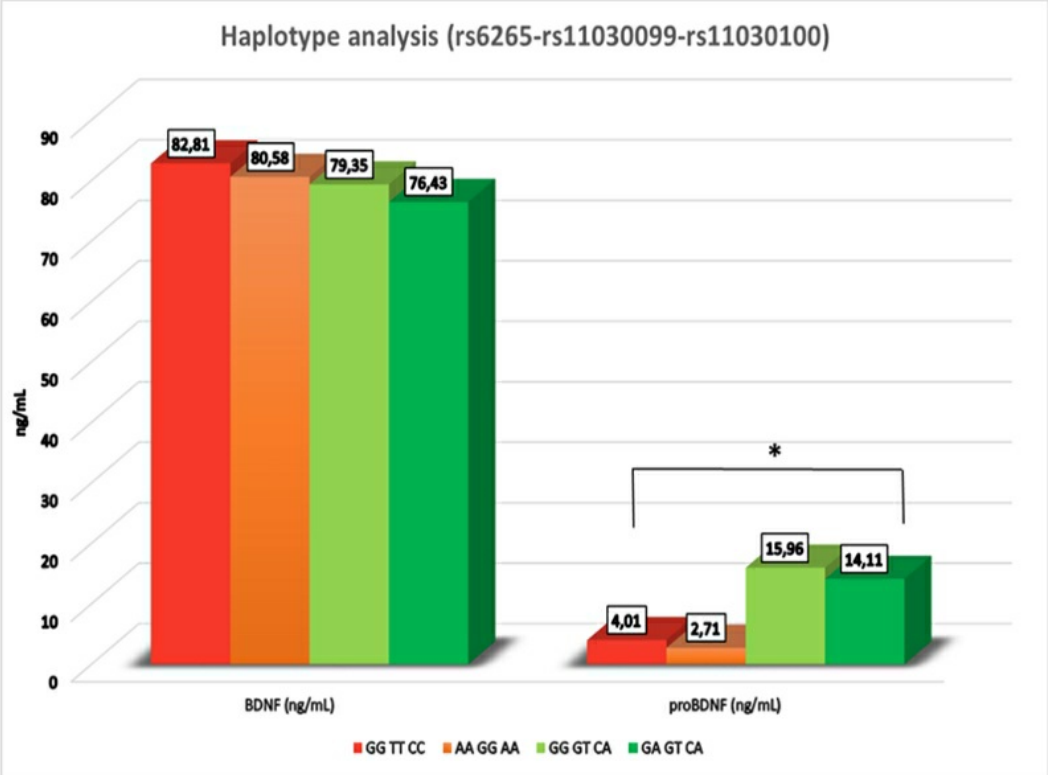

Figure 3. Basal serum proBDNF and BDNF levels in reference to the most significant and frequent SNP haplotype rs6265/rs11030099/rs11030100, GGTTCC N=51, AAGGAA N=8, GGGTCA N=6, GAGTCA N=21)

Serum hGH Biomarkers

SNP polymorphisms rs1520220 located in the IGF1 gene on human chromosome 12 determines effects on basal serum IGF1 concentration only. Individuals carrying the polymorphism in homozygous form (CC) show levels of serum IGF1 lower than GG or GC genotypes. The observation is corroborated by statistical significance (refer to Figure 4). SNP IGF1 Polymorphism rs1520220 seems not to have effect at the heterozygous level. Two SNP polymorphisms of the human Growth Hormone Receptor (GHR) exert their effects on the basal Rec/Pit Ratio that is currently used as threshold of Recombinant GH abuse in sport doping. SNP rs6873545, in his full polymorphic form (CC) is linked to the Exon 3 deletion of the human GHR and SNP rs7703713 is an intronic variant of the GHR itself. GHR exon 3 deletion seems to lower basal Rec/Pit ratio only in the full polymorphic form (CC) while SNP rs7703713 determines lowering of basal Rec/Pit ratio in the heterozygous form already. No fully polymorphic individuals were detected for this SNP (Figure 4). As far as we know, this is the first study to investigate the variability in serum of the basal values of the two natural isoforms of GH and I the IGF1, both of which are used in anti-doping investigations as direct and indirect markers of recombinant GH abuse for doping purposes. The results obtained here indicate that the underlying inter-individual variability due to these polymorphisms should be taken into consideration as an additional factor in determining the population cutoffs that are routinely used to discriminate abuse.

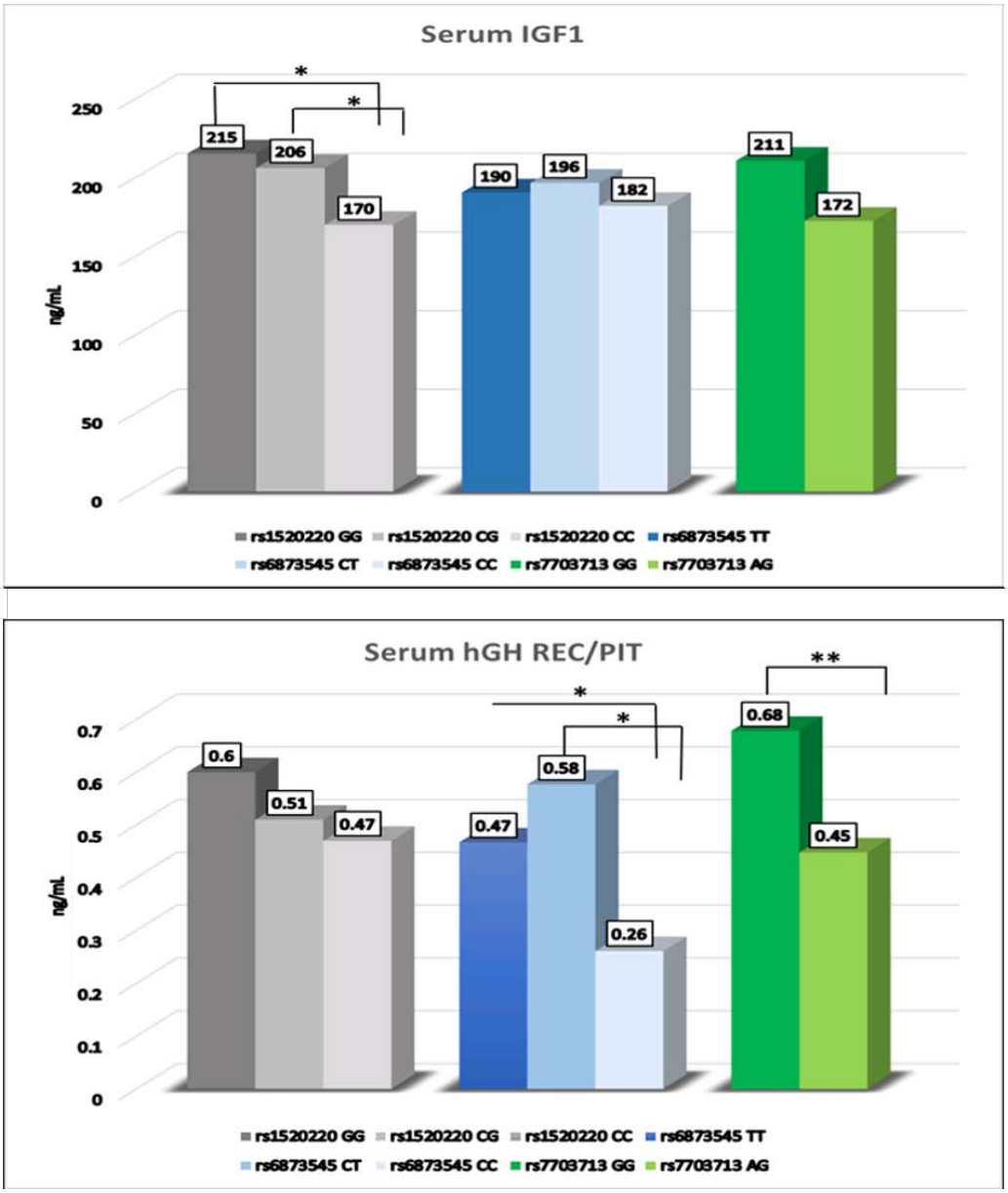

Figure 4. Athlete basal IGF1 serum levels in reference to SNP rs1520220 (up) and basal GH Rec/Pit ratio in reference to SNPs rs6873545 and rs7703713

Conclusions

SNP and CNV genetic polymorphisms may affect the basal values of endogenous biomarkers and metabolites relevant for the anti-doping analyzes as well as the different outcomes due to drugs administration. In doping control, genotyping of SNP and CNV polymorphisms may help in, 1.) the evaluation of the test results for the detection of the abuse of exogenous substances, 2.) the evaluation of markers related to all three modules of the athlete biological passport and 3.) the setting of more reliable and accurate population thresholds of abuse for endogenous compounds. Accurate and sensitive genotyping screening procedures need to be implemented to full investigate the impact of relevant polymorphisms and to improve the effectiveness of anti-doping analyzes.

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Detection of homologous blood transfused erythrocytes by means of a two color fluorescence flow cytometric analysis

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Abstract

Homologous blood transfusion (HBT) is a traditional prohibited method to increase the hemoglobin level of athletes. The analysis of red blood cell (RBC) surface antigen expression profiles by flow cytometry allows to reveal the mixed RBC population, which was developed in 2008. Although HBT prevalence has been considered recently as fairly low compared to autologous blood transfusion, testing is still needed to prevent athletes to return to this "old" doping practice. Indeed, the number of adverse analytical findings for HBT has increased recently. In this study, we attempted to change the conventional single-color blood group antigen distribution analysis to a two-color analysis of simultaneous staining of two antigens to improve unclear double population observed when the separation pattern was insufficient. Moreover, the method was applied to blood samples collected from three actual transfused patients. The improved HBT detection method allows to separate mixed RBC populations more clearly and to discriminate RBCs derived from multiple donors.

Introduction

In doping control, homologous blood transfusion (HBT) can be revealed by the analysis of the red blood cell surface antigen population with flow cytometry [1]. In the current published method, the expression profiles are analyzed with a single color staining [1-3]. In transfused blood, a double population (DP) is observed in the histogram. But the weakness is that it is difficult to detect a clear DP when the difference in the amount of expressed antigen or the number of mixed RBCs is small. In this study, we attempted to change to simultaneous staining of two antigens by two color analysis in order to improve the unclear DP.

Experimental

Material

Surgiscreen® was purchased from Ortho Clinical Diagnostics (Tokyo, Japan). Two types RBCs mixed transfusion model (mixed 2 different vials of Surgiscreen®) and 3 types RBCs mixed transfusion model (mixed 3 different vials of Surgiscreen®) were prepared as shown in Table 1. Blood specimens from transfused patients were EDTA-2K blood specimens collected at day 17 (patient 1), day 21 (patient 2), and day 32 (patient 3) after transfusion. Primary antibodies and antisera were anti-C, c, E, e, Jka, Jkb, Fya, Fyb, S and s (BIO-RAD,CA,USA). Fluorescent labeled secondary antibodies were Goat anti-Human IgM FITC, Goat anti-Human IgG FITC and Goat anti-Human IgG PE (Thermofisher, MA,USA) to be a dye that could be excited by a 488 nm laser only.

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	ratio (%)	C	c	E	e	Jka	Jkb	Fya	Fyb	S	s
2 types RBCs mixed transfusion model	99.5	+	0	0	+	0	+	0	+	+	0
	0.5	0	+	+	0	0	+	+	0	+	+
3 types RBCs mixed transfusion model	99.5	0	+	0	+	+	0	+	0	+	+
	0.25	+	0	0	+	0	+	0	+	+	0
	0.25	0	+	+	0	0	+	+	0	+	+

TABLE 1. Surgiscreen® antigen profile (RBCs mixed transfusion model)

Analytical method

The RBCs were washed by centrifugation and the concentration was adjusted to 5 × 10^7 RBCs/mL. 100 µL of the adjusted RBCs suspension was dispensed into a tube and centrifuged with PBS. 50 µL of various human IgM antibodies (anti-C, c, E, e, Jka, Jkb) and human IgG antiserum (anti-Fya, Fyb, S, s) adjusted to optimal concentrations were added to each tube. After mixing, they were incubated for 90 min at room temperature. In the two color analysis, the panels were assembled so that the antibody isotypes were different from each other and reacted. After the primary reaction, the cells were centrifuged with PBS. 50 µL of the fluorescent-labeled secondary antibody, which hab been adjusted to an optimal concentration, was added to correspond to the isotype of the antibody used in the primary reaction. Single color analysis was performed using FITC-labeled Goat anti-human IgM antibody and anti-human IgG antibody, and two color analysis was performed using FITC-labeled Goat anti-human IgM antibody and PE-labeled Goat anti-human IgG antibody. The reaction was performed for 45 min in the refrigerator. After the secondary reaction, cells were washed twice and re-suspended in 1 mL of PBS. Then, they were measured and analyzed using a flow cytometer (Navios EX, BECKMAN COULTER).

Results and Discussion

RBCs mixed transfusion model

In the histogram of 2 types RBCs mixed transfusion model, DP was confirmed, but the shape of the minor peak was hard to find because the ratio of mixed RBCs was only 0.5 %. A clear DP was not obtained for C, e, and Fya, and no DP was observed at the s antigen. The same was true for 3 types RBCs mixed transfusion model (Figure 1).

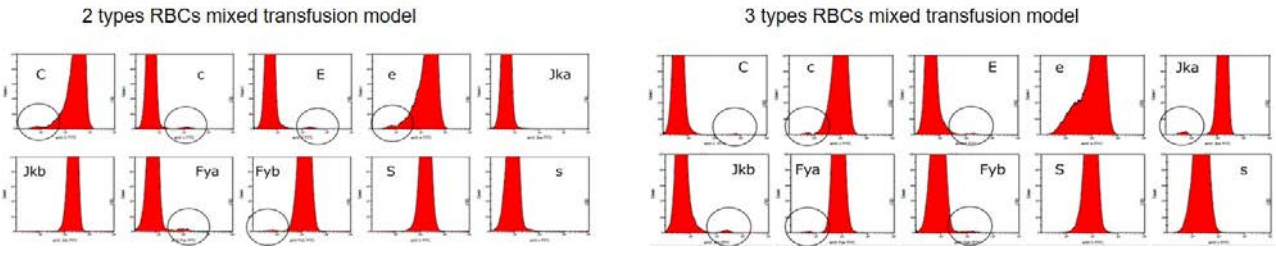

FIGURE 1. Single color analysis in simulated transfusion samples

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On the other hand, in the two color analysis, the partially overlapping peaks as described above clearly showed another population in the negative direction (C, e) or the positive direction (Fya, s) of the main population. The Jkb / s panel in 3 types RBCs mixed transfusion model was separated into three populations. The s-antigen expression level was weak in the main blood cells of 3 types RBCs mixed transfusion model, and the s-antigen negative population was confirmed only in the Jkb / s panel using two color analysis (Figure 2). However, the expression level of the e antigen varied widely, and no negative population could be detected in this panel.

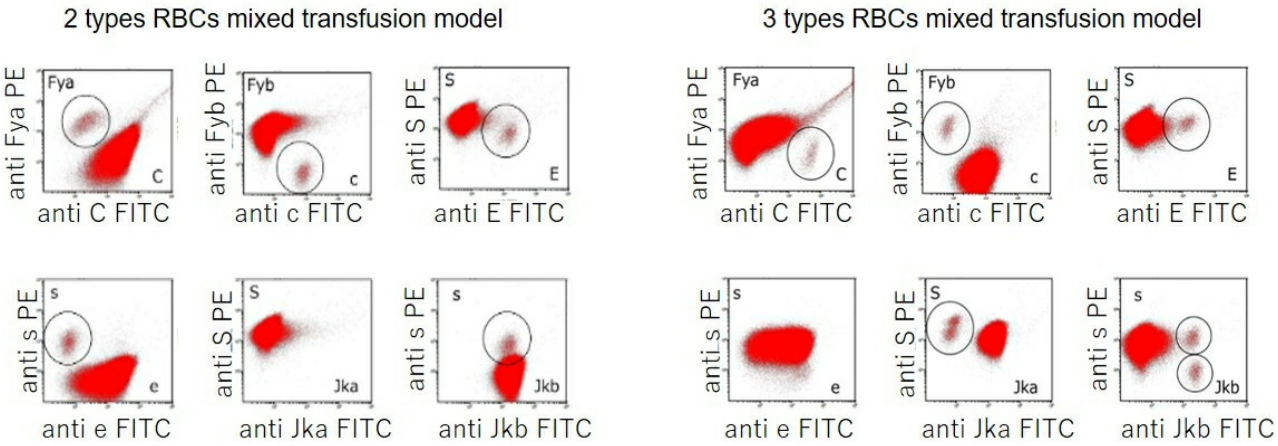

FIGURE 2. Two color analysis dot plot of simulated transfusion samples

Transfused patient

In patient's sample, DPs could be detected by single-color analysis because the transfused red blood cell count was sufficient to form a minor population histogram peak. However, the area of the minor peak was different for each antigen since HBT is generally performed from multiple donors. In two-color analysis, a two-dimensional expansion was possible to further separate such differences (Figure 3).

In addition, when two-color analysis was performed on antigens that became unclear-DP due to the small expression level difference (s of patient 2 and E of patient 3) and other DP antigens, another population was recognized in the positive or negative directions, respectively (Figure 4).

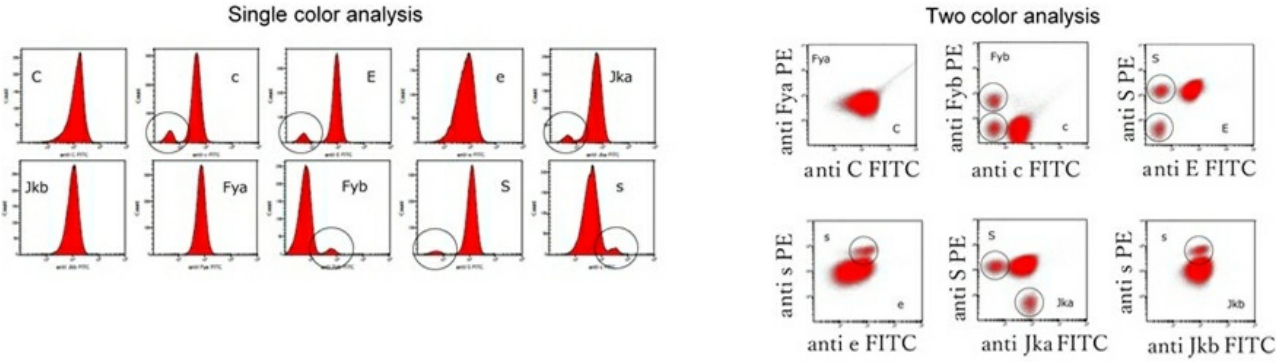

FIGURE 3. Single color analysis histogram and two color analysis dot plot of transfused patient 1 sample

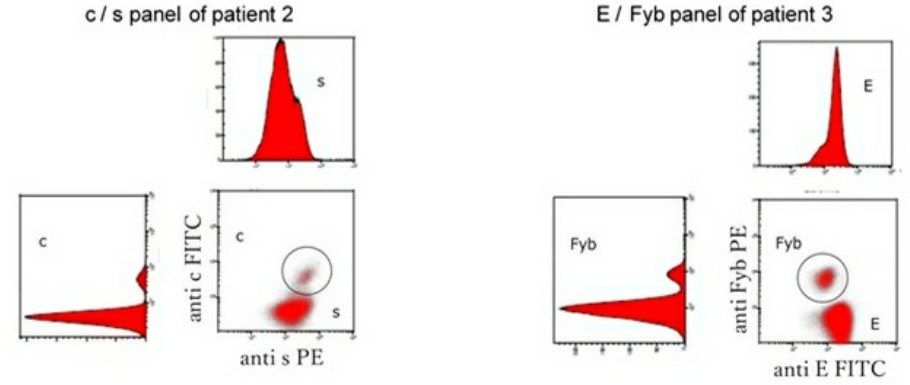

FIGURE 4. Two color analysis by panels with unclear DP and other DP antigens

Conclusions

The two-dimensional dot plot displays of the two-color analysis made it easy to visually confirm minute populations, and was particularly effective when analyzing minute amounts of mixed red blood cells. Performing two-color analysis with other DP markers when unclear DP was recognized, it was confirmed that part of unclear peak was composed of mixed red blood cells. When DP is detected in the ITP, reproducibility is confirmed by changing the antibody concentration to 0.5 or 2.0-fold dilution in the current confirmation analysis. Two-color analysis may be a more appropriate confirmation analysis.

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Zhou X, He S, Zhang L, Shen L, He C

Administration Study of rEPOs circulated in China

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Abstract

As the patent period expired, many brands of recombinant erythropoietins (rEPOs) have been produced from different companies all over the world. Due to differences in glycosylation, molecular weights (MW) and isoelectric points (pI), rEPOs are not exactly the same, which cause different electrophoretic behaviors on both SAR- and IEF-PAGE. Since multiple kinds of EPOs have been found in doping control samples in recent years, it is necessary to collect the image of all types of rEPOs in order to confirm the results. In this project nine different brands of rEPOs which circulated on the Chinese market were collected. Electrophoretic behaviors were compared. Administration studies were conducted with microdoses, i.e. 10 mIU/kg of each rEPO was injected intravenously. Urine and blood samples were collected 1 h pre- and 8 h, 16 h, 22 h and 32 h post-administration. All these samples were analyzed by SAR-PAGE and IEF methods. In summary, the results of urine and serum samples collected from the same person are nearly the same. However, for some rEPOs, time window of detection between SAR-PAGE and IEF are different. It depends on its MW or pI. SAR-PAGE is more effective for rEPOs which are larger but less basic, while IEF analysis is more suitable for those are more basic but relatively small. Above all, for the samples of which the diffuse area above endogenous band is faint, IEF could be an additional evidence for the judgement of rEPO which has a smaller MW.

Introduction

As the patent period expired, many brands of recombinant erythropoietins (rEPOs) have been produced by different companies all over the world, which were referred to as “biosimilars” of rEPOs [1]. Due to differences in glycosylation resulting from different cell-lines and the entire biotechnological process [2], molecular weights (MW) and isoelectric points (pI) of biosimilars are not exactly the same. This leads to different electrophoretic behaviors on both SAR- and IEF-PAGE. As in recent years various types of bands have been found in doping control samples, it is necessary to collect the electrophoretic profile of each biosimilar in order to confirm the results. For this reason, administration studies of rEPO biosimilars were conducted, which aimed to compare the efficiency between different analytical methods or matrices.

Experimental

Study Design

Nine different brands of rEPO and biosimilar preparations circulated on the Chinese market were collected. Electrophoretic behaviors of each preparation were compared with SAR-PAGE and IEF method, including syringe alone, spiked in blank urine sample and spiked in serum sample, respectively.

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Administration studies of rEPOs were conducted with micro-doses. Six male volunteers and three female volunteers were recruited in this study. All volunteers were healthy non-athletes, age from 18 to 40 years, without chronic illness history, allergic history and doping history. Each volunteer was administrated one brand of rEPO by intravenous injection with a dosage of 10 mIU per kg body weight. Blood samples were collected 1 hour pre injection, and 8, 16, 22 and 32 hours post injection. Urine samples were also collected 30 minutes around each venipuncture.
All the urine and serum samples collected in the administration study were analyzed for EPO using SAR- and IEF-PAGE, respectively.

Sample Analysis

10 mL Urine sample was used for each analysis of EPO, while 0.5 mL serum sample was used for each blood analysis. The methods of immunopurification, SAR-PAGE and western blotting were summarized in Table 1. Details of the whole procedure were described before [3,4]. IEF and western blotting was conducted as Lasne described before [5], with a minor change in immunopurification [6].
Urine samples were immunopurified with StemCell ELISA plates after ultrafiltration, while serum samples were immunopurified with anti-EPO polyclonal antibody LS-C26017 coupled to Dynabeads streptavidin C1 beads.
Anti-EPO monoclonal antibody AE7A5 (MAB2871) was used as primary antibody for western blotting. Single blotting was applied for urine analysis with SAR-PAGE only, while double blotting was used for all IEF tests and serum analysis with SAR-PAGE.

	SAR-PAGE		IEF	
	urine	**serum**	**urine**	**serum**
Immunopurification	StemCell ELISA	magnetic beads	StemCell ELISA	magnetic beads
Elution	SAR sampling Buffer (0.1M DTT)		4.4% CHAPS	
Western Blot	single blotting	double blotting	double blotting	

Table 1. Method summary of each analysis

Results and Discussion

Comparison of Electrophoretic Behavior from each rEPO

Based on the criteria of TD2014EPO [7], rEPO could be detected from all urine and serum samples which were collected 8 h after injection, no matter wether with SAR-PAGE or IEF analysis. In addition, the electrophoretic profiles of the rEPOs from spiked samples were nearly identical to the profiles obtained from the wash-out samples (data not shown). However, though all these rEPOs and biosimilars are produced in Chinese hamster ovary (CHO) cell lines, the electrophoretic behaviors between the individual rEPOs vary largely (Figure 1).
Beside that, for some rEPOs, the electrophoretic behaviors of the syringes are not accordant to the behaviors of the resulting urine and serum samples from the administration study, such as Jimaixin and its respective samples examined with IEF analysis.

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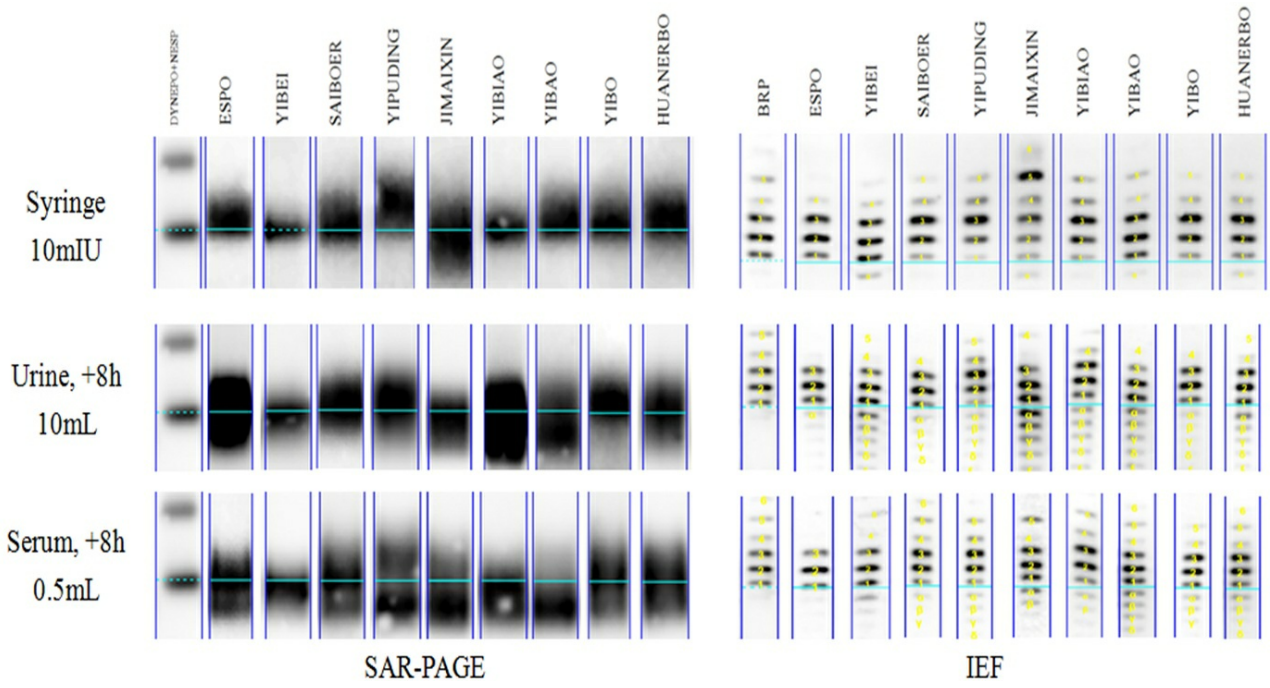

Figure 1. Comparison of biosimilars in SAR-PAGE and IEF

Time Window of Detection with rEPO

The time window of detection with each rEPO was summarized in Table 2.

rEPO	Electrophoresis	8h		16h		22h		32h	
		Urine	Serum	Urine	Serum	Urine	Serum	Urine	Serum
Huanerbo	SAR-PAGE	+	+	+	+	+	+	+	+
	IEF	+	+	+	+	+	+	+	+
Yibao	SAR-PAGE	+	+	+	+	+	+	-	-
	IEF	+	+	+	-	-	-	-	-
Yibo	SAR-PAGE	+	+	+	+	+	-	-	-
	IEF	+	+	+	+	+	-	-	-
Yipuding	SAR-PAGE	+	+	+	+	+	+	+	+
	IEF	+	+	+	+	+	+	+	+
Saiboer	SAR-PAGE	+	+	+	+	+	+	+	+
	IEF	+	+	+	+	+	+	-	+
Yibiao	SAR-PAGE	+	+	+	+	-	-	-	-
	IEF	+	+	+	+	-	-	-	-
Yibei	SAR-PAGE	+	+	-	-	-	-	-	-
	IEF	+	+	+	+	+	+	-	-
Jimaixin	SAR-PAGE	+	+	+	+	+	+	-	-
	IEF	+	+	+	-	-	-	-	-
ESPO	SAR-PAGE	+	+	+	+	+	+	+	+
	IEF	+	+	+	+	-	+	-	-

Table 2. Results of the administration study

As shown, the efficiency of detection between urine and serum samples are similar. But for each rEPO, the efficiency of the detection between SAR-PAGE and IEF analysis is different, which depends on the MW and pI of each one (Figure 2).

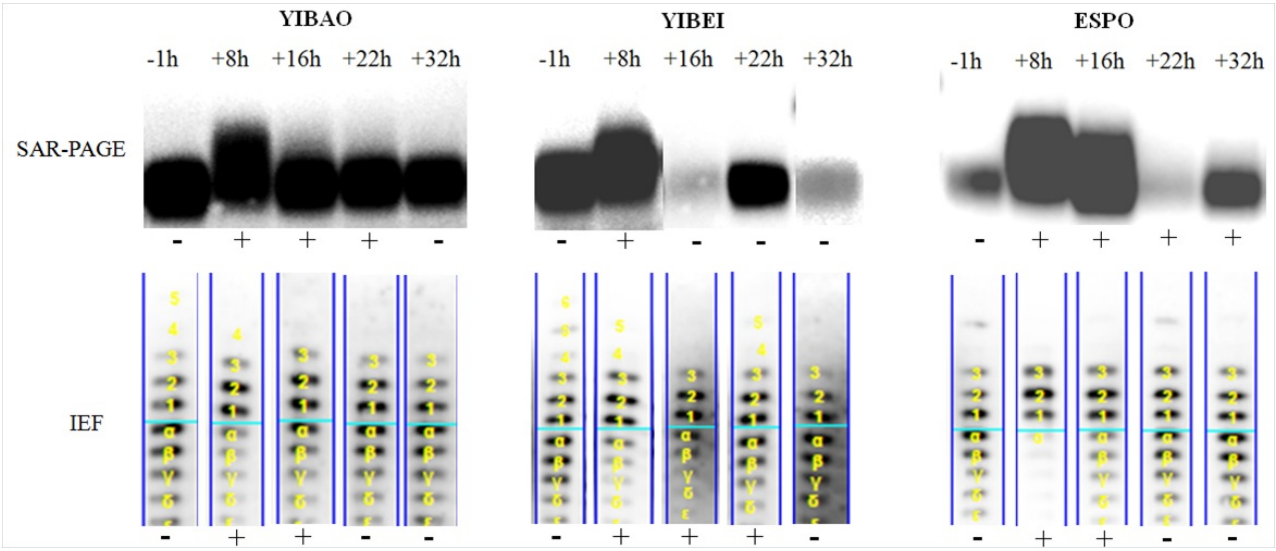

Figure 2. Comparison of urine samples from the excretion study

Due to the requirements of the TD and the high throughput of analysis, SAR-PAGE is now commonly used for the detection of rEPOs both in the initial testing procedure and CP. Though the diffuse area above endogenous band could be more obvious if more volume of sample is used, it is still difficult to judge the samples with SAR-PAGE results on the borderline between “negative” and “positive”, which contains the biosimilars with smaller MW. For these samples, IEF analysis would be a better method. However, as it is prone to false positive due to active and/or effort urine, it can only be an additional evidence to judge these “suspicious” samples, not a confirmation method.

Conclusions

Summarizing the results of the presented study, SAR-PAGE is more effective for analyzing biosimilars which are larger but less basic, while IEF analysis is much better for those more basic but relatively smaller.

Considering the requirements of the TD and the possibility of false positive with IEF analysis, SAR-PAGE is a suitable method both in the initial testing procedure and CP. However, IEF analysis could be an additional evidence to judge the suspicious samples in which the diffuse area above endogenous band is faint and short.

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Troubleshooting for doping analysis of EPO

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Abstract

Electrophoretic techniques followed by Western Blot and chemoluminescence detection are the prioritized choices to detect erythropoietins (EPO) and their analogues in anti-doping laboratories. Although the requirements on these techniques are described in a Technical Document (TD2014EPO) published by the World Anti-Doping Agency (WADA) in order to harmonize the analysis procedures in different anti-doping laboratories, the complex nature of these techniques could introduce various manual operation errors; and even manufacturer batches of the reagents or consumables could result in unacceptable results (“failed gels”). Hence, the cause of “failed gels” is usually implicit and depends mostly on the experience of the analyst.
To ease the burden of repeating the analysis with different hypothetical conditions looking for the cause of “failed gel” during routine analysis, we summarized the images of failed gels and relevant causes which were listed in our laboratory’s Annex of Standard Operation Procedure (SOP) on EPO analysis.
Hopefully, the results and conclusions presented in this paper could provide ideas on troubleshooting in EPO routine analysis for anti-doping laboratories.

Introduction

Electrophoretic techniques followed by Western Blotting and chemiluminescence detection are the prioritized choices to detect erythropoietins (EPO) and their analogues in anti-doping laboratories. Although the requirements on these techniques are described in a Technical Document (TD2014EPO)[1] published by the World Anti-Doping Agency in order to harmonize the analysis procedures in different anti-doping laboratories, the complex steps in the experiment could introduce various errors. In order to determine the possible reason causing the unacceptable result (“failed gels”), we analyzed the images obtained from a large number of routine gels.

Experimental

SAR-PAGE is applied for routine analysis of EPO for both ITP and CP in our lab. All the examples listed in this paper were conducted with this method. For urine samples, StemCell ELISA plates were used for immunopurification after ultrafiltration and Western single or double blotting was applied. For blood samples, double blotting was conducted after immunopurification with MAIIA columns. For single blotting, goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody HRP (Thermo,31432) was used after incubation with clone AE7A5 anti-EPO antibody. For double blotting, goat anti-mouse IgG (H+L) secondary antibody biotin (Thermo, 31800) and streptavidin horseradish peroxidase conjugate (Pierce, 21126) were used for incubation following blocking after the second blotting. The whole experimental

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procedure was conducted as we described before [2].

The reasons which caused “failed gels” were investigated by changing only one reagent or consumable in sequence. Once the image became satisfactory, the reason would be confirmed by running two gels with different batches of “problematic reagent or consumable” in parallel. This was used to determine if one is satisfactory, while the other which using the “problematic reagent or consumable” is also a “failed gel”.

Results and Discussion

All the examples listed below (Table 1) are summarized from routine analyses performed in our lab. The “possible reason” are the only ones we have found with compared experiments, it might not conclude all possible reasons. “Solution” is focused to the corresponding reason which was shown, while “Remedy” is the solution of saving the membrane without having to start from the very beginning of the analysis.

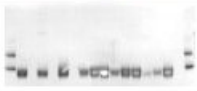
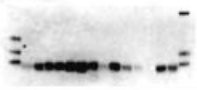
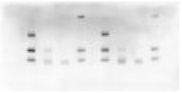
No.	Images of “failed gel”	Description of “failed gel”	Possible reason	Solution	Remedy
1		CERA and EPO-Fc were not detected in EPO reference peparation.	Low purity of Sarcosyl	99% HPLC grade Sarcosyl is compulsory	None
2		The migrant distance of capture antibody light chain changed.	The concentration of SAR in running buffer is seriously below normal	Accurately confirm the SAR concentration in running buffer	None
3	blank	Nothing was detected and background was very clean with single blotting.	2nd Ab-HRP is seriously below normal.	Confirm that 2nd-HRP is in good condition	Wash the membrane with PBS 3 times, incubate the membrane with new 2nd Ab-HRP.
4		Uneven gray spots were spread all over the membrane in double blotting	Worn roller surface	Use a new roller	The first membrane can be re-conducted starting with blocking.
5		White irregular patches distributed over the membrane	Using the Extra thick blotting paper from BioRad in double blotting	Electrode thin blotting paper is necessary in double blotting	The first membrane can be re-conducted starting with blocking.
6		The background of the membrane was smeared	The quality of non-fat milk can influence the background of double-blotting membrane	Change the non-fat milk powder	The first membrane can be re-conducted starting with blocking.
7		Tiny spots spread all over the membrane	**For single blotting**: 1st Ab has degraded. **For double blotting**: 1st Ab has degraded or Incubation time of HRP is too long.	Make sure the condition of incubation with HRP should be 1h at RT, if it has been done like this, change the 1st Ab.	**For single blotting**: The membrane can be stripped, then re-conducted starting with the incubation of 1st Ab. **For double blotting**: The first membrane can be re-conducted starting with blocking.
8		The signal of all bands are weak, while multiple spots are detected at the edge of membrane.	1st Ab has degraded.	Change the 1st Ab.	**For single blotting**: The membrane can be stripped, then re-conducted starting with the incubation of 1st Ab. **For double blotting**: The first membrane can be re-conducted starting with blocking.

Table 1. Troubleshooting for doping analysis of EPO

Conclusions

The procedure of EPO analysis consists of multiple steps, which could introduce various manual operation errors. Reagents and consumables which are most likely effecting the quality of the result are listed in Table 2. It is practical to analyze the cause of “failed gel” by check them.

Reagents	Antibody and relevant kits for immunopurification, Sarcosyl, slim milk powder, 1st Ab, 2nd Ab-HRP, 2nd Ab coupled with biotin,streptavidin-HRP, substrate
Consumables	Blotting paper, Roller, Precast gel

Table 2. Important reagents and consumables

References

1. WADA. WADA Technical Document-TD2014EPO, Sep 2014.
2. X. Zhou, L. Zhang, S. He, et al. Comparison and optimization of SAR-PAGE tests for erythropoietins in doping analysis. *Drug Test. Anal.* 2020, 1, 109-118.

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Effects of protease inhibitors on stability of urine hCG and LH in doping analyses

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Abstract

Molecular stability of peptides in urine samples could be an important issue in sports drug testing since heat and proteolytic enyzme activities induced by microorganism might result in decomposition of them. Furthermore, adulteration of urine using proteases have been reported to impede the detection of drugs. The aim of present study was to investigate the effects of various proteases and microorganisms on the stability of human chorionic gonadotropin (hCG) and luteinizing hormone (LH) in urine samples. In the first set of experiments, we tested whether three different proteases in urine samples with or without protease inhibitors lead to a reduction in urinary hCG and LH levels at -20°C, 4°C, and 37°C for one or four days. In the second set of the experiments we have tested the effect of two different bacteria (E.coli and E.faecalis), which are the most frequent pathogens found in urinary tract infections, on urine hCG and LH levels at 37°C. Intact hCG and total LH levels were analysed by using the AutoDELFIA hCG and AutoDELFIA hLH spec kits. All three proteases resulted in decreased hCG and LH levels in urine samples at 37°C and the addition of protease inhibitors mixture prevented these decreases. In the second set of the experiments, four days of incubation with E.Faecalis resulted in decreased levels of both hCG and LH. E.coli resulted in an increase in hCG levels both on day one and day four and a decrease in LH levels on day four. These results show that proteases result in a temperature dependent decomposition of hCG and LH. Addition of protease inhibitors to urine samples might protect protease induced degradation of hCG and LH.

Introduction

Human chorionic gonadotropin (hCG) and Luteinizing hormone (LH) are glycoproteins consisting of two polypeptide chains, α- and β-subunits [1]. The desired effects of hCG and LH in sport are due to the enhancement of testosterone by Leydig cells [2]. In addition, hCG can be used by male athletes to normalize testicular testosterone production that is suppressed during and after prolonged use of anabolic steroids. Both hCG and LH are listed by the WADA as a prohibited substance in male athletes and by using Delfia assay, urinary concentrations of hCG > 5 IU/L and LHf> 40 IU/L may be an indicator of an adverse analytical finding. Molecular stability of peptides in urine samples could be an important issue since heat and proteolytic enyzme activities induced by microorganism might result in a decomposition of the peptides [1-3]. Furthermore, adulteration of urine by using proteases have been reported [4]. Therefore, the aim of the present study was to investigate the effects of various proteases and microorganisms on the stability of hCG and LH in urine samples.

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Experimental

Urine specimen which an approximate concentration of 150 IU/L LH obtained from a pregnant woman and a male urine sample spiked with hCG (hCG:99/688 NIBSC, Potters Bar, Hertfordshire, UK) to achieve final concentration of 10 IU/L were prepared. Both samples were divided to different tubes and 150 µg/mL of trypsin, pepsin or papain (Sigma Aldrich Chemie, Steinheim, Germany) spiked with or without having 2% of cocktails of protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany) and 4.6 mM of pepstatin A (Serva Electrophoresis, Heidelberg, Germany). Samples were incubated at three different temperature conditions (-20°C, 4°C, or 37°C) for four consecutive days and tested at the end of the day one and four. In a second set of experiments, both urine samples were divided to separate tubes and inoculated with either gram (-) E.coli or gram (+) E.faecalis (10^5 -10^6 cfu mL^{-1}) and incubated at 37°C for four days. Intact hCG and total LH levels were measured by using the AutoDELFIA hCG and AutoDELFIA hLH spec kits.

Results and Discussion

Proteases did not have any effect on hCG and LH levels over the four days at -20°C. Figure 1 and 2 show urine hCG and LH levels at 4°C and 37°C with or without protease inhibitors. All three proteases resulted in decreased hCG and LH levels at 37°C ($p<0.05$).

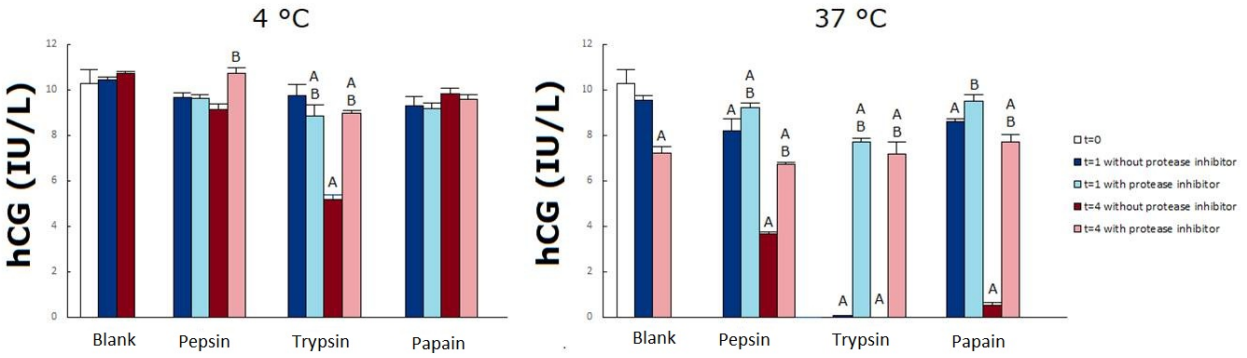

Figure 1. Changes in hCG concentration (IU/L, n=6). A, different than blank ($p<0.05$) B, different than without protease inhibitor at the same time point ($p<0.05$)

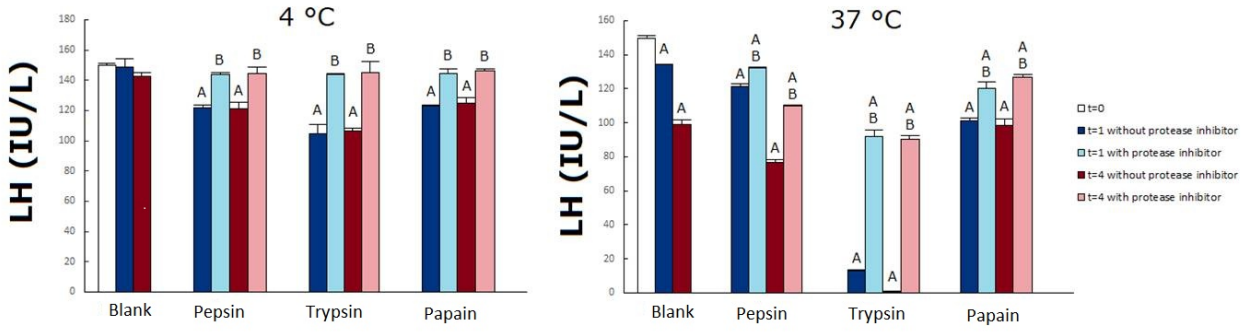

Figure 2. Changes in LH concentration (IU/L, n=6). A, different than blank ($p<0.05$) B, different than without protease inhibitor at the same time point ($p<0.05$)

Figure 3 shows the effects of E.faecalis and E.coli on hCG and LH levels at 37°C. While four days of incubation with E.faecalis resulted in decreased levels of both hCG and LH, E.coli resulted in increased levels of hCG starting at day one and decreased levels of LH at day four ($p<0.05$).

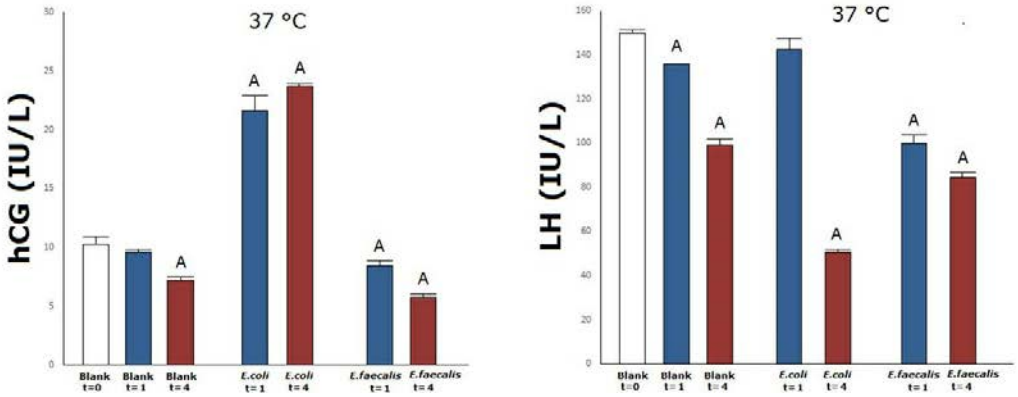

Figure 3. The effects of E.faecalis and E.coli inoculation on hCG and LH levels. A, different than blank ($p<0.05$).

Conclusions

These results have shown that proteases result in a temperature dependent decomposition of the hCG and LH, since urine levels of hCG and LH were not affected by adding proteases at -20°C, but decreased at 37°C. The addition of protease inhibitors to urine samples might protect against protease induced degradation of hCG and LH. Since the effect of proteases was seen at 37°C for all proteases applied, we decided to test specifically if E.coli and E.faecalis - the most frequent pathogens found in urinary tract infections - would result in decomposition of urine at 37°C by means of decreased levels of hCG and LH. Our results showed that both microorganisms have resulted in decreased levels of urinary LH. Therefore, this preliminary study supports previous findings that adultering of urine samples by adding proteases to urine, or having a urinary tract enfection, may effect the results of sports drug testing.

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Direct and indirect detection of pseudo-endogenous steroids in plasma

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Abstract

The detection of the abuse of pseudo-endogenous steroids represent a complex analytical challenge for the accredited laboratories, mostly for the need to discriminating between their endogenous or exogenous origin and for the use of “micro-dosing”. It is based on the longitudinal monitoring of six urinary steroidal markers and their relative ratios, described in Steroidal Module of the Athlete Biological Passport (ABP), by the application of a Bayesian adaptive model that is able to outline atypical results. Although the introduction of the longitudinal evaluation of the markers of the steroid profile clearly improved the detection of the pseudo-endogenous steroid doping, it does not allow to gather any information on the occurrence of atypical profiles due to the presence of endogenous (i.e. enzyme induction or inhibition, genetic polymorphisms) or exogenous (i.e. banned drugs, masking agents, ethanol, bacterial contamination) confounding factors affecting the urinary steroid profile. To overcome these drawbacks, the evaluation of a parallel “blood steroid profile” has been proposed in the last years. On the other hand, to improve the detection of testosterone (T) and other anabolic steroids (i.e. nandrolone (NAND)) different research groups developed chromatographic-mass spectrometric methods for the analysis of intact steroid esters, the most common administration form.
In this work we present a liquid chromatographic-tandem mass spectrometric method for the analysis of T, its major precursors and metabolites, T esters (14) and NAND esters (2) in serum/plasma with the aim to extend the analytical strategies followed by the accredited laboratories for the detection of the pseudo-endogenous steroid doping.

Introduction

Since 2014, the pseudo-endogenous steroid doping is detected thanks to the longitudinal monitoring of six urinary markers and their relative ratios measured by gas-chromatography mass spectrometry (GC-MS^n) techniques, as reported in the Steroidal Module of the Athlete Biological Passport (ABP)[1]. Although the longitudinal approach clearly improved the detection of the pseudo-endogenous steroid abuse, in comparison with the former testosterone/epitestosterone (T/E) threshold of 4 [2], it is still subject to various drawbacks related to the nature of urine matrix and to the presence of endogenous and exogenous confounding factors [3-5]. To overcome these drawbacks, in this work we propose the evaluation by liquid chromatohraphy tandem mass spectrometry (LC-MS/MS) of a parallel "blood steroid profile" [6] and the analysis of intact anabolic steroids esters [7-12].

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Experimental

Chemicals and reagents

Standards and solvents were from Sigma Aldrich (Milano, Italy), Steraloids (Newport, USA) and National Measurement Institute (Pymble, Australia).

Sample pre-treatment

To assess the protein precipitation, 500 µL of a mixture of acetonitrile/1% formic acid (ACN/1% HCOOH) were added to 200 µL of serum/plasma fortified with 20 µL of internal standard (deuterated testosterone (T-d_3) and trenbolone enanthate, 5 ng/mL). Samples were centrifuged (10000 rpm, 10 min) and the liquid phase was transferred in a glass tube. 100 µL of carbonate/bicarbonate buffer (20% w/V, pH 9) were added and a liquid-liquid extraction was carried out with 5 mL of a mixture of n-hexane/ethylacetate (70/30 V/V) for 5 minutes on a mechanical shaker. Samples were centrifuged (3000 rpm, 2 min) and the organic layer was transferred in a glass tube and evaporated to dryness under nitrogen steam at 75°C. The residue was reconstituted in 50 µL of mobile phase (water/methanol 50/50 V/V), transferred in a glass vial and a 5-µL aliquot was injected into the LC-MS/MS system.

Instrumental analysis

Liquid chromatographic-mass spectrometric analysis was performed on an Agilent 1290 Infinity II LC system instrument coupled to an Agilent Ultivo triple quadrupole (TQ) mass spectrometer (Agilent Technologies, Cernusco sul Naviglio, Italy). The analytical column used was an Eclipse Plus C18 (l: 50 mm; i. d: 2.1 mm; particle size: 1.8 µm, CPS Analitica, Milano, Italy) operated at 30°C. The mobile phase (A: water/1% formic acid, B: methanol/1% formic acid) flow rate was 0.3 mL/min and 5 µL were injected. An Agilent Technologies Jet Stream electrospray ionization (AJS ESI) ion source was used. All compounds were monitored in positive ionization using a multiple reaction monitoring (MRM) mode. The capillary voltage, drying gas flow, drying gas temperature, nebulizer, noozle voltage, sheat gas flow and sheat gas temperature were set at 3500 V, 8 L/min, 200°C, 40 psi, 500 V, 11 L/min, 375°C, respectively.

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Analyte	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision energy (eV)
17αOHP	331	109; 97; 79	136; 136; 136	32; 28; 60
AED	287	109; 97; 79	131; 131; 131	28; 24; 56
DHEA	289	271; 253	103; 103	4; 8
T	289	109; 97; 79	126; 126; 126	28; 28; 56
DHT	291	273; 255	126; 126	12; 16
Trenbolone	271	253; 199; 165	146; 146; 146	20; 24; 50
T acetate	331	271; 109; 97	131; 131; 131	16; 32; 28
T propionate	345	271; 109; 97	131; 131; 131	16; 32; 28
T valerate	373	271; 109; 97	131; 131; 131	16; 36; 28
T isocaproate	387	271; 109; 97	141; 141; 141	16; 36; 16
T caproate	387	271; 109; 97	141; 141; 141	16; 36; 32
T benzoate	393	271; 109; 97	146; 146; 146	16; 40; 32
T hexahydrobenzoate	399	271; 109; 97	146; 146; 146	16; 40; 32
T enanthate	401	271; 109; 97	146; 146; 146	16; 40; 28
T cypionate	413	271; 109; 97	156; 156; 156	16; 40; 24
T octanoate	415	271; 109; 97	151; 151; 151	16; 36; 32
T decanoate	443	271; 109; 97	164; 164; 164	16; 44; 32
T phenylpropionate	421	271; 109; 97	141; 141; 141	16; 40; 32
T undecanoate	457	271; 109; 97	164; 164; 164	16; 44; 32
T dodecanoate	471	271; 109; 97	169; 169; 169	20; 44; 32
NA decanoate	425	257; 155; 85	169; 169; 169	16; 20; 28
NA phenylpropionate	407	257; 133; 109	156; 156; 156	16; 16; 40
ISTDs				
T-*d3*	292	109; 97	131; 131	32; 28
Trenbolone enanthate	383	253; 225	164; 164	24; 32

Table 1. Selected tandem mass spectrometry transitions for steroid analysis

Results and Discussion

The detection of an intact steroid ester in blood matrices is an unequivocal proof of its exogenous administration, since the esters used in drug formulation are not synthesized in the body. Despite this, when T esters preparations are applied, both orally or as a depot injection, the cleavege process by esterase enzymes starts immediately and a detectable proportion of exogenous T diffuses slowly into the blood stream, suggesting the identification and quantification of its major precursors and metabolites.

In this work we propose an LC-MS/MS method for the simultaneous analysis of T, its major precursors and metabolite (17α-hydroxyprogesterone (17αOHP), androstenedione (AED), dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT)), 14 T esters (acetate, propionate, valerate, caproate, isocaproate, benzoate, hexahydrobenzoate, enanthate, cypionate, octanoate, decanoate, phenylpropionate, undecanoate, dodecanoate) and 2 nandrolone (NAND) esters (decanoate, phenylpropionate) in serum/plasma (Figure 1).

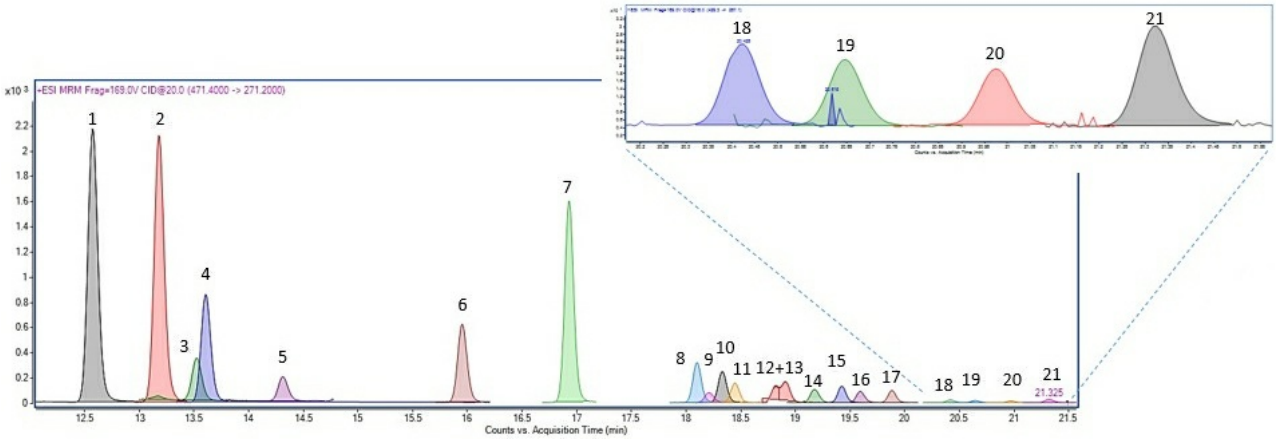

Figure 1. Extracted ion chromatogram for all considered compounds. 1: AED (207-109 m/z); 2: T (289-109 m/z); 3: DHEA (289-271 m/z); 4: 17αOHP (331-109 m/z); 5: DHT (291-273 m/z); 6: T acetate (331-271 m/z); 7: T propionate (345-271 m/z); 8: NAND phenylpropionate (407-257 m/z); 9: T benzoate (393-271 m/z); 10: T valerate (373-271 m/z); 11: T phenylpropionate (421-271 m/z); 12: T isocaproate (387-271 m/z); 13: T caproate (387-271 m/z); 14: T hexahydrobenzoate (399-271 m/z); 15: T enanthate (401-271 m/z); 16: T cypionate (413-271 m/z); 17: T octanoate (415-271 m/z); 18: NAND decanoate (429-257 m/z); 19: T decanoate (443-271 m/z); 20: T undecanoate (457-271 m/z); 21: T dodecanoate (471-271 m/z)

The sentitivity of the method is estimated at 0.1 ng/mL for each selected pseudo-endogenous compound, except for DHEA, for which the sensitivity is estimated at 0.5 ng/mL. According to already published data [6], the presentend method is linear in the range of 0.1 ng/mL - 20 ng/mL for 17αOHP, AED, T, DHT, and in the range of 0.5 ng/mL - 20 ng/mL for DHEA (Figure 2).

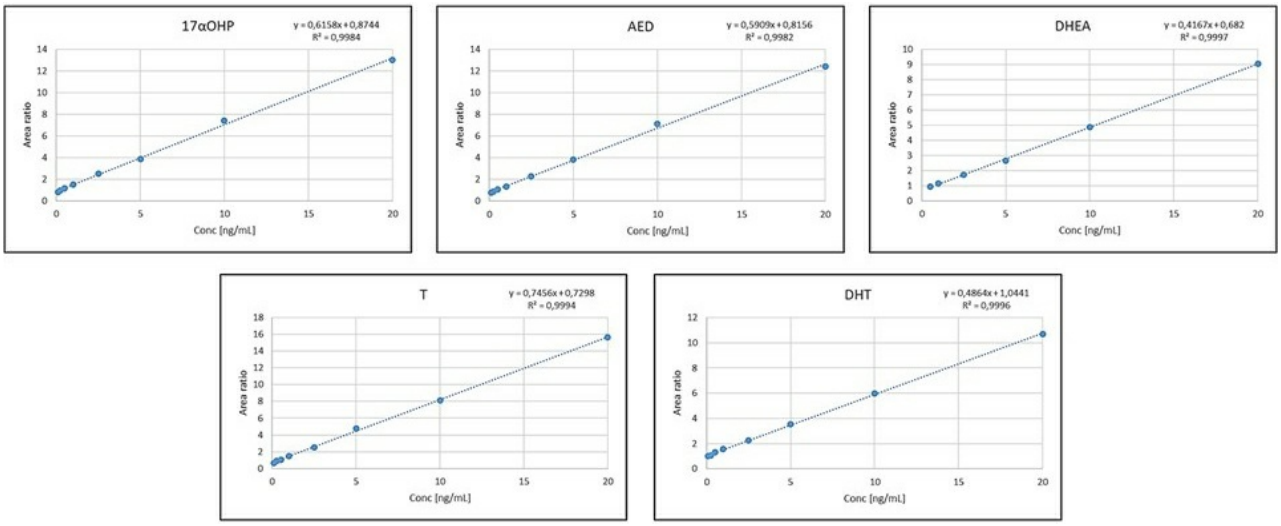

Figure 2. Calibration curves for 17αOHP (0.1 ng/mL - 20 ng/mL), AED (0.1 ng/mL - 20 ng/mL), DHEA (0.5 ng/mL - 20 ng/mL), T (0.1 ng/mL - 20 ng/mL) and DHT (0.1 ng/mL - 20 ng/mL)

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For all the considered esters the sensitivity of the method is estimated at 0.2 - 0.5 ng/mL (Figure 3).

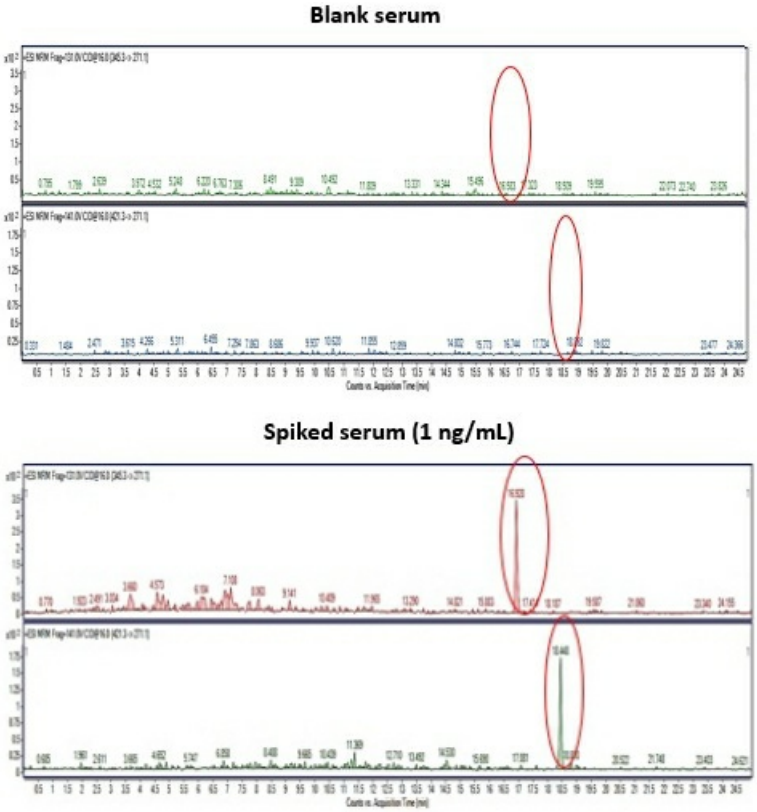

Figure 3. Comparison between an extracted ion chromatogram of a blank serum and an extracted ion chromatogram of a spiked serum at 1 ng/mL with T propionate (345-271 m/z) and T phenylpropionate (421-271 m/z). The results obtained are overlapped to those obtained in plasma samples.

These values are in accordance with the sensitivity obtained by other research groups [7-12] and are adequate for the detection of the compounds over the range in which the urinary parameters of the steroid profile are affected [13]. Furthermore, to monitor the activity of blood esterase enzymes, we propose to evalaute the ratio between trenbolone, an exogenous anabolic steroid not phisiologically present in blood matrices, and trenbolone enanthate, used as internal standard.

Conclusions

In this work we present a LC-MS/MS method for the analysis of 5 pseudo-endogenous steroids (17α-hydroxyprogesterone, androstenedione, dehydroepiandrosterone, testosterone and dihydrotestosterone) and 16 esters of anabolic steroids (T acetate, propionate, valerate, caproate, isocaproate, benzoate, hexahydrobenzoate, enanthate, cypionate, octanoate, decanoate, phenylpropionate, undecanoate, dodecanoate and NAND decanoate, phenylpropionate) in blood matrix (serum and/or plasma). The method is suitable for the direct detection of the pseudo-endogenous steroid doping and potentially for the indirect detection trough the alterations of key compounds of the blood steroid profile, offering an alternative to routinely used methods (analysis of the longitudinal urinary steroid profile by GC-MS^n and analysis of T esters in serum/plasma by GC-MS^n) in the WADA accredited laboratories.

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Multiple Biomarkers of Anabolic Agent Administration in Urine as Candidates for Doping Diagnosis

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Abstract

By certain drugs, biochemical change occurs and remains in our bodies. It is expected that when we discover metabolomic traces, we can apply them to the diagnosis of doping. We performed a metabolomics study using a liquid chromatography quadrupole / orbitrap mass spectrometer (Shimadzu LC - Q-Exactive Plus™, ThermoFisher Scientific) and Compound Discoverer™ 2.1 software (ThermoFisher Scientific). We prepared 15 urine samples from cases with adverse analytical findings (AAF) of anabolic steroids, another 15 negative case samples, and a “pooled urine” in which all 30 urine samples were mixed in equal volumes. Deionized water, urine samples, and pooled urine were analyzed by Q-Exactive™ in Full MS and ddMS^2 mode, and then raw files were processed in Compound Discoverer™ for data mining. As a result, 241 positive ions and 559 negative ions showed *p*-values less than 0.05 and an absolute value of Log2 fold change of more than 1. Bilirubin concentrations were elevated in AAF cases and that implies that one of the adverse effects of anabolic agents is hepatotoxicity. Concentrations of conjugated metabolites of endogenous testosterone and dihydrotestosterone were lower in AAF cases, indicating that exogenous anabolic compounds were administered, so that the internal anabolic hormone secretion was reduced by negative feedback for homeostasis. Also, concentrations of estradiol glucuronides were higher in AAF cases, corresponding with clinical observations of AAS users. Obvious differences of metabolomic profiles between AAF and control urines were observed, implying that doping diagnosis via urine metabolomic profiles is possible.

Introduction

Currently, the anti-doping community is demanding more indirect and comprehensive doping control strategies to detect unestablished drugs. The current doping diagnosis method is based on ‘target analysis’, a method comparing each established doping substance’s analytical data with athletes’ samples. However, such an approach has limited ability to detect ‘designer drugs’, secretly developed drugs to evade doping detection.

As one of the strategies, we focused on the fact that a metabolomic profile alters after drug administration. For example, metabolomic profiles of rosuvastatin administration were investigated, suggesting that they may act as biomarkers of monitoring dyslipidemia treatment or occurrence of adverse effect [1]. We also investigated metabolomic profiles of anabolic androgenic steroid (AAS) users and found possible biomarkers that indicate AAS administration.

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Experimental

Shimadzu UHPLC-Q Exactive Plus was used for analysis. As stationary phase, Imtakt Scherzo SW-C18 100 × 2 mm, 3 µm coupled with a guard cartridge UK-C18 (5 × 2 mm) and a guard holder were used. As mobile phase, 0.1% formic acid in deionized water (A) and 0.1% formic acid in acetonitrile (B) was used.
15 male urines of negative cases and 15 male urines of adverse analytical finding (AAF) cases caught up with AAS administration, in the sport disciplines of baseball, bodybuilding, and wrestling, were involved in the metabolomic study. Pooled urine was prepared by taking equal volumes of the samples and mixing them together. Each aliquot of sample including pooled urine was mixed with deionized water in the same volume and centrifuged at 4°C, 12,500 rpm (17,643 rcf) for 15 minutes. Supernatants of the samples were analyzed.
Injection volume was 6 µL. Flow rate was 0.3 mL/min. Solvent gradient was as follows: 0 to 4 minutes: 1% B, 4 to 7 minutes: 1% to 15% B, 7 to 12 minutes: 15% to 40% B, 12 to 16 minutes: 40% to 98% B, 16 to 18 minutes: 98% B, 18 to 18.5 minutes: 98% to 1% B, 18.5 to 21 minutes: 1% B. Pooled urine and samples were analyzed in full scan and $ddMS^2$ mode, with a scan range from 80 to 1,200 *m/z* and picking up the 5 most intensive ions in each scan. Positive ions and negative ions were scanned separately. Data were mined with Compound Discoverer 2.1 using the workflow 'Untargeted Metabolomics workflow: Find and identify the differences between samples' and the setting to calculate concentration ratios of each identified ion in average AAF cases divided by the negative cases. The detailed handling process was done according to Compound Discoverer 3.1 Metabolomics Tutorial [2].

Results and Discussion

A total of 14,396 ions were identified for the positive ions, and 564 ions showed a p-value of less than 0.05 in the concentration ratio (Fig. 1). 1,233 positive ions showed a Log2 fold change (FC) of the concentration ratio of more than 1 or less than -1.

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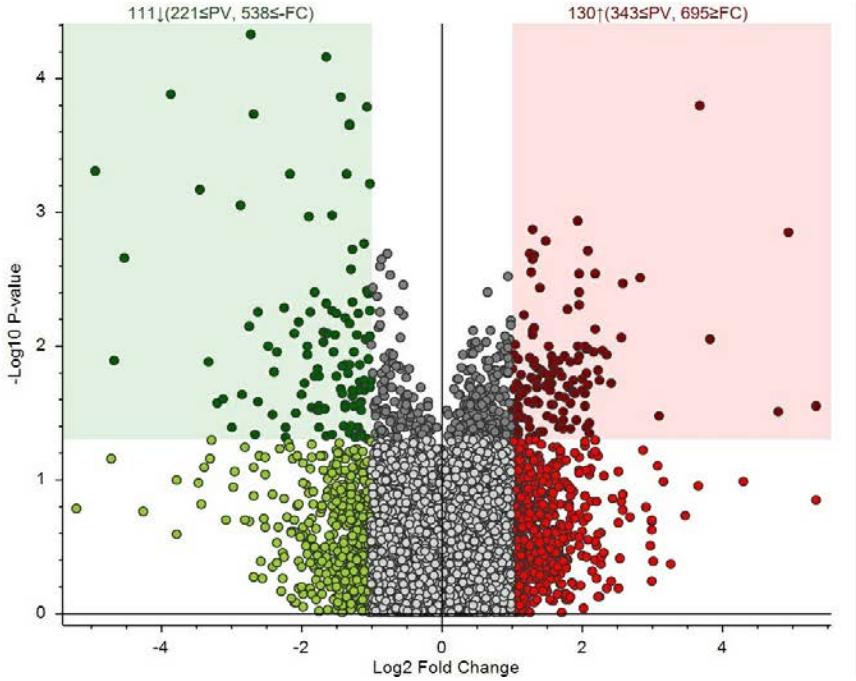

Figure 1. Volcano Plot of the Positive Ions. Among 14,396 identified ions, 564 ions showed*p*-value less than 0.05. 538 ions showed FC less than -1, and 695 ions showed FC more than 1.

A total of 27,319 ions were identified for the negative ions, and 1,077 ions showed a *p*-value of less than 0.05 in the concentration ratio (Fig. 2). 3,704 negative ions showed a FC of more than 1 or less than -1.

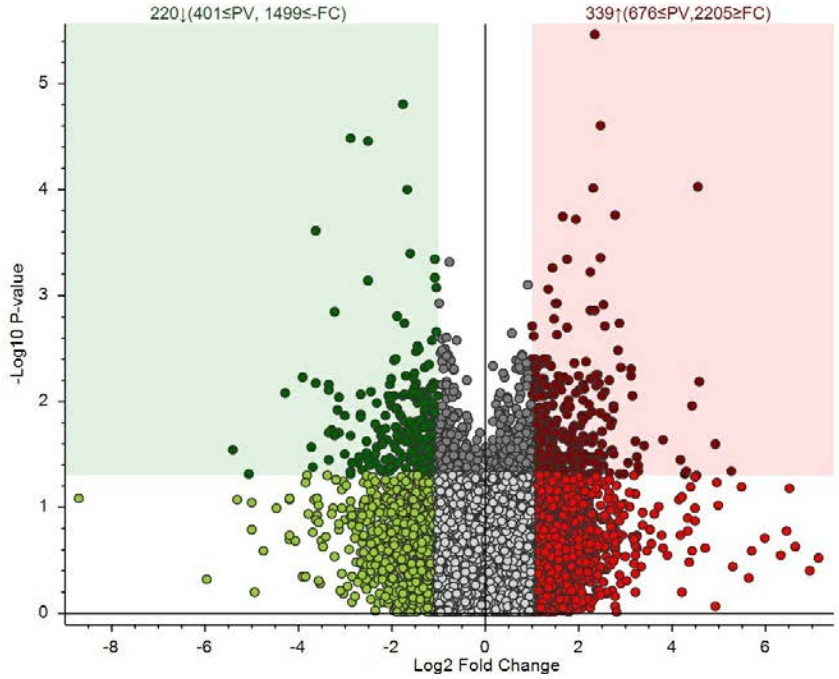

Figure 2. Volcano Plot of the Negative Ions. Among 27,319 identified ions, 1,077 ions showed*p*-value less than 0.05. 1,499 ions showed FC less than -1, and 2,205 ions showed FC more than 1.

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Comprehensively, 241 positive ions and 559 negative ions showed *p*-value of less than 0.05 and an absolute value of FC of more than 1. Principal component analysis showed well separated plots (Fig. 3 and 4).

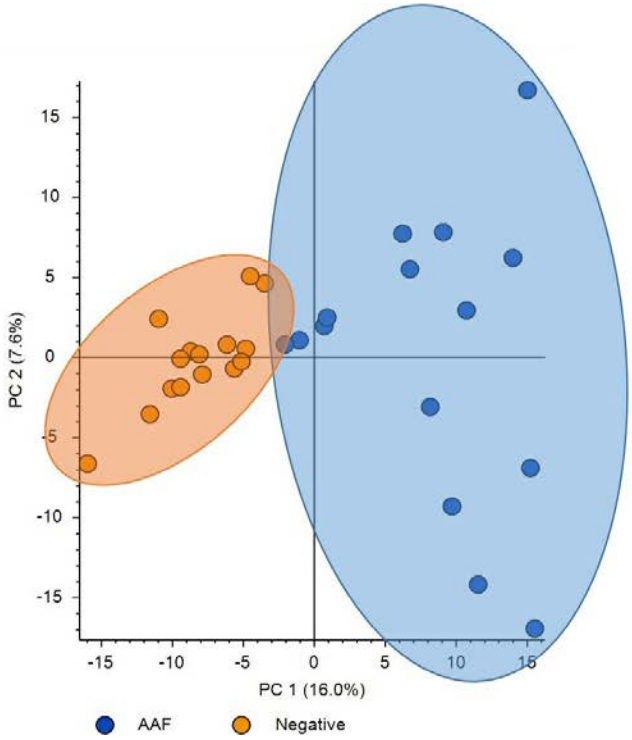

Figure 3. Principal Component Analysis of the Positive Ions whose *p*-value less than 0.05

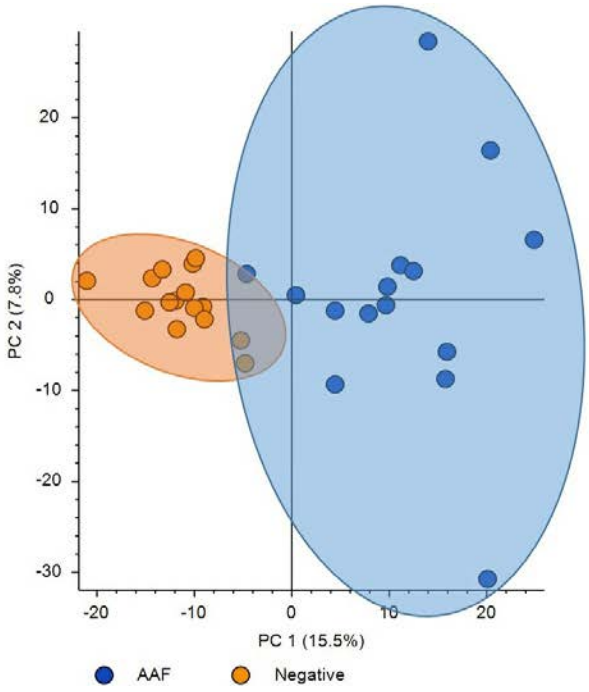

Figure 4. Prinicipal Component Analysis of the Negative Ions whose *p*-value less than 0.05

Bilirubin concentration increased in AAF cases (FC 0.54, *p*-value 0.014). It supports the fact that AAS administration is highly associated with bland cholestasis, a drug-induced hepatotoxicity with obstructed bile acid flow and finally leading to jaundice [3]. We assume that congested bile juice may have mixed into the sinusoidal bloodstream and finally excreted with urine.

The concentration of testosterone metabolites decreased (5α-dihydrotestosterone glucuronide FC -0.76, *p*-value 0.033; testosterone glucuronide FC -0.82, *p*-value 0.035). The secretion of testosterone, the endogenous AAS, is strictly regulated to maintain homeostasis [4]. We suggest that if exogenous AAS is brought into a human body, the endocrinology system reduces the secretion of endogenous testosterone.

The concentration of the estrogen metabolite increased (β-Estradiol-17β-glucuronide FC 1.18, *p*-value 0.016). As anabolic steroids act as a precursor of the female hormone, the circulating amount of estradiol in AAS users may have increased [5]. It corresponds with clinical observations that many AAS abusers eventually suffer from gynecomastia and testicular atrophy.

We suggest to test urinary levels of bilirubin and the excreted metabolites of testosterone and estradiol, to diagnose whether an athlete has administered AAS. As metabolomic approaches have been suggested and actually applied in the Athlete Biological Passport, our investigation may be worth applying to doping diagnosis.

Conclusions

In urine of AAS users, the concentrations of bilirubin and the estradiol metabolite are increased, whereas testosterone metabolites are decreased. Alteration of those three compounds supports humans' biochemical actions and clinical observations related to AAS administration. Therefore, we suggest that examining the concentrations of urine bilirubin, testosterone metabolites, and the estradiol metabolite to determine AAS administration is meaningful. As a part of indirect doping diagnosis which can supplement target analysis, investigating metabolomic profiles in urine may be one way.

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Danila G, Pop A, Toboc A, Stan C

7-Keto-DHEA – a case study

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Abstract

Among the urine samples analyzed in 2019, an unusual case from a male urine sample was identified. The presence of Arimistane metabolite (7-hydroxyandrost-3,5-dien-17-one) was detected without its parent compound, in conjunction with a low concentration of 6α-hydroxy-androstenedione (<10 ng/mL) and a very high concentration of 7β-hydroxy-DHEA (~1400 ng/mL). The athlete declared in his doping control form the use of *Anadrox*, a 3-acetyl-7-keto-DHEA containing nutritional supplement.

In order to elucidate the excretion profile after oral administration of *Anadrox* (easily available on the internet with no specified steroid quantity) and to identify possible markers for 7-keto-DHEA misuse, an excretion study was performed based on the administration of *Anadrox* supplement to two male volunteers.

We observed that the T/E ratios were not significantly affected in the present study, but administration of 3-acetyl-7-keto-DHEA affects the profile parameters in the same way by increasing their concentrations.

The results of the excretion study are confirming the results obtained in the athlete's sample which presented elevated concentration of 7α-hydroxy-dehydroepiandrosterone, 7β-hydroxy-dehydroepiandrosterone and 6α-hydroxy-androstenedione and the data is in accordance with the metabolism of 7-keto-DHEA which is partially metabolized into 7-hydroxylated DHEA.

Introduction

7-Keto-DHEA (5-androsten-3β-ol-7,17-dione) is included in the Prohibited List of the World Anti-Doping Agency (WADA) in the section S1.1 Anabolic Androgenic Steroids [1]. It can be produce endogenously as metabolite of dehydroepiandrosterone [2] but it also can be administered exogenously as nutritional supplement [3] as itself or as 3-acetyl-7-keto-DHEA. The main path of biotransformation in humans leads to the formation of 7β- and 7α- hydroxylated metabolites by reduction of the C7 keto group [4].

A sample analyzed during 2019 presented an elevated amount of Arimistane metabolite (7-hydroxy-androst-3,5-dien-17-one), but no sign of parent compound (3,5-androstadien-7,17-dione). The steroid profile did not trigger any suspicion but some other endogenous metabolites showed relatively high values. In his doping control form, the athlete declared a supplement called *Anadrox* which contains 3-acetylated-7-keto-DHEA. The supplement was bought from the internet and an excretion study was conducted with two healthy male volunteers (28 years, 178 cm, 78k g and 48 years, 195 cm, 130 kg).

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Experimental

Reagents

The enzyme β-glucuronidase from *E.coli*, used for the enzymatic hydrolysis, was purchased from Roche Diagnostics Division (Romania). Tert-butyl-methyl-ether, potassium hydrogen carbonate ($KHCO_3$), potassium carbonate (K_2CO_3), monosodium phosphate (NaH_2PO_4), disodium phosphate (Na_2HPO_4), ammonium iodide (NH_4I), N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) and ethanethiol (C_2H_6S) were purchased from Merck (Chimexim, Romania).

Testosterone (T), epitestosterone (E), androsterone (A), etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5α-Adiol), 5β-androstane-3α,17β-diol (5β-Adiol), dehydroepiandrosterone (DHEA), 7α-hydroxy-DHEA were purchased from Steraloids (USA), 4-androstenedione, 7β-hydroxy-DHEA and 7-keto-DHEA from NMI (Pymble, Australia), 6α-hydroxy-androstenedione from TRC (Toronto, Canada).

Instrumentation

The quantification of steroid profile parameters was performed on a Thermo Scientific GC Trace 1310 gas chromatograph system coupled with a TSQ Quantum XLS Ultra and a TriPlus RSH autosampler (all from Thermo Scientific, USA). The GC column – 17 m x 200 µm and 0.11 µm film thickness – was an HP-Ultra 1 from J&W Scientific (Agilent Technologies, USA). The temperature program was as follows: the initial temperature was 160°C (min), hold time 2 min, increased at 255°C with 5°C/min (held 0 min), then the temperature was increased to 285°C with 30 °C/min (held 5min), then to 300°C with 60°C/min (held 3.75 min). The transfer line temperature was set at 310°C. Helium 6.0 was used as carrier gas at a flow rate of approximately 1 mL/min. 2 µL of derivatized sample were injected in split mode (1:10) into GC. The acquisition was made in SRM mode and the collision gas was Argon at 1 mTorr.

Sample preparation

Common procedures for the detection of anabolic and endogenous steroids in doping control were applied: hydrolysis with β-glucuronidase from *E.coli* at pH=7 followed by a tert-butyl-methyl-ether extraction at pH=9, evaporation under oxygen-free nitrogen and desiccation, then derivatization with MSTFA/NH_4I/Ethanethiol (1000/2/3; v/w/v) [5].

Steroid profile parameters monitored in the study, including the ion transitions used for the quantification of steroids are presented in Table 1.

Parameter	Monitored transition (collision energy, eV)
Androsterone, Etiocholanolone	434>239 (17)
5α-,5β-Androstane-3α, 17β-diol	256>185 (15)
Testosterone, Epitestosterone	432>209 (9)
6α-hydroxy-androstenedione	518>319 (20)
4-androstenedione	430>209 (13)
Dehydroepiandrosterone (DHEA)	432>327 (13)
7-keto-DHEA	428>413 (9)
7α-hydroxy-DHEA	415>169 (8)
7β-hydroxy-DHEA	415>235 (18)

Table 1. Steroid profile parameters used in the study and the monitored transitions for the quantification

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Excretion study

The collection schedule included four negative urines and two types of administration according to the plan showed in Figure 1:

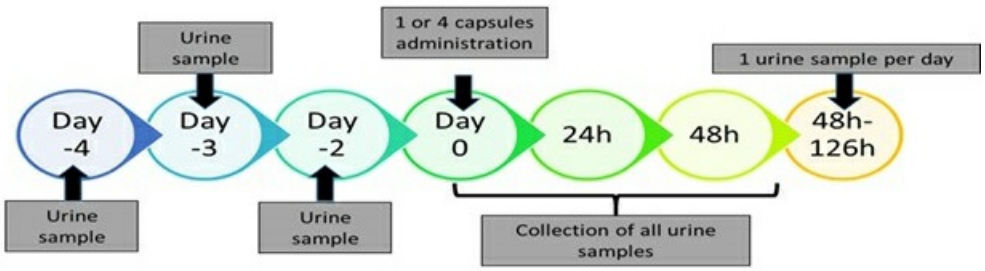

Figure 1. Timeline of the study: Administration of ANADROX capsules and collection of urine sample during the study

- One single administration of a single capsule and collection of all urine samples for the next 48 hours after ingestion. Morning urines were collected from 48 hours to 126 hours after administration.
- One single administration of the recommended dose (4 capsules) and collection of all urine samples after administration. One daily urine was collected every morning up to 48 hours after the ingestion of the dose.

All studies were approved by the Ethical Committee of the National Institute of Sports of Romania (Bucharest, Romania, approval #2283/2016) and written informed consent was provided by the volunteers.

Results and Discussion

The results obtained for the excretion study are presented in Figure 2.

- Markers of the steroidal passport showed no significant variations after *Anadrox* administration regardless of the number of capsules administered, with one exception for one volunteer at 13h after administration of one capsule. This could be more a disturbance than a tendency.
- Arimistane metabolite peaks are present up to 24 h when 1 capsule was administered and was still present after 28.5 h in case of 4 capsules administration.
- DHEA is not significantly influenced by 7-keto-DHEA administration and the results are in accordance with literature data [4].
- 7-Keto-DHEA is below 50 ng/mL for more than 90% of samples analyzed in 2019. In the excretion study, 7-keto-DHEA did not exeed 50 ng/mL for 1 or 4 capsules of *Anadrox* administered.

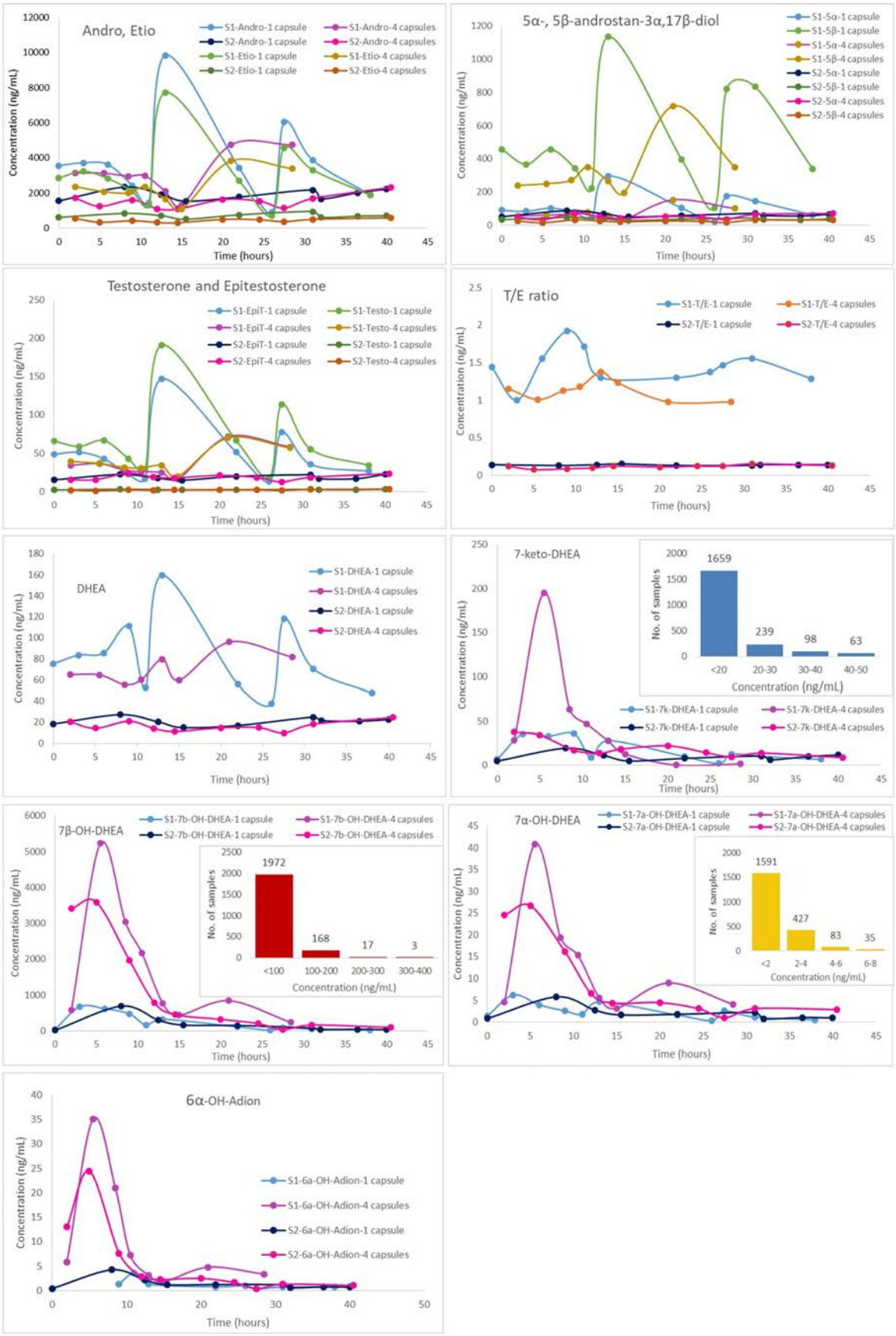

Figure 2. Obtained results for the excretion study

- 7α-OH-DHEA concentration is less than 4 ng/mL for ~90% of the samples analyzed for doping control purposes in 2019 in our lab. In the excretion study, 7α-OH-DHEA concentrations barely exceeded 5 ng/mL for 1 capsule and is greater than 10 ng/mL only up to 12h for 4 capsules.
- 7β-OH-DHEA concentration is usually less than 100 ng/mL. In the present excretion study, 7β-OH-DHEA concentration reaches a maximum after ~8 h after administration. This maximum corresponds to 650 ng/mL for 1 capsule and 5200 ng/mL for 4 capsules. In both cases concentration stays over 500 ng/mL for at least 12 h proving to be the most affected metabolite after oral administration of 3-acetyl-7-keto-DHEA. For this reason, 7β-OH-DHEA might be the most promising candidate to detect the misuse of 7-keto-DHEA.
- The doping control sample with similar behavior as the samples collected during the excretion study was analyzed in the Cologne Doping Control Laboratory based on a validated GC/C/IRMS method for 7-keto-DHEA and 7b-OH-DHEA [6] and the exogenous origin of 7-keto-DHEA was confirmed.

Conclusions

- The results obtained in the excretion study of *Anadrox* are similar to those obtained for the routine control urine sample which proves that the administration of the supplement could lead in a very high concentration of 7β-OH-DHEA and the presence of 6α-OH-androstenedione at a concentration unusually high, along to ion traces corresponding to Arimistane metabolite.
- Administration of *Anadrox* does not influence significantly the concentration of neither steroidal passport parameters nor DHEA.
- This excretion study shows that doping control laboratories should screen for 7-keto-DHEA (in accordance with The Prohibited List), and also for 7β-OH-DHEA together with 7α-OH-DHEA as additional target compounds.
- As long as there is no concentration threshold for this analytes, a GC/C/IRMS analysis to confirm the exogenous origin of 7-keto-DHEA is necessary.

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Geyer H, Fusshöller G, Haenelt N, Mareck U, Blatt C, Gougoulidis V, Hülsemann F, Piper T, Thevis M

The ratio 5aAdiol/E as indicator for the detection of testosterone doping in athletes with naturally low T/E – a case study

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Abstract

At the end of 2018, the Cologne Athlete Passport Management Unit (APMU) was able to detect testosterone doping in more than 30 doping control samples from athletes (mainly female athletes) with naturally low T/E ratios between 0.05 and 0.15 via the steroidal athlete's biological passport (steroidal ABP). The detection of these cases was mainly triggered by the increase of the ratios of 5aAdiol/E. The detection of these cases via the T/E ratios was difficult due to the low concentrations of testosterone. In all samples the T-doping could be proven by IRMS.

Introduction

Case report

At the end of 2018, the Cologne APMU became aware of several ABP cases with the following characteristics (see Figure 1):

- No or incomplete reference limits were available in the ABP graphs of the T/E ratio.
- At least one of the last samples were invalidated. In nearly all cases, the first invalidated sample has received an ATPF-CPR before invalidation.
- Samples, analysed after the invalidated samples, did not receive an ATPF-CPR although a clear increase of the T/E ratio was obvious.
- After the invalidation, the APMU did not receive any notification about an atypical sequence of T/E, although a clear increaseof the T/E ratio was obvious.

Experimental

The analyses of the steroid profiles were conducted according to the method described by Thevis [1]. The IRMS analyses were conducted according to the method described by Thomas et al. [2].

Results and Discussion

Results of further investigations:

- The missing reference limits in the T/E-graphs were due to many samples with T concentrations LOD ≤ [T] < LOQ.
- The invalidation of samples was due to an ATPF-CPR. The confirmation of T led to a ratio T_{free}/T_{total}

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>0.05, which led to an invalidation of a sample according to TDEAAS [3]. The particular laboratory did not conduct IRMS analyses of the invalidated samples.

- The consideration of the T/E data of the steroid profile table of the ABP case gave a clear picture of the increased T/E ratios of the invalidated samples (see Figure 2).
- For the samples with the increased T/Es, APMU-CPRs were sent to the passport custodian. All samples led to positive IRMS results (red bars in Figure 2).

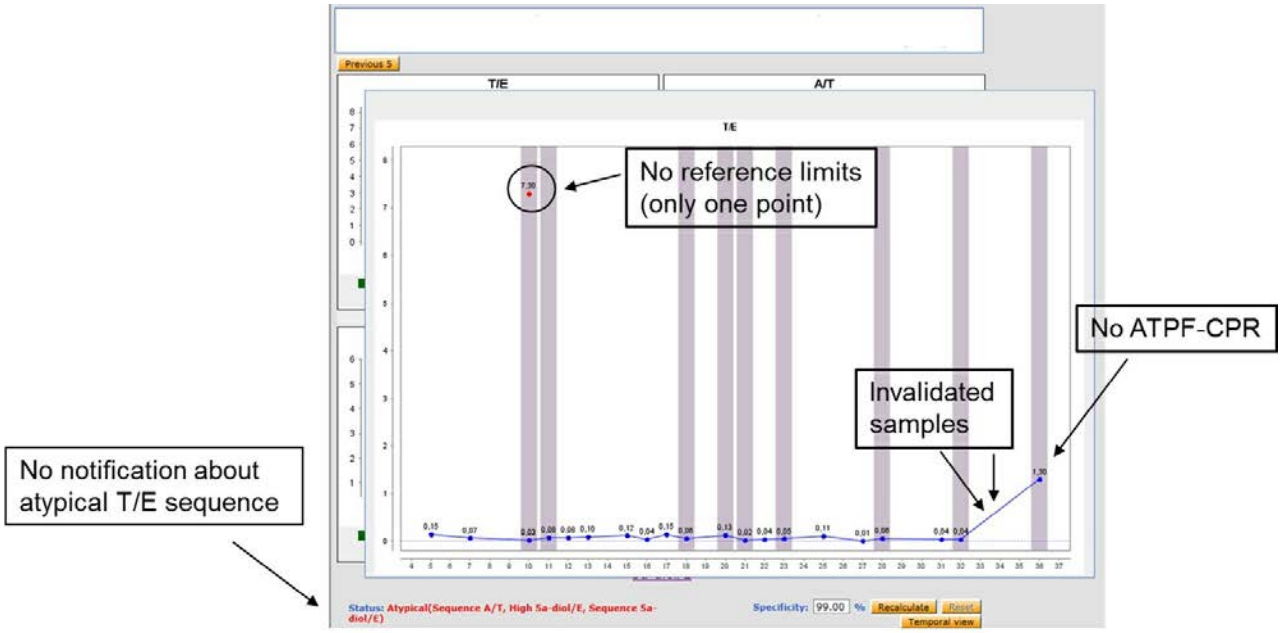

Figure 1. T/E graph of the steroidal ABP of an athlete with a naturally low T/E ratio. The last 3 samples (two invalidated samples and the last sample) led to positive IRMS results.

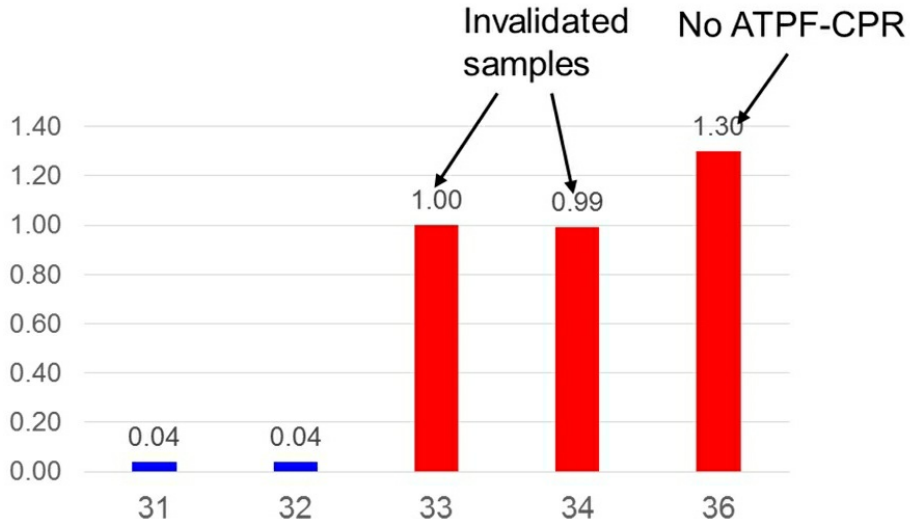

Figure 2. T/E ratios extracted from the ABP table of the case of Figure 1. Sample numbers correspond to last sample numbers of graph in Figure 1.

Consideration of the ratio 5aAdiol/E

The consideration of the graph of the 5aAdiol/E ratio leads to a clearer picture than the T/E graph (see Figure 3).

- Reference limits are available. The exceeding of the reference limits is clearly visible.
- Notifications of high 5aAdiol/E and atypical sequences of 5aAdiol/E are available

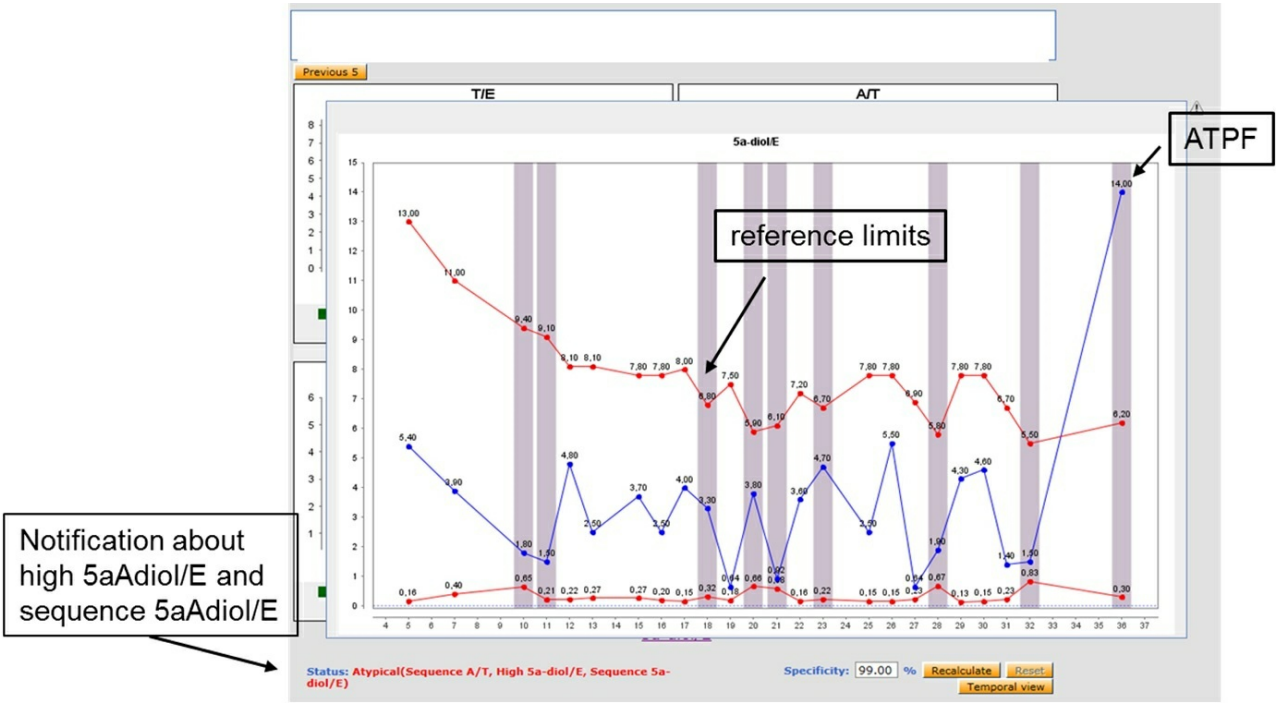

Figure 3. Graph of 5aAdiol/E ratios from the same ABP case as the T/E graph in Figure1

In Table 1, the ratios of T/E and 5aAdiol/E of the samples are shown, which led to positive IRMS results. All cases originate from athletes with a naturally low T/E ratio between 0.05-1.5. The suspicious samples were mainly detected via the increased 5aAdiol/E ratio.

T/E	5aAdiol/E
0.7	16
0.6	13
1.2	6.1
1.7	14
1.0	11
1.3	15
2.2	16
2.2	21
0.9	26
0.6	20

Table 1. Ratios of T/E and 5aAdiol/E of samples which led to positive IRMS results. All cases originate from female athletes with naturally low T/E ratios beween 0.05 - 0.15. The suspicious samples were mainly detected via the increased 5aAdiol/E ratios.

Conclusions

In cases with naturally low T/E ratios (most probably UGT2B17 del/del genotypes), the graph for the 5aAdiol/E may be the most discriminative tool to detect an application of T, especially T-gel. This observation is in consistence with publications, which describe the ratio 5aAdiol/E as most discriminative parameter together with the T/E ratio [4,5].

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Wen C, Liu X, Tianshuo Z, Jingzhu W

Isolation of boldenone and its metabolite in urine sample and further determination of the $^{13}C/^{12}C$ ratios by gas chromatography-combustion-isotope ratio mass spectrometry

National Anti-Doping Laboratory, China Anti-Doping Agency, Beijing, China

Abstract

Gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) is widely used by anti-doping laboratories to detect the synthetic form of endogenous anabolic androgenic steroids (EAAS)[1-4]. Boldenone (Bo) is one of the anabolic androgenic steroids which can occasionally be found in human urine in trace amounts. Bo and its metabolites belong to the list of prohibited substances of the World Anti-Doping Agency because they can be used as doping agents by athletes to enhance performance. Exogenous Bo and its main metabolite 5β-androst-1-en-17β-ol-3-one (BoM) can be differentiated from the synthetic drug by comparing the $^{13}C/^{12}C$ ratios (expressed as $\delta^{13}C_{VPDB}$ value of Bo and BoM to the endogenous reference compounds (ERCs). Sample purification process plays important roles in GC-C-IRMS analysis of Bo and BoM. The present method provides an easy approach to analyze Bo or BoM at concentration from 4 to 30 ng/mL in urine samples, which covers the mandatory requirements of the World Anti-Doping Agency. Only one HPLC purification procedure is required to isolate the target compounds and no derivatization is involved.

Introduction

Boldenone (androst-1,4-dien-17β-o-3-one, Bo) and its main metabolite 5β-androst-1-en-17β-ol-3-one (BoM) belong to class S1 substances in the prohibited list issued by World Anti-Doping Agency (WADA). When the concentrations of Bo or BoM are between 5 to 30 ng/mL in urine, gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) is carried out to determine the possible origin of the substance due to its endogenous production in human urine. The present work aims to develop a sample pretreatment approach with high isolation and enrichment capacity for GC-C-IRMS analysis. Only one HPLC purification procedure is involved and no derivatization is used. The present method provides an easy approach to analyze Bo or BoM at concentration from 4 to 30 ng ng/mL in urine sample.

Experimental

- Solid phase extraction: Sep-Pak C18 6 cc Vac Cartridge (9-15 mL of urine), 4 mL of MeOH and 6 mL of water for activation. 6 mL of water and 4 mL of MeOH for elution
- Enzymatic hydrolysis
- Liquid-liquid extraction: 4 mL of solvent (3/1 of n-Hexane to tert-Butyl methyl ether) for 2 times
- HPLC purification: column Kinetex 5 µm Biphenyl 100 Å 250 x 4.6 mm
- GC-C-IRMS analysis: column: DB-5MS (30 m x 0.25 mm i.d., 0.25 µm film thickness)

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Results and Discussion

Selectivity

One phenyl HPLC column with core-shell structure is selected instead of the common used C18 column. Figure 1 illustrates the GC chromatograms of the Bo fraction isolated from the C18 column and the phenyl column. The strong interfering compounds wrapped Bo completely indicating the poor isolation efficiency of the C18 column. The interfering compounds can be eliminated when the chromatography column is switched to a phenyl column in the present method. Ten aliquots of positive urine samples with 4 ng/mL of Bo and BoM are used to confirm the selectivity of the present method. As illustrated in Fig. 2, both Bo and BoM could be identified without any co-elution on GC chromatography, the $\delta^{13}C$ values are listed in Table 1. All the measured $\delta^{13}C$ values are similar indicating that the present method is stable.

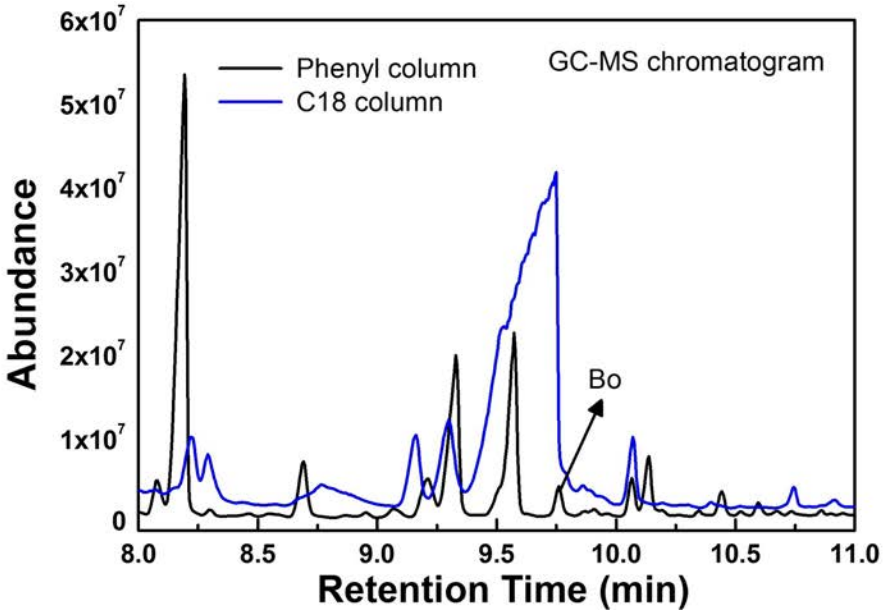
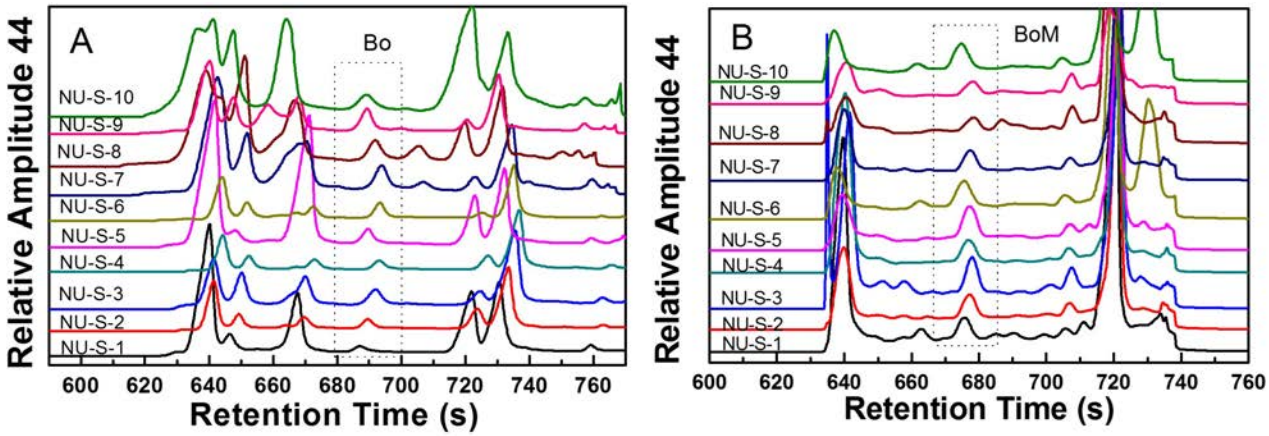


Figure 1. Gas chromatogram of same urine sample containing 4 ng/mL of Bo separated by two type of HPLC columns



Figue 2. GC-C-IRMS chromatograms for the ten spiked positive samples

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Repeatability and Method Linearity

Three aliquots of urine Bo and BoM at concentration of 4 ng/mL are used to determine the repeatability of the method. The urine samples are analyzed in three consecutive days. The results are listed in Table 2. Both Bo and BoM can be analyzed without any interfering co-elution and the values are quite similar to each other indicating the high repeatability of the present method.

Six aliquots of urine with Bo and BoM concentration ranged from 4 to 30 ng/mL are also analyzed to evaluate the method linearity. The measured SD of Bo and BoM are 0.34 and 0.29.

Sample	Bo		BoM	
	Ampl. 44 (mV)	$\delta^{13}C$ (‰)	Ampl. 44 (mV)	$\delta^{13}C$ (‰)
NU-S-1	414	-30.79	522	-30.82
NU-S-2	565	-30.15	555	-30.49
NU-S-3	611	-31.7	636	-30.82
NU-S-4	505	-31.72	448	-30.78
NU-S-5	555	-30.71	556	-31.34
NU-S-6	582	-31.21	509	-30.7
NU-S-7	711	-31.68	471	-30.8
NU-S-8	615	-30.9	425	-30.9
NU-S-9	605	-31.3	432	-30.81
NU-S-10	488	-31	559	-30.49
Mean		-31.12		-30.80
SD		0.48		0.22

Teble 1. Selectivity testing results

Conc.	Bo		BoM	
	Ampl. 44 (mV)	$\delta^{13}C$ (‰)	Ampl. 44 (mV)	$\delta^{13}C$ (‰)
4ng mL^{-1}	561	-30.95	814	-30.71
4ng mL^{-1}	615	-30.32	689	-30.61
4ng mL^{-1}	655	-30.88	713	-30.68
8ng mL^{-1}	915	-31.04	1068	-31.52
10ng mL^{-1}	1365	-30.55	1154	-30.85
20ng mL^{-1}	1621	-31.46	1030	-31.05
30ng mL^{-1}	1541	-30.78	1226	-31.08
Mean		-30.85		-30.93
SD		0.34		0.29

Table 2. Linearity and repeatability testing results

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Conclusions

The present method provides a straightforward GC-C-IRMS analysis strategy for Bo and BoM with only one-step HPLC purification process and no derivatization procedure involved. SPE procedure is conducted in the first step to remove some of the impurities in urine and enrich the sample into a small volume for further steps. The combined solvent used in the LLE process keeps high recovery of the analytes and avoid over extraction of urine matrix caused by MTBE. Selectivity, lowest detectable concentration, linearity and repeatability have been validated. 4 ng/mL of Bo or BoM can be measured by the present method using 24 mL of urine.

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Proposed suppression of phentermine derivatization by concomitant medication: a GC-MS case study and suggested solution

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Abstract

An in-competition female athlete sample arrived at the laboratory for which the doping control form (DCF) declared the following list of medications taken:

- metformin
- isotretinoin
- ***phentermine***
- “PAINKILLER”, most probably NSAID since no codeine was detected

Initial testing procedures (ITP) on GC-MS and HR-LCMS indicated a high concentration of phentermine. LC-MS/MS analysis proved no ortetamine (isobaric to phentermine) nor the presence of oxethazaine and metabolites. The presence of phentermine indicated by the ITP was thus as expected and confirmed the declaration on the DCF.
Confirmation analysis for phentermine is performed on GC-MS by repeating underivatized analysis and by trifluoro-acetic anhydride (TFAA) derivatization. In this case, however, the derivatization was not successful to confirm the presence of phentermine – no phentermine was detected. Repeat derivatization analyses on undiluted and different dilutions of the sample were also unsuccessful. Carbon disulfide derivatization was successful to indicate phentermine but since it was not the validated method, this could not be used to report an AAF.
Back extraction into an acidic aqueous phase succeeded in observing both underivatized and derivatized phentermine, adequate for positive identification so that an AAF could be reported. Performing the same back extraction on the reference standard in blank urine did not cause the same increase in peak intensity. Furthermore, the intensity of the underivatized phentermine in the sample following back extraction was slightly less than that in the original single extraction. This proves that the back extraction removed some factor(s) in the sample, which interfered with the TFAA derivatization process.
The conclusion of this observation is a probable competition for TFAA derivatization between compounds from the concomitant medication and phentermine. This effect could mostly be cancelled by acidic aqueous back extraction. Studies to identify a possible substance/s responsible for this effect and to develop optimal extraction procedures are being planned.

Introduction

Phentermine is a sympathomimetic anorectic that stimulates the hypothalamic release of norepinephrine. Phentermine was approved by the Food and Drug Administration (FDA) as an appetite-suppressing drug for short-term weight management in 1959 [1]. It is included in the Prohibited List of

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the World-Anti-doping Agency (WADA) as a non-specified stimulant (S6a)[2].The majority of phentermine suspects identified at SADoCoL came from the athletics sport discipline. The DCF of the sample received from this female athlete showed a few drugs, including Duromine, whose active ingredient is phentermine. The ITP for stimulants involves GC/MS analysis of the underivatized sample while the confirmation procedure (CP) for phentermine also includes derivatization with TFA-A. As expected, the ITP indicated a high concentration of phentermine, but it could not be confirmed using the usual validated CP method. Only following an additional clean-up step involving acidic back extraction, the derivatised phentermine could be identified for confirmation.

Experimental

Chemicals and reagents

Amber ampoules (5 mL and 10 mL) were purchased from Schott Kaisha PvT.LTD, India). Short Thread amber GC vials (1.5 mL) and 0.5 mL Micro-Inserts were from La-Pha-Pack, Thermo Fisher Scientific (USA). Sodium Hydroxide (NaOH), Sodium Sulfate ($NaSO_4$), Diphenylamine and Hydrochloric acid (HCL) were all purchased from Merk KGaA (Germany). Toluene and Ethylacetate were from Honeywell-Burdick & Jackson, (USA). *Tert*-Butyl Methyl Ether (TBME) and TFAA were purchased from Sigma-Aldrich (USA).

Analytical procedures

GC-MS ITP extraction procedure

Three milliliters (3.0 mL) of urine sample was pipetted into a 10 mL ampoule, to that 300 µL of 5M NaOH, 200 µL Toluene, 20 µL Diphenylamine (internal standard), 1-2 g $NaSO_4$ and 0.5 mL TBME were added. The ampoules were vortexed for 2 minutes and centrifuged for 5 minutes at 3000 rpm. The aqueous phase was separated by freezing and the TBME layer was transferred into GC vials and 2 µL injected onto GC-MS.

GC-MS CP for phentermine with TFAA derivatization

After performing the ITP extraction, the TBME phase was transferred to a 5 mL ampoule, evaporated to dryness under N_2 at room temperature. Dry ethyl acetate (50 µL) and 15 µL TFAA were added to the ampoules, the ampoules were sealed, vortexed briefly and allowed to derivatize at 60°C for 20 minutes. The seal was broken and contents evaporated to dryness at 60°C under N_2. The residue was dissolved in 100 µL toluene, vortexed and transferred into GC vials. Two microlitres were injected onto GC/MS.

Acidic back extraction of phentermine before TFAA derivatization

After performing the ITP extraction, the TBME phase was transferred to a 5 mL ampoule, 1.5 mL 0.07 M HCL was added and the mixture was vortexed for 2 minutes and centrifuged for 5 minutes at 3000 rpm to separate the phases. TBME phase was discarded and 2 mL clean TBME was added to the aqueous phase. After vortexing and centrifuging, the TBME phase was discarded again and 1.5 mL 5M NaOH was added to the aqueous phase to adjust the pH. Extraction into TBME using this new aqueous phase was done as in the ITP, after discarding the aqueous phase the TBME phase was derivatized as in CP above and 2 µL were injected onto GC/MS.

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Results and Discussion

Applying original methodology

Confirmation analysis for phentermine was performed on GC-MS by repeating underivatized analysis and by trifluoroacetic anhydride (TFAA) derivatization. In this case, however, the derivatization was not successful to confirm the presence of phentermine – no phentermine was detected in the sample (Figure 1). Repeated derivatization analyses on undiluted and different dilutions of the sample were also unsuccessful. Carbon disulfide (CS_2) derivatization was successful to indicate phentermine but since it was not the validated method, this could not be used to report an AAF. Initial testing procedures (ITP) on GC-MS and HR-LCMS indicated a high concentration of phentermine (Figure 2). LC-MS/MS analysis proved no ortetamine (isobaric to phentermine), nor the presence of oxethazaine and metabolites; β-hydroxy-phentermine and β-hydroxymephentermine (results not shown). The presence of phentermine indicated by the ITP was thus as expected and confirmed the declaration on the DCF.

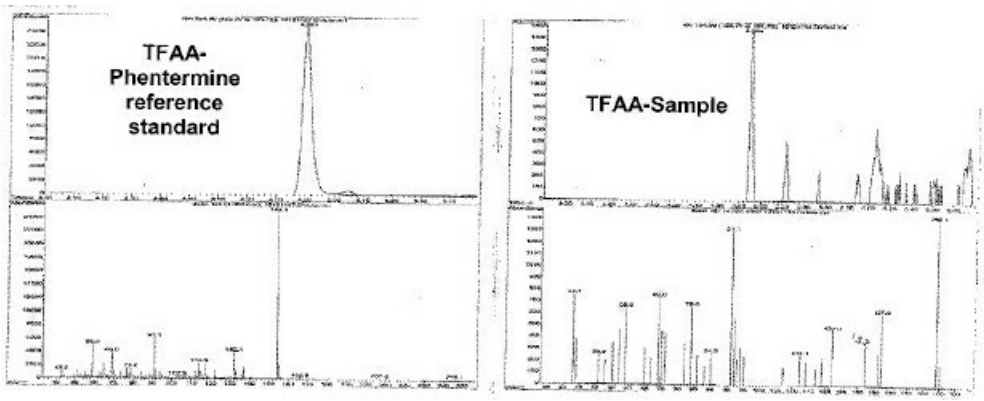

Figure 1. GC-MS CP results of Phentermine Reference Standard and the sample (derivatized with TFAA)

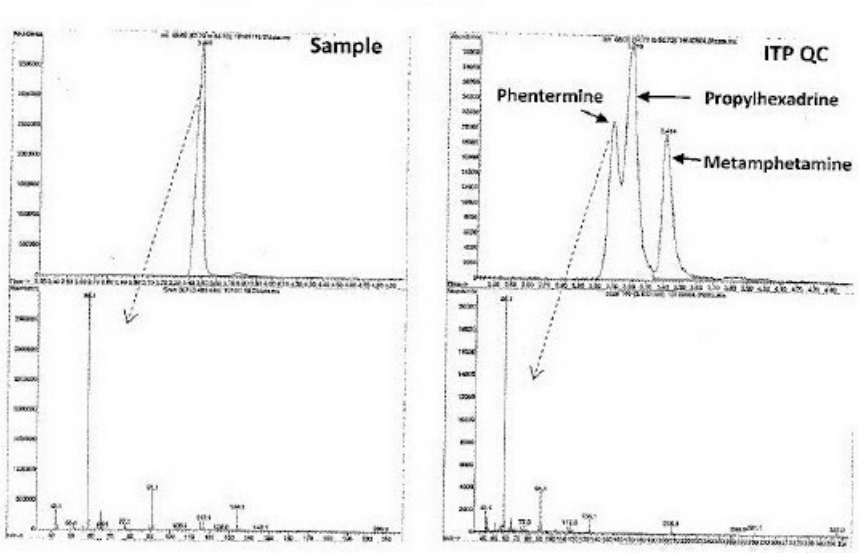

Figure 2: GC-MS ITP results of the sample and QC (underivatized)

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Implementing a clean-up step using acidic back extraction

Back extraction into an acidic aqueous phase [3] succeeded in observing both underivatized and derivatized phentermine (see Figure 3 for the derivatized product). These results provided a clear enough positive identification of phentermine which allowed an AAF to be reported. Performing the same back extraction on the reference standard in blank urine, did not cause the same increase in peak intensity. Furthermore, the intensity of the underivatized phentermine in the sample following back extraction was slightly less than that in the original single extraction. This proves that the back extraction removed some factor(s) in the sample, which interfered with the TFAA derivatization process (see also Table 1).

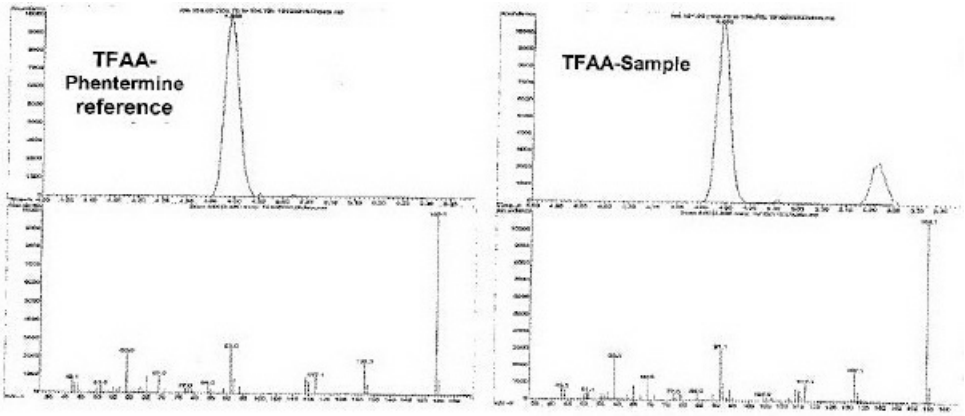

Figure 3. Following an acidic back extraction: GC-MS CP results of Phentermine Reference Standard and the sample (derivatized with TFAA)

	ITP without TFAA derivatization	CP with TFAA derivatization	Acidic Back Extraction before TFAA derivatization
Reference/QC in urine	+	+	+
Sample	+	-	+

+: Phentermine observed
-: Phentermine not observed

Table 1. Summary of results

Conclusions

It can be concluded that the sample contained a factor or factors that totally inhibited the TFAA derivatization of phentermine. These factor(s) were able to be removed by a clean-up step using an acidic back extraction. The factors may originate from the concomitant medication taken by the athlete as indicated on the DCF. Further studies are underway to investigate the nature of these factor(s). Preliminary investigations also indicate that an extended incubation period (60 minutes instead of 20

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minutes) for derivatization could abrogate the inhibitory effect observed during derivatization of the sample. This will also be further investigated.

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Kaewklum M, Kaewklum S, Dithayam S, Wilairat P, Kongpatanakul S

Separation and identification of regioisomers of fluoroamphetamine and fluoromethamphetamine in doping control by gas chromatography-mass spectrometry

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Abstract

4-Fluoroamphetamine (4-FA) and other positional isomers are psychoactive designer drugs often used for recreational purposes. At NDCC, using the validated ITP of liquid chromatography-electrospray mass spectrometry (LC-ESI-MS/MS), with Hypersil™ BDS C8 column, it was possible to detect 4-FA and the regioisomer (positional isomer) 3-fluoroamphetaminen (3-FA) but not their separation. This study was carried out to develop a method to separate and identify the regioisomers of fluoroamphetamine, i.e. 3-FA and 4-FA, and fluoromethamphetamine, i.e. 3-FMA and 4-FMA,and their trifluoroacetamides by GC-MS. The results showed that the developed GC-MS method could separate and identify all the regioisomers. The method was validated as confirmation procedure (CP) of these four stimulants. The limit of identification (LOI) of 3-FA/4-FA is 37.5 ng/mL and of 3-FMA/4-FMA is 50.0 ng/mL, respectively.

Introduction

4-Fluoroamphetamine (4-FA) (Fig.1b) and its positional isomers are psychoactive designer drugs often used as recreational drugs. These substances have a similar chemical structure to that of traditional stimulants with a change or addition of various chemical groups on the aromatic ring to evade the controlled drug group or drug laws [1,2]. At NDCC, using the validated ITP of liquid chromatography-electrospray mass spectrometry (LC-ESI-MS/MS) for analysis of stimulants, it was possible to detect 4-FA or the regioisomer (positional isomer) 3-fluoroamphetaminen (3-FA) (Fig.1a), but not their separation. This study was to develop a GC-MS method for the separation and identification of the regioisomers of fluoroamphetamine (3-FA and 4-FA) and fluoromethamphetamine (3-FMA and 4-FMA) (see Figure 1) as their trifluoroacetamide derivatives.

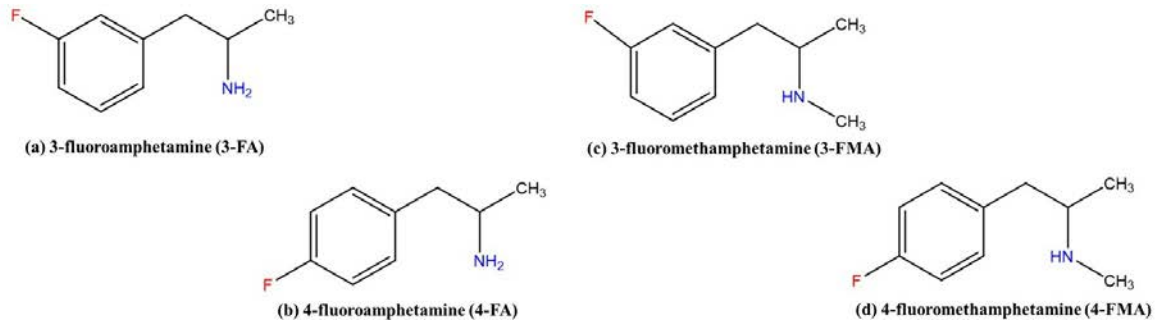

Figure 1. Chemical structure of regioisomers of fluoroamphetamine (FA) and fluoromethamphetamine (FMA): (a) 3-FA, (b) 4-FA, (c) 3-FMA, and (d) 4-FMA

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Experimental

Materials

The reference compounds were obtained from NMI (Australia). Diphenylamine (ISTD) was obtained from Fluka Chemika (Switzerland), and N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) and N-methyl-bis-trifluoroacetamide (MBTFA) were from Macherey-Nagel (Germany).

Sample preparation

A 10 µL aliquot of the ISTD (50 µg/mL), 0.2 mL of 5M KOH, 3 g anhydrous Na_2SO_4 were added to 2.5 mL urine. The sample was extracted with 5 mL of *tert*-butyl methyl ether, the organic layer separated and evaporated to dryness with nitrogen gas at 25°C. Derivatization was carried out with 100 µL of MSTFA (10 min, 80°C) followed by 10 µL of MBTFA (10 min, 80°C). The final solution was transferred into a GC insert vial for GC-MS analysis.

GC-MS analysis

Agilent GC 6890, 5975N MSD, and 7673 autosamplers were used. The capillary column was Phenomenex ZB-1 (100% dimethylpolysiloxane, 20 m, 0.25 mm id, 0.10 µm film thickness) with He gas at a constant flow rate of 1.0 mL/min. The inlet was at 280°C, with 2 µL injection at a split ratio of 10:1. The MSD employed electron ionization (EI) at 70eV, source temperature at 230°C, and SCAN/SIM switching mode. The GC temperature program was the initial temperature at 80 C, held for 1.2 min, raised to 103°C at 120°C/min, held for 5.0 min, raised to 300°C at 50°C/min, and held for 3 min, with total runtime of 12.33 min.

Method validation

Selectivity was performed with negative control urines from 10 different volunteers. The limit of identification (LOI) is the lowest concentration of the analyte which complies with the TD IDCR criteria for all 10 samples, after at least three serial dilutions from the MRL (50 ng/mL) to less than 50% of the MRL (25 ng/mL). The percent recovery was calculated from the peak areas of three negative control samples spiked at 100 ng/mL before and after extraction.

Results and Discussion

Figure 2 shows the mass spectra of the trifluoroacetamide derivatives of the four standard fluoro-compounds. The fluoroamphetamines have fragment ions at *m/z* 140, 136, and 109, and the fluoromethamphetamines at *m/z* 154, 136, and 110, respectively. Extract ion chromatograms of a negative urine sample spiked at 100 ng/mL (MRPL) of 3-FA, 4-FA, 3-FMA, and 4-FMA and negative control urine are shown in Figure 3. The peaks of 3-FMA and 4-FMA mono-TFA are completely separated. The peaks 3-FA mono-TFA and 4-FA mono-TFA are clearly identified (R=1.2), although not baseline separated. Diagnostic ions and mass ratios are shown in Table 1. The limits of identification (LOI) are less than or equal to 50 ng/mL and recoveries of all compounds are in the range 31 – 45%. No interfering signals were present in all 10 negative control urine samples at the retention times of the analytes and no carry-over was detected at 4x MRPL.

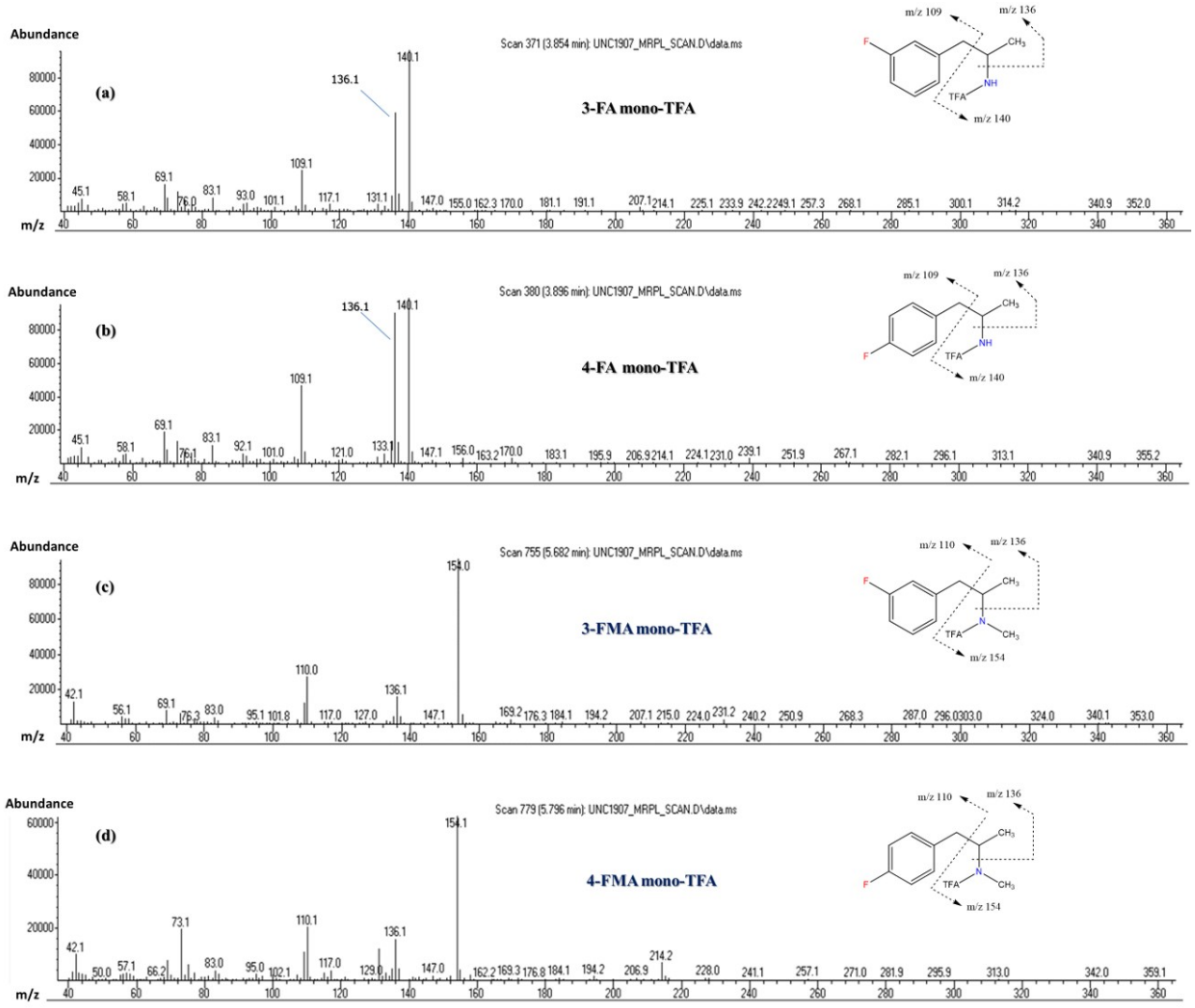

Figure 2. EI mass spectra of (a) 3-FA mono-TFA, (b) 4-FA mono-TFA, (c) 3-FMA mono-TFA and (d) 4-FMA mono-TFA and proposed fragmenatation.

Compound	Retention time (min)	Relative retention time	Molecular weight	Diagnostic ions (ratio) m/z 1	m/z 2	m/z 3	LOI (ng/mL)	Percent Recovery
3-FA mono-TFA	3.85	0.488	249	140 (100)	136 (59)	109 (23)	37.5	40
4-FA mono-TFA	3.89	0.493	249	140 (100)	136 (75)	109 (43)	37.5	45
3-FMA mono-TFA	5.68	0.721	263	154 (100)	136 (15)	110 (28)	50	31
4-FMA mono-TFA	5.79	0.735	263	154 (100)	136 (26)	110 (31)	50	37

Table 1. Retention and relative retention times, the molecular weight of trifluoroacetamides, diagnostic ions, mass ratios, the limit of identification (LOI) and percent recovery for GC-MS analysis of 3-, 4-fluoroamphetamines (3-FA, 4-FA) and 3-, 4-fluoromethamphetamines (3-FMA, 4-FMA) as trifluoroacetamide derivatives

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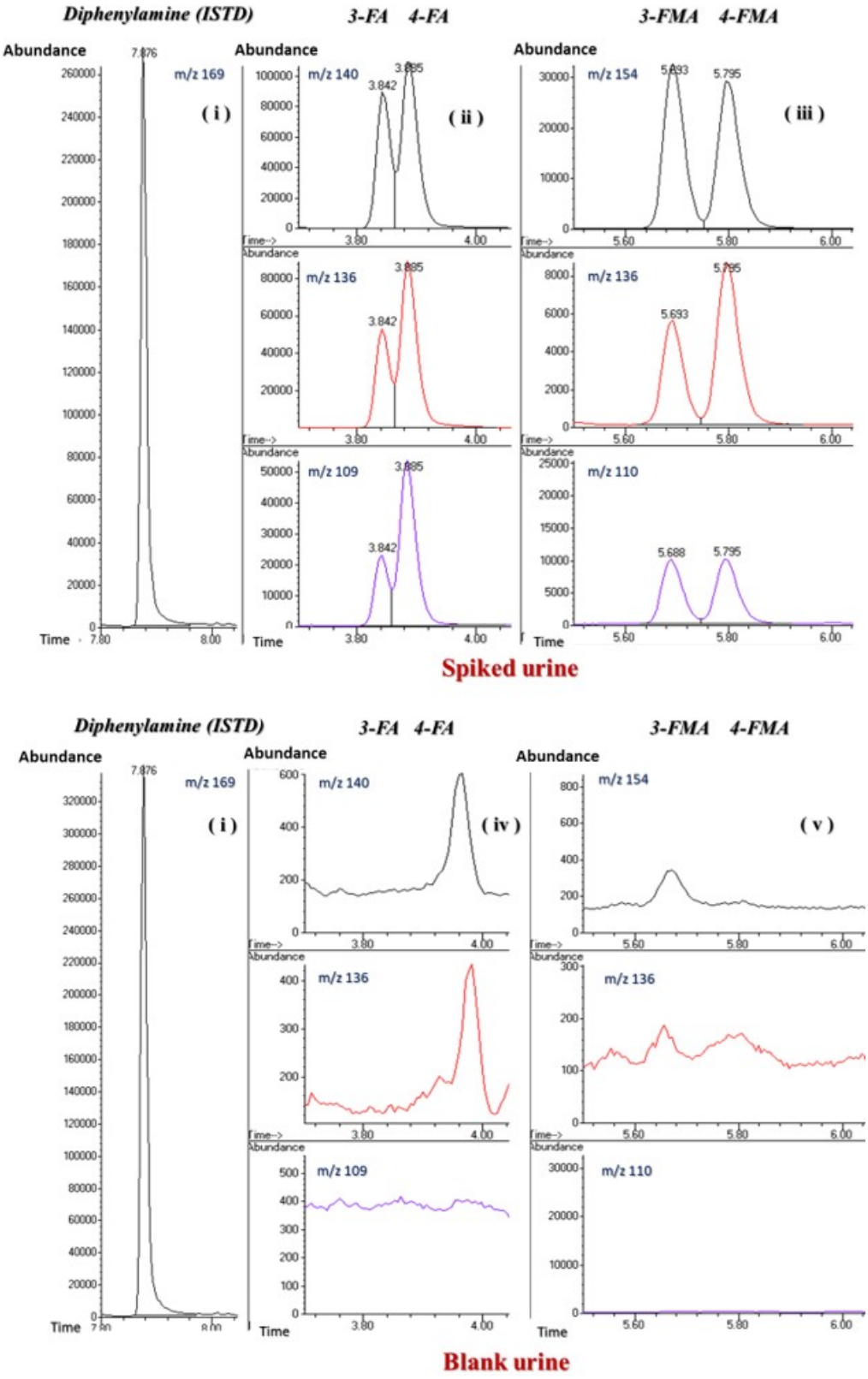

Figure 3. Extracted ion chromatograms of (i) diphenylamine (ISTD) for *m/z* 169 (upper spiked urine, lower blank urine), (ii) TFA-derivatives of 3-FA and 4-FA for *m/z* 140, 136 and 109 (retention time 3-FA 3.85 min, 4-FA 3.89 min), (iii) TFA-derivatives of 3-FMA and 4-FMA for *m/z* 154, 136 and 110 (retention time 3-FMA 5.68 min, 4-FMA 5.79 min), (iv) and (v) corresponding ions of blank urine sample.

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A previous work using LC-ESI-MS/MS, with a biphenyl column, separated all regioisomers of 2-, 3-, and 4-FA and 2-, 3-, and 4-FMA [6]. Nakazono et al. also separated all six isomers as their trifluoroacetamide derivatives by GC-MS using the DB-5 column [3]. Rösner et al., using GC-MS, could only achieve complete separation of 2-FA from the partial separation of 3-and 4- FA, as pure compounds and as acetylated and trifluoroacetylated derivatives [4]. They did not study the fluoromethylamphetamines. Similar results were reported by Weiß et al. for the fluoroamphetamines using chiral derivatizing agents [5]. The enantiomeric separation was achieved for each positional isomers.

Since it was not possible to separate these isomers in the initial testing procedure using the LC-MS/MS method for stimulants, the GC-MS separation of the trifluoroacetamide derivatives was developed as the confirmation procedure. The laboratory had previous experience with GC-MS for analysis of trifluoroacetylated derivatives of stimulants and diuretics.

Conclusions

Using GC-MS analysis of the trifluoroacetamide derivatives, it was possible to separate and identify the regioisomeric pairs 3-FA/4-FA and 3-FMA/4-FMA. The method is simple and validation complies with WADA criteria for the confirmation of non-threshold substances [7,8].

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In vitro studies of the metabolic profile of different classes of SARMs using liquid chromatographic-mass spectrometric techniques

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Abstract

The purpose of the present study was to characterize the most appropriate marker(s) of misuse of several SARMs, including steroidal and non-steroidal derivatives (arylpropionamide, isoquinoline-carbonyle and pyrrolidinyl-benzonitrile), by liquid chromatography coupled to mass spectrometry. The *in vitro* assays based on the use of pooled human liver microsomes were performed to identify the phase I metabolic profiles. The single recombinant cytochrome P450 isoforms, the influence of genetic polymorphism and possible drug-drug interactions were also considered. Samples were extracted with *tert*-butyl methyl ether and analysed by liquid chromatography coupled to mass spectrometry using both the time of flight and the triple quadrupole as mass analysers, in different acquisition modes. Our results showed that the SARMs under investigation were extensively metabolized by CYP3A4, subject to drug-drug interactions, followed by CYP2D6, characterized by genetic polymorphism. Particularly, the metabolic profiles of the steroidal, isoquinoline-carbonyle and pyrrolidinyl-benzonitrile derivatives have shown hydroxylation and dealkylation as the main biotransformation reactions; several metabolites were identified for arylpropionamide derivatives, corresponding to hydroxylation, *O*-dephenylation and nitro reduction. Our study also showed that antifungals cause a significant modification in the production of the metabolites; moderate or no significant variations were observed with antiacids or progestins.

Introduction

Selective androgen receptor modulators (SARMs) are androgen receptor ligands characterized by their ability to selectively modulate the activity of the receptor. They are classified into steroidal and nonsteroidal derivatives, taking into account the pharmacophore (see Table 1)[1,2]. Although they are not approved as drugs yet, they are sold on the black market and may be taken by athletes to increase their sport performance. Therefore, SARMs were included in 2008 in the section S1 of the Prohibited List [3]. We focused our study on the characterization of the most appropriate marker(s) of misuse of SARMs by LC-MS. We have considered compounds belonging to different groups in order to define a common metabolic pathway for each class, using *in vitro* assays based on the use of human liver microsomes (HLM).

Chemical Classes	*Compounds under investigation*
Steroidal derivatives	MK0773, TFM-4AS-1, Cl-4AS-1
Pyrrolidinyl-benzonitrile derivative	LGD4033
Isoquinoline-carbonyle derivative	PF06260414
Quinolinone derivative	LGD2226
Arylpropionamide derivatives	S6, Andarine, Ostarine, S9, S23, S1

Table 1. SARMs considered in this study, divided according to the chemical class

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Experimental

Instrumental conditions

The unknown metabolites were detected by a high-resolution mass spectrometry system in full scan mode to identify the characteristic *m/z*. The separation was performed with an Agilent 1290 Infinity II system (Zorbax Eclipse Plus C18). The mobile phase composition was ultrapurified water (A) and acetonitrile (B), both containing 0.1% formic acid. The gradient program starts with 5% B to 60% in 6 min after 4 min, to 100% in 4 min; the column was flushed for 2 min at 100% B and re-equilibrated at 5% B for 2 min (0.4 mL/min). Experiments were performed using an Agilent 6545 time-of-flight equipped with an ESI source operating in positive and negative. The fragmentation profiles of the metabolites were studied in product ion scan and an MRM method for metabolites and parent compounds with a triple-quadrupole system was developed: Acquity Waters I-Class (Supelco Ascentis® C18) coupled with an API 5500 ABSciex triple-quadrupole equipped with an ESI source operating in positive and negative were used. The separation was performed with the same gradient program described above.

In vitro studies and sample pre-treatment

The incubation medium contained 250 µL phosphate buffer 0.1 M and NADPH Regenerating System, 2.5 µL substrate (1 mg/mL), for the *in vitro* predictions of drug-drug interactions also 2.5 µL of the inhibitors (1 mg/mL); HLM or P450 isoforms (0.5 mg/mL) were added and the incubations were performed at 37°C (4 hours); 200 µL of acetonitrile were added; centrifugation at 21.000 g at room temperature for 5 min. Negative control samples were prepared to evaluate the potential non-enzymatic reactions. Phosphate buffer (200 µL, 0.8 M pH 7.4) and ISTD (17α-methyl-testosterone, 0.5 µg/mL) were added; analytes were extracted with 5 mL of *tert*-butylmethylether; the organic layer was separated and dried under nitrogen flow at 75°C. The residue was reconstituted in 50 µL of mobile phase (initial composition) and injected into the LC-MS systems.

Results and Discussion

The best condition for understanding the metabolic behaviour of the compounds under investigation were obtained using a substrate concentration of 30 µM and an incubation time of 4 h [4]. Regarding the steroidal derivatives, the main metabolic reactions involved are hydroxylation and demethylation: particularly, three mono-hydroxylated and one dealkylated metabolite were identified for Cl-4AS-1; three metabolites were found for TFM-4AS-1, two mono-hydroxylated and one dealkylated; six mono-hydroxylated metabolites and the dealkylated one were detected for MK0773. The CYP450 isoforms mainly involved are CYP3A4, CYP3A5, CYP2D6. The pyrrolidinyl-benzonitrile derivatives undergo hydroxylation and oxidation reactions, mediated mainly by CYP3A4 and CYP3A5 isoforms. The isoquinoline-carbonyle derivative PF-06260414 is characterized by hydroxylation and dealkylation reactions, mediated mainly by CYP3A4 and CYP1A2 isoforms; specifically, three hydroxylated and one de-alkylated metabolite were detected. The metabolic profile of the quinolinone derivative LGD2226 is characterized by the dealkylation as main metabolic reaction, whereas for LGD3303 no metabolites were detected.

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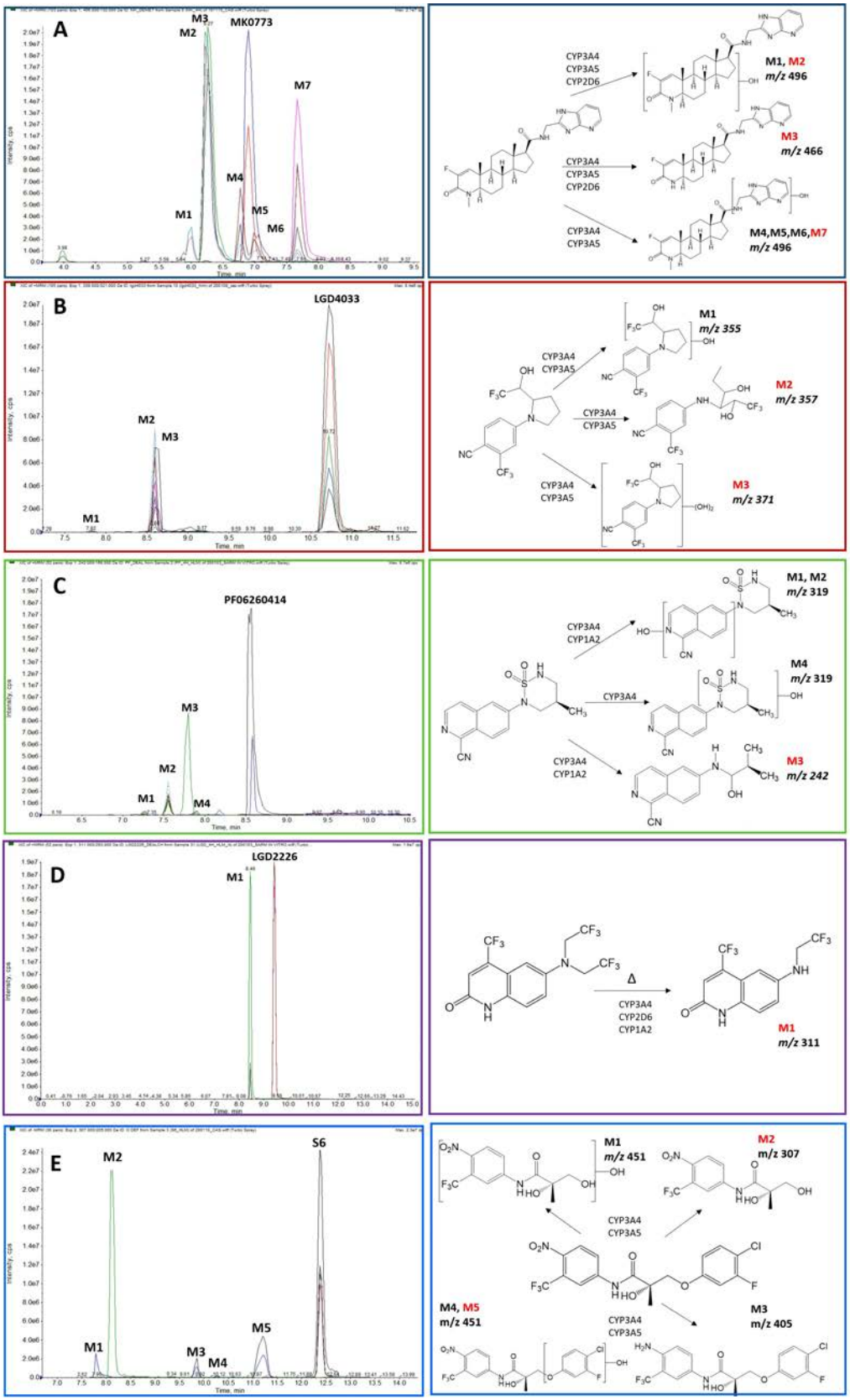

Figure 1. EIC of the *in vitro* incubations and hypothesized molecular structures of the metabolites of the prototype compounds of each chemical class under investigation: (A) MK0773; (B) LGD4033; (C) PF-06260414; (D) LGD2226; (E) S6

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The main reactions involved in the biotransformation of LGD4033, the pyrrolidinyl-benzonitrile, are hydroxylation and oxidation [5,6]. S6 has shown the same metabolic profile as the other, already studied, arylpropionamide derivatives [7-11]. The main reactions involved in the metabolism are hydroxylation, nitro-reduction and dephenylation. The results of the *in vitro* incubations and the postulated molecular structures of the metabolites of the prototype compounds of each chemical class under investigation are shown in Figure 1; the metabolites highlighted are the proposed markers of intake of the prototype compounds.

Preliminary data have shown inhibition of the metabolic reactions for the steroidal, pyrrolidinyl-benzonitrile and arylpropionamide derivatives by antifungals, specifically econazole, miconazole, tioconazole and ketoconazole; a partial inhibition was shown with itraconazole and posaconazole. Isoquinoline-carbonyle derivatives have shown a partial inhibition of the metabolic profile with some of the antifungal tested (i.e. econazole, miconazole, tioconazole, ketoconazole). Partial inhibition with progestin was shown for quinolinone derivatives.

Conclusions

The main metabolic reactions involved in steroidal derivatives are hydroxylation and demethylation, hydroxylation and oxidation for pyrrolidinyl-benzonitrile derivatives, hydroxylation and dealkylation for isoquinoline-carbonyle derivatives, dealkylation for quinolinone derivatives and hydroxylation, nitro-reduction and dephenylation for arylpropionamide derivatives. The single enzymatic isoforms mainly involved are CYP3A4 and CYP3A5, therefore, drug-drug interactions with CYP3A4 inhibitors, such antifungals, could alter the metabolic behaviour of these compound and consequently their detection in urine samples. Regarding the metabolic profile of arylpropionamide derivatives, S6 and andarine and S23 and ostarine undergo the same metabolic pathways with the most abundant metabolite (the *O*-dephenylated), in common with the non banned compounds flutamide and bicalutamide, respectively; this observation imposes a thorough examination of the entire metabolic profile before reporting an adverse analytical finding.

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Songsaeng R, Saardpun N, Kusamran T, Kongpatanakul S

Method Development for Identification of Growth Hormone Releasing Peptides (GHRPs) in Doping Control by UFLC/MS-IT-TOF

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Abstract

Growth hormone releasing peptides (GHRPs) are synthetic peptides that stimulate growth hormone secretion. GHRPs are illicitly used for enhancing sports performance, especially for muscle growth. Different methods have been developed for identification of the prohibited GHRPs and their metabolites using liquid chromatography (LC) coupled with various types of mass spectrometers (MS). Recently, the detection capability of these small peptides has been improved by the addition of dimethylsulfoxide (DMSO) to the mobile phase. In this study, a method was developed for identification of GHRPs using solid-phase extraction followed by UFLC separation and DMSO assisted electrospray ionization coupled with an IT-TOF mass spectrometer. The benefit of DMSO in increasing detection sensitivity was observed similarly to other previous reports. Twenty-three GHRPs and their metabolites included in the WADA Prohibited List were identified with a limit of detection (LOD) compliant to the minimum required performance levels (MRPL) established by WADA. The method was validated and found fit-for-purpose in routine initial screening and confirmation for doping control analysis. This investigation demonstrated the establishment of a validated methods fit-for-purpose in the screening and confirmation of GHRPs by IT-TOF mass spectrometry.

Introduction

GHRPs have been included in section S2 of the World Anti-Doping Agency (WADA) Prohibited List since 2015. Different analytical methods employing LC and tandem mass spectrometry techniques have been reported to detect these compounds [1]. A method using ultra-fast liquid chromatography (UFLC) coupled with an Ion-Trap Time of Flight (IT-TOF) has been developed at the NDCC and implemented for routine screening and confirmation [2]. However, the method has been constrained by inverse association between increasing number of the target analytes and detection sensitivity. Recently, improved sensitivity for the detection of small doping peptides has been demonstrated by addition of DMSO in the mobile phase [3,4]. Therefore, the aim of this work was to investigate the DMSO-enhancement on the detection sensitivity of the doping peptides by the UFLC-MS/IT-TOF.

Experimental

Sample preparation

Three mL of blank urine was spiked with GHRPs standard solution to the total final concentration of 2 ng/mL (MRPL [5]), followed by adding 200 µL of phosphate buffer (0.8 M, pH 6.8), before centrifugation

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at 5,000 rpm for 5 min. The supernatant was collected, added with the ISTD ([deamino-Cys^{1}, Val^{4}, D-Arg^{8}]-Vasopressin) and loaded into the WCX (Oasis®) cartridge preconditioned with methanol (1 mL) and ultra-pure water (1 mL). The cartridge was washed with 1 mL each of water and methanol-water (10%, v/v). The sample was eluted with 1 mL of 10% formic acid in methanol (freshly prepared). The eluate was collected and dried in a vacuum centrifuge at 45°C for 2 h. The residue was reconstituted with 50 µL of 0.2% formic acid in water (freshly prepared), and injected into the UFLC-MS/IT-TOF.

Instrumentation

The sample was analyzed by a UFLC/MS-IT-TOF (Shimadzu) using the analytical column Poroshell 120 EC-C18 (2.1 mm x 100 mm x 2.7 µm) at flow rate of 0.2 mL/min with 12 min of total run time. Mobile phase A was 0.2% formic acid in water containing 1% DMSO (v/v) with acetonitrile as mobile phase B. The gradient started at 20% B, raised to 30% in 2 min, then to 45% in 4 min, and finally to 100% in 0.5 min, and staying for 1 min, followed by re-equilibration at 20% B for 4.5 min.

Method validation

For determination of the LOD, 10 different sources of human urine spiked with the GHRPs target compounds at concentrations between 0.2 – 2.0 ng/mL were analyzed. Three sources of blank urines were used for determination of the recovery and matrix effect at the MRPL (2 ng/mL), and 10 repeated injections for the instrumental precision.

Results and Discussion

A significantly increased signal abundance of the target analytes was observed between 2 – 20 fold after the addition of 1% DMSO in the mobile phase A (Figure 1). This observation is similar to other previous reports which used dilute-and-shoot sample preparation coupled with LC-HRMS (Orbitrap) since adding DMSO into the mobile phase helps more effective ionization in the electrospray process [3,4]. However, decreasing abundances (0.90 – 0.03 fold) was observed in the present investigation for desmopressin, GHRP-3, GHRP-1 (2-4) free acid, and GHRP-6 (2-6) free acid. Whereas felypressin and hexarelin (1-3) free acid showed unchanged abundance using DMSO. Different enhancement efficiency patternby DMSO of some peptides observed in this study from the previous reports [3,4,6] may reflect the detection capability of different types of mass analyzers, ex. orbitrap and triple quadrupole.The charge state reduction from double charge (+2) to single charge (+1) was observed for desmopressin and alexamorelin similar to the previous report.This is probably because the high gas-phase basicity of DMSO (charge stripping) [3]. In addition, charge state reduction was also observed for GHRP-2, AOD9604 and Ipamorelin (1-4) free acid. This may be due to the ion accumulation in the ion-trap mass analyzer. By using high resolution mass spectrometry, recent developments show that the direct injection of native urine enables to screen for small peptides in urine samples with also meet the criteria of the WADA [3,4]. A similar approach will be further explored to escalate efficiency of the herewith presented method.

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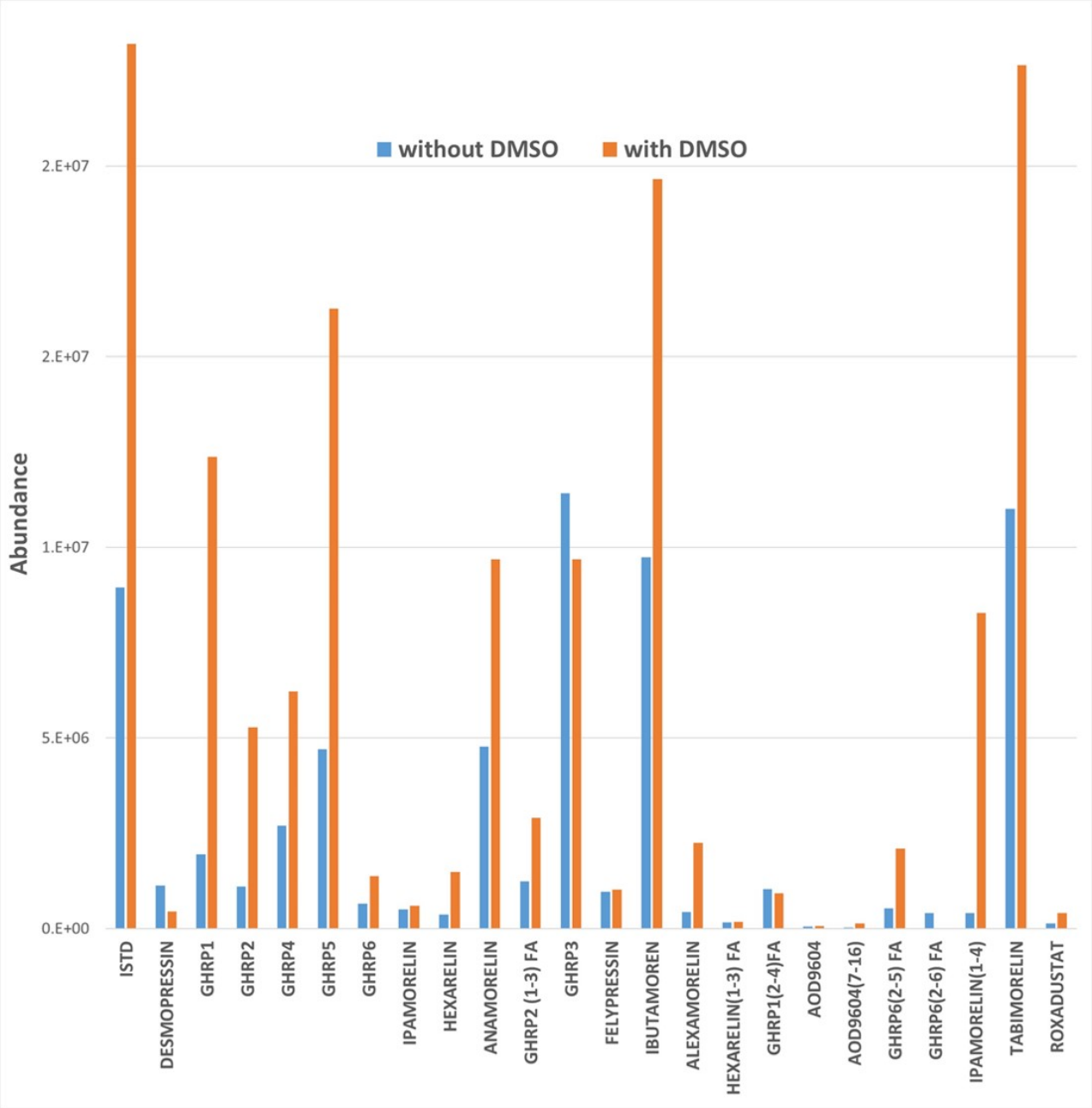

Figure 1. Comparison of MS signal intensities of different 23 GHRPs by addition of 1% DMSO in the mobile phase

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The method validation results showed that the limit of detection (LOD) was in the range of 0.2 – 1 ng/mL. The instrument precision and the recovery were in the range of 4 – 15 % and 27 – 96 %, respectively (Table 1). This indicates that this method is fit-for-purpose and complies with WADA requirements.

No	Compound	Ion transition	RT (min)	LOD (ng/ml)	Recovery (%)	Matrix effect (%)	Instrument precision (%CV)
1	GHRP1	478.2 → 209.1	4.25	0.2	45	40	4
2	GHRP1(2-4) FA	424.2 → 335.1	2.44	0.5	74	91	9
3	GHRP2	819.4 → 673.3	5.42	0.2	58	112	5
4	GHRP2(1-3) FA	358.2 → 269.1	4.53	0.2	55	33	12
5	GHRP3	328.2 → 384.3	2.31	1.0	86	12	9
6	GHRP4	608.3 → 444.2	6.38	0.5	64	49	4
7	GHRP5	771.3 → 754.3	7.10	0.2	73	92	6
8	GHRP6	437.2 → 324.1	3.45	1.0	61	23	12
9	GHRP6(2-5) FA	609.2 → 335.1	6.83	0.2	88	30	8
10	GHRP6(2-6) FA	369.2 → 346.7	5.09	1.0	27	14	13
11	Ipamorelin	356.7 → 223.1	1.98	1.0	96	5	12
12	Ipamorelin(1-4) FA	586.3 → 420.2	4.50	0.2	96	65	8
13	Hexarelin	444.2 → 248.1	3.60	0.5	48	26	13
14	Hexarelin(1-3) FA	427.2 → 273.1	1.75	1.0	63	15	8
15	Alexamorelin	480.8 → 209.1	3.68	1.0	59	27	8
16	Felypressin	520.7 → 226.1	2.46	1.0	56	11	7
17	Ibutamoren	529.2 → 267.1	7.08	0.2	80	79	5
18	AOD9604	907.9 → 835.4	3.53	0.5	78	61	13
19	AOD9604(7-16)	521.7 → 879.3	2.47	1.0	91	4	13
20	Anamorelin	547.3 → 276.2	7.97	0.2	70	64	4
21	Desmopressin	1070.0 → 328.2	3.85	0.5	45	66	9
22	Tabimorelin	529.2 → 280.1	7.08	1.0	61	35	12
23	Roxadustat	353.1 → 278.1	9.36	0.5	63	68	15

Table 1. Method validation for GHRPs identification (2 ng/mL) with DMSO in the mobile phase

Conclusions

Addition of DMSO to the mobile phase markedly improved detection sensitivity of most of the GHRPs investigated in doping control by using UFLC coupled with a tandem Ion-Trap Time of Flight mass spectrometer. However, detection sensitivity of a few compounds was found unchanged. Charge state reduction was commonly observed in many target compounds. The validated method was established and found fit-for-purpose for routine screening analysis. Although these new methods are suitable for initial testing procedures, the herewith presented report is a good alternative for a confirmation procedure.

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Ahi S, Dubey S, Bhardwaj A, Sahu PL

Fast and sensitive detection of small peptides in human urine using liquid chromatography tandem mass spectrometry

National Dope Testing Laboratory, New Delhi, India

Abstract

The detection of small peptides in doping control urines remains a challenge for the anti doping laboratories due to the required detection limit and effect of urine matrix on extraction efficiency. Though in the last decade, many advances have been made, yet their analysis in routine samples remains critical and sensitive. National Dope Testing Laboratory India incorporated small peptides in its test menu in the year 2016. The method currently in use comprises solid phase extraction followed by liquid chromatographic tandem mass spectrometric (LC-MS/MS) analysis. Although the method was fit for use in routine analysis according to the minimum requirements of World Anti Doping Agency (WADA), there were few impediments like longer run-time and less sensitivity for few peptides like Alexamorelin, AOD9604 etc.

Considering the need of improving the existing screening method, a fast and sensitive method for the detection of thirty eight small peptides (including desmopressin, felypressin, GHS, GHRPs, GnRHs and their target metabolites) was developed on LC-MS/MS. The main objective of this study was to reduce the analysis turn-around-time while preserving the required sensitivity and specificity. The new method had half of runtime as compared to the older method and also provided increased sensitivity for most of the target analytes. The method was successfully validated according to the prevalent WADA requirements. The limits of detection (LODs) were in agreement to the requirements of WADA. The applicability of the method was studied by analyzing the retained positives urine and WADA EQAS samples.

Introduction

The small peptides have paved their way in sports doping almost a decade back. The use of substances is forbidden in sports by the WADA [1]. In spite of considerable advancements in detection strategies for small peptides, their identification in doping control samples is a challenge for the anti-doping laboratories [2,3]. The existing initial testing method for small peptide in NDTL included the detection of 32 analytes using liquid chromatography tandem mass spectrometry in a 20 minutes run. The method had few impediments like a longer run time and less sensitivity for few analytes *i.e.* Alexamorelin, AOD9604, etc. Hence, the aim of this study was to improve the existing screening method for small peptides by reducing the analysis turn-around-time and preserving highest possible sensitivity and specificity.

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Experimental

Reagents and standards

The reference standard were purchased from different sources viz. Sigma Aldrich (St. Louis, USA), AB Bio Technologies, Inc (Bloomington, USA), Toronto Research Chemical (Canada, USA), MedChem Express (USA) & Auspep (Melbourne, Australia). All reagents and chemicals used were of analytical or mass spectrometric grade. Mixed-mode solid-phase extraction cartridges (Oasis WCX 3cc) were obtained from Waters Corporation, (Milford, USA). The deionised water was obtained from in-house Millipore system. The standard stock solutions were prepared in peptide carrier (bovine insulin 1 nmol/mL in 0.2% formic acid). Further three different working mixtures were prepared using the same carrier at required concentrations to prepare quality controls.

Sample extraction and instrumentation

The extraction involved the steps presented in Figure 1. The analysis was performed on Waters XEVO-TQ-XS coupled to Acquity UPLC using the analytical conditions laid down in Table 1.

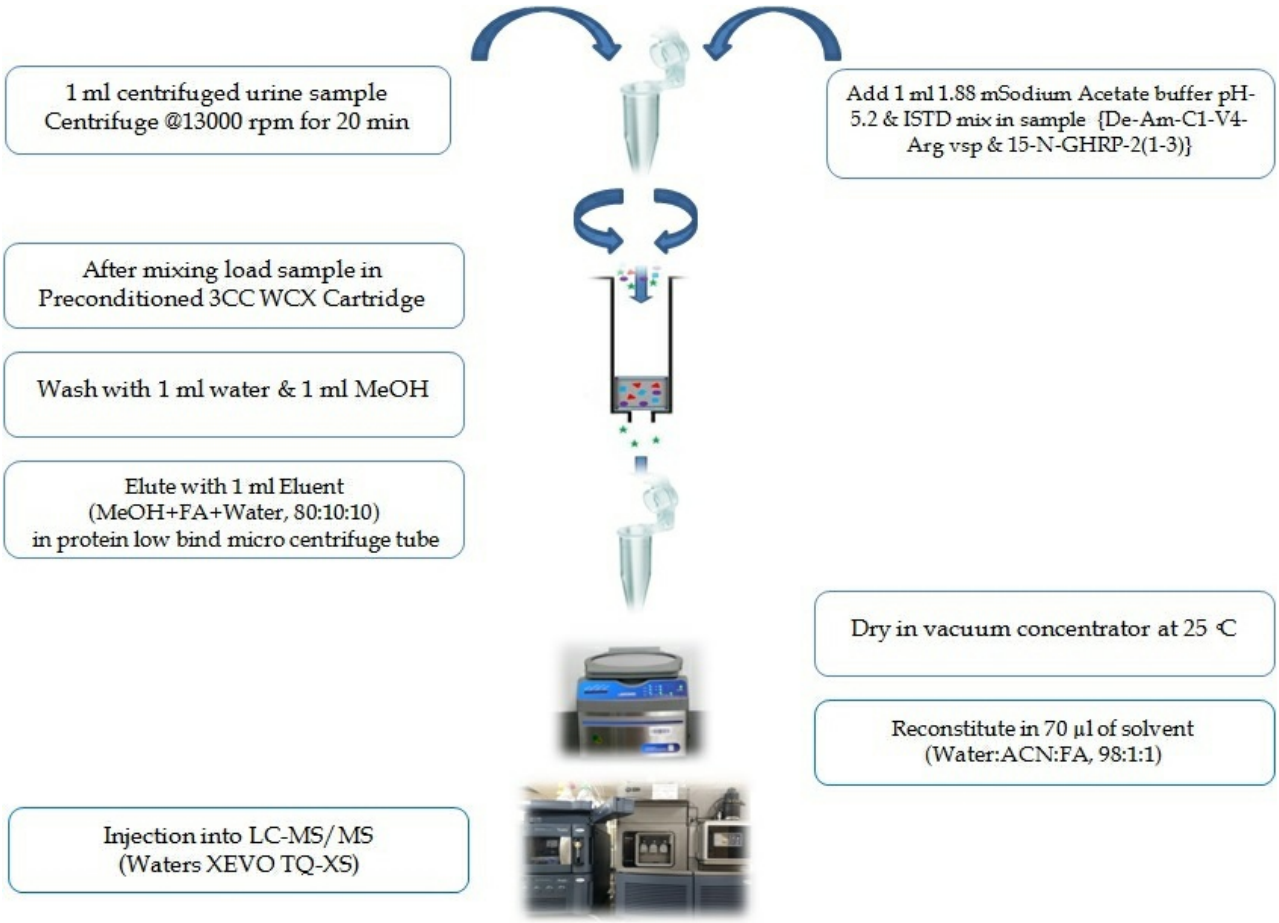

Figure 1. Details of sample preparation protocol

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Acquisition method ID	SMALL PEPTIDE ITP_01L
Tune method ID	LCMS_POS_NEG2
Column	Aeris 3.6 um peptide XB-C-18-100*2.1 mm (Phenomenex)
Solvent A	0.1 % Formic acid in water
Solvent B	0.1 % Formic acid in ACN
Flow rate	0.5 ml/min
Run time	10 mins
Injection volume	10 µl
Gradient	The gradient started at 0% B for 2 mins, raised to 15% B in 4 mins, hold for 1 min, raised to 60% B in 6 mins, hold for 0.5 mins followed by re-equilibration at 0% B in 10 mins
Ionization/ polarity	ESI positive
Collision gas	Argon
Source temperature	450
Scan Mode	MRM
Mass Resolution	Q1 : 1.2 amu ; Q3 - 0.7 amu

Table 1. Details of instrument parameters

Method validation

The method validation was performed as per the requirements of effective WADA ISL [4]. For identification capability, 10 different blank urines were fortified with peptide standards at 100%, 50% and 25% of the minimum required performance limit (MRPL). For selectivity and specificity 20 different blank urine samples were analyzed and monitored for interfering signals at the expected retention of target peptides. The extraction recoveries (n=5) were estimated by comparing analyte peak area in pre-extraction QCs with that of post extraction QC for each analyte at MRPL. The LODs were estimated by estimating S/N of each analyte spiked in ten different urines at different concentrations. For carryover QCs were prepared and injected at higher levels *i.e.* of 200% and 500% of MRPL followed by three blank urines. For robustness and stability six different urine samples were spiked at MRPL with slight modification in the sample extraction step.

Applicability of the method

The applicability of the developed method was assessed by analysing the retained positive urine samples from WADA external quality assurance scheme (EQAS) and routine doping control samples available in the laboratory.

Results and Discussion

The developed analytical method was able to detect thirty eight small peptides belonging to different categories (Gonadotrophin-releasing factors (GnRHs)-10, Growth hormone Secretagogues (GHS)-6, Growth hormone releasing peptides (GHRPs)-15, hGH fragments & Mechano Growth Factors (MGFs)-5 and masking agents-2 in a runtime of 10 minutes. The weak ion exchange mixed-mode sorbent cartridges used for sample extraction have provided selectivity in removing interfering matrix

components from urine samples to give relatively clean extracts. The use of peptide separation technology C-18 Column (100 Å pore size) and combination of 0.1% formic acid in water: 0.1% formic acid in acetonitrile in gradient mixing at a constant flow rate of 500 µL/minutes yielded sharp and symmetrical peaks for all the peptides in ten minutes of chromatographic run.

DRUG	MOL WT.	CHARGE STATE	MRM TRANSITIONS (PARENT/DAUGHTER IONS)	CONE (V)	COLLISION (eV)	RT (mins)	CV% (RT)	LOD ng/ml	RECO-VERY %
ALEXAMORELIN	958.1	3+	320/144	4	12	5.53	0.09	0.5	76
ANAMORELIN	547	1+	547.48/276.2	18	16	6.14	0.00	0.1	112
AOD 9604	1815	3+	605.96/797.29	46	16	5.73	0.09	0.5	58
AOD 9604 7_16	1042	2+	521.84/166.11	2	20	4.30	0.18	0.5	85
BUSERELIN	1239	2+	620.61/592.4	6	16	5.85	0.00	0.2	125
DESLORELIN	1281.64	2+	642.11/249.09	2	30	5.85	0.10	1	122
DESMOPRESSIN	1069.2	2+	535.38/526.70	38	12	5.77	0.25	1	109
FELYPRESSIN	1040.2	2+	520.93/120.09	4	40	4.58	0.13	0.2	99
GHRP 3 FREE ACID	656	2+	328.78/385.30	2	10	4.92	0.33	0.5	97
GHRP-1	955	3+	319.36/129.1	16	14	5.79	0.09	1	65
GHRP-1 (2-4)	423	1+	424.29/307.20	26	20	4.53	0.20	0.2	92
GHRP-2	818	2+	409.87/170.0	2	28	5.87	0.08	0.5	81
GHRP-2 (1-3)	357	1+	358.26/170.16	14	26	5.83	0.10	0.2	56
GHRP-3	655	2+	328.30/384.3	8	10	4.56	0.13	0.2	96
GHRP-4	607.2	1+	608.34/159.04	48	32	6.00	0.08	0.5	157
GHRP-5	770.3	1+	771.41/159.06	70	64	6.07	0.09	1	126
GHRP-6	873	2+	437.43/129.15	4	18	5.19	0.69	1	80
GHRP-6 (2-5)	609	1+	609.37/159.05	2	30	6.07	0.08	0.5	93
GHRP-6 (2-6)	736	2+	369.35/346.74	4	12	5.87	0.00	0.5	152
GOSERELIN	1269	2+	635.55/607.36	28	18	5.82	0.00	0.2	122
HEXARELIN	886.46	2+	444.40/129.13	4	18	5.44	0.79	0.5	82
HEXARELIN FREE ACID	888	2+	444.90/144.10	2	46	5.78	0.10	1	48
HEXARELIN- (1-3)	426	1+	427.29/273.17	6	20	4.06	0.15	0.5	21
HISTRELIN	1324	3+	442.16/200.20	2	31	5.69	0.16	0.5	85
IBUTAMOREN	528.2	1+	529.35/267.16	2	20	6.07	0.16	0.5	125
IPAMORELIN	712	2+	356.83/110.0	4	32	4.39	0.21	0.5	74
IPAMORELIN FREE ACID	713	2+	357.29/223.12	2	14	4.81	0.23	0.5	94
IPAMORELIN (1-4)	585	2+	293.37/270.15	40	10	5.79	0.00	0.5	149
LEUPRORELIN	1209	.2+	605.56/249.09	24	28	5.81	0.10	0.2	123
LEUPRORELIN (5-9)	688	2+	344.88/136.0	14	18	5.58	0.64	0.1	96
LH RH 1_3_OH	452	1+	453.22/249.15	2	18	4.53	0.12	0.2	23
LHRH (Gonadorelin)	1182	2+	592.03/249.0	48	26	5.05	0.44	0.1	91
NAFARELIN	1321.6	2+	662.12/249.15	50	32	5.93	0.00	1	125
TABIMORELIN	528.3	1+	529.41/280.13	8	12	6.08	0.08	0.1	116
TB-500N-AC LKKTETQ	889	2+	445.41/147.12	2	18	3.61	0.20	1	19
TB-500-M 1	301	1+	302.26/147.16	6	14	3.69	0.16	1	25
TB-500-M 3	330.1	1+	330.80/84.10	2	32	3.52	0.20	1	22
TRIPTORELIN	1311	2+	656.60/249.1	50	28	5.83	0.09	0.5	123
15_N_GHRP-2 (1-3) IS	361	1+	362.23/170.07	10	24	5.82	0.08	--	--
De-Am-C1-V4-D-Arg VSP (IS)	1040	1+	1040.42/120.15	100	80	5.88	0.08	--	--

Table 2. Showcasing details of acquisition and validation results for target peptides

All analytes were efficiently ionised under positive ESI using 0.1% formic acid as modifier agent in the mobile phase. The multiple reaction monitoring ions were obtained by infusing an individual standard solution of peptides prepared in mobile phases, in different acquisition modes. The method performance was evaluated based on validation parameters. The validation results demonstrated detection of the target analytes well within the requirement set by the WADA.

The estimated LODs (based on S/N>3 for lowest abundant monitored MRM) were within minimum required performance limit imposed by WADA [5]. The LODs were estimated in the range of 100 pg/mL to 1 ng/mL across all analytes. The method was found to be specific and selective as no significant interfering signals were observed at the expected RT of analytes. The extraction recoveries were in the range of 50% to 5% of MRPL (Table 2).

Though the recovery for few analytes were low, it was possible to detect the analytes at 50% of MRPL consistently. The analysis result of retained positive control and WADA EQAS samples were comparable to earlier results, which endorses suitability of this method for the use in routine doping control analysis for small peptides (Figure 2).

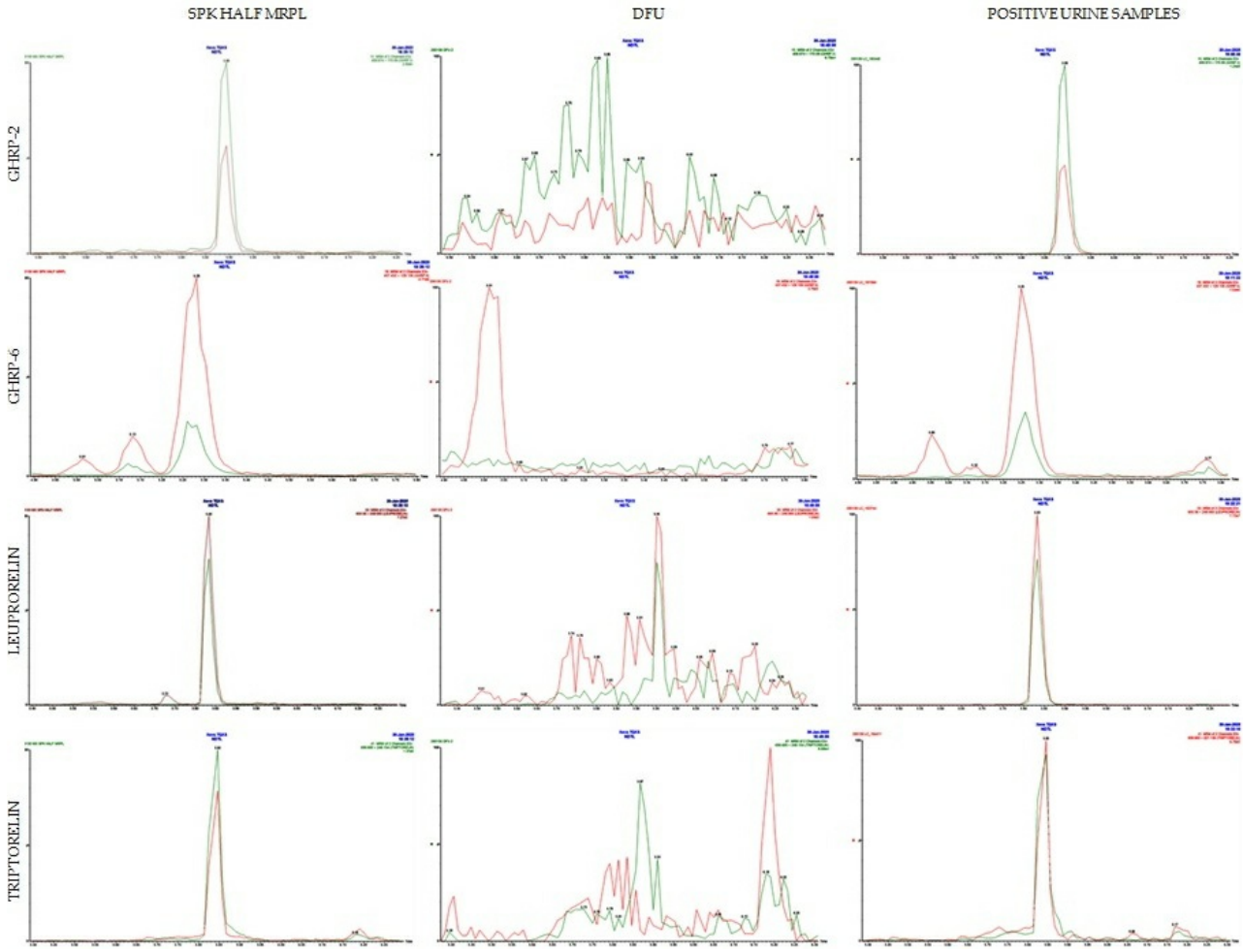

Figure 2. Extract ion chromatogram of slected peptides in retained positive control urines, QCs and blank urines

Conclusions

The presented work provides a fast and sensitive method for the detection of small peptides in urine and fulfils the aim of testing a maximum number of target analytes in a single run without compromising the sensitivity and selectivity in a shorter runtime. Sufficient LODs were obtained and the method was found to be fit-for-purpose as per requirement of WADA ISL.

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Validation of HIF activating agents confirmation methods

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Abstract

Hypoxia-inducible factors (HIF) activating agents such as Daprodustat, Daprodustat bishydroxylated metabolite, Roxadustat, Vadadustat and Molidustat glucuronide are included in the Prohibited List of the World Anti-Doping Agency (WADA) because they can induce hypoxia, that results in increased red blood cells and erythropoietin (EPO) production. The aim of this study is to establish reliable confirmation methods for doping control in urine samples. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) on Agilent 1290 Infinity/ABSciex QTrap 5500 was used for confirmation methods. Sample preparation was performed by liquid-liquid extraction for Roxadustat, Daprodustat and Daprodustat bishydroxylated metabolite and solid phase extraction for Vadadustat and Molidustat glucuronide. The methods were validated for the parameters specificity and matrix effect, limit of identification (2 ng/mL Molidustat glucuronide, 1 ng/mL Daprodustat metabolite and Vadadustat and 0.5 ng/mL Roxadustat and Daprodustat), carry-over and robustness. The results of the methods presented were found to be suitable for the confirmation and are compliant with the requirements of WADA technical documents TD2019MRPL and TD2015IDCR. ITP for all HIF's is performed by dilute and shoot method on an Agilent 1290 Infinity/ABSciex 5500 QTrap. The different aproaches were chosen after tests for sensitivity and specificity enhancement (data not shown). The methods that achieved the best sensitivity and specificity have been selected and validated and are presented in this paper.

Introduction

HIF stabilizers are an emerging class of drugs for the treatment of low blood haemoglobin levels. Hypoxia-inducible factors (HIFs), proteins composed of two subunits (HIF-α and HIF-β), play a central role in blood oxygen sensing. HIFs are transcriptional activators of hypoxia inducible genes, the most prominent being the erythropoietin (EPO) gene. The erythropoiesis-stimulating effect of HIF makes them a promising drug target for the treatment of hypoxia and anaemia, but also has great potential for abuse for performance enhancing purposes in sports since this class of therapeutics increases the capacity for oxygen transport [1-4]. Due to their performance enhancing effects hypoxia-inducible factor (HIF) activating agents are banned in sport and are included in the WADA Prohibited List in section S2.1.2 [5].

Experimental

Materials and methods

Roxadustat and Vadadustat were purchased from TRC Canada, Molidustat Glucuronide, Mefruside and Desoximethasone from LGC Standards, Germany, Daprodustat and Daprodustat bishydroxilated metabolite were provided by WADA. All reagents used were obtained from Merck (Chimexim, Romania).

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SPE X-CW-STRATA cartridges (30 mg, 30 µm particles, 1 mL) were purchased from Phenomenex (SC Muso SRL, Romania), XAD-2 resin from Supelco (Redox, Romania) and beta-glucuronidase from Helix pomatia from Roche (Roche Romania, Romania).

Sample preparation methods are presented in Table 1. Equipment used was an Agilent 1290 Infinity/ABSciex Qtrap 5500. The chromatographic column was Zorbax SB-C18 2.1 x 50 mm, 5 µm with SecurityGuard ULTRA Cartridges UHPLC C18 guard column. The A solvent was 5mM amonium formate, 1‰ formic acid in water, B solvent 5mM amonium formate, 1‰ formic acid in 90% acetonitrile + 10% water. Chormatographic and MS conditions and MRM transitions are presented in Table 2.

VADADUSTAT SAMPLE PREPARATION	ROXADUSTAT, DAPRODUSTAT+METABOLIT SAMPLE PREPARATION	MOLIDUSTAT GLUCURONIDE SAMPLE PREAPARATION
2mL doping control sample	2mL doping control sample	2mL doping control sample
50µL SI-Mefruside 0,5µg/mL	40µL SI-Dezoximetasone 1µg/mL	20µL SI-Mefruside 500ng/mL
XAD-2 column preparation (2cm)	1mL phosphate buffer 0.8M, pH 7,0	200µL sodium acetate/acetic acid buffer 1M, pH 5,2
Wash column with 2mL ultrapure water	25µL β-glucuronidase from E. Coli	Centrifuge 5min at 6000rpm
2mL sample on XAD-2 column	Homogenisation on vibro-mixer 5 sec. at 2400rpm	prepare Phenomenex Strata X-CW column
Wash column with 2x1mL ultrapure water	Hydrolysis 1,5h at 50°±1°C	Condition column with 1mL methanol
Elute with 2x1mL methanol	Adjust pH at 2 with formic acid	Condition column with 1mL ultrapure water
Evaporate methanol on block-heater at 50C under nitrogen stream	6mL tert-butyl-methil-ether	Add 2mL sample on Phenomenex Strata X-CW column (1mL at a time)
Reconstitute with 100µL solvent B:solvent A 1:4	shake15min. at 100 oscilations/minute	Wash with 1mL ultrapure water
Transfer in vial	Centrifuge 10min at 2500 rpm	Elute with 2x0,5mL 5% formic acid in methanol
	Evaporate the ether on block heater at 40±5°C under nitrogen stream	Evaporate methanol on roatary evaporator (5mbar; 40±5°C)
	Reconstitute with 100µL nethabol	Reconstitution with 100µL 0,3% formic acid in acetonitrile:water 5:95
	Transfer in vial	Transfer in vial

Table 1. Sample preparation protocols

MOLIDUSTAT GLUCURONIDE					DAPRODUSTAT+METABOLITE					ROXADUSTAT					VADADUSTAT				
LC PROGRAM			MS PARAMETERS		LC PROGRAM			MS PARAMETERS		LC PROGRAM			MS PARAMETERS		LC PROGRAM			MS PARAMETERS	
Time (min)	B%	Flow (µL/min)	Scan Type	MRM	Time (min)	B%	Flow (µL/min)	Scan Type	MRM	Time (min)	B%	Flow (µL/min)	Scan Type	MRM	Time (min)	B%	Flow (µL/min)	Scan Type	MRM
0	0	250	Polarity	Positive	0	20	250	Polarity	Negative	0	0	300	Polarity	Positive	0	10	300	Polarity	Negative
1	0	250	CUR	25	5.5	100	250	CUR	25	4.5	90	300	CUR	25	5.5	100	300	CUR	25
9	90	250	IS	5500	8.5	100	250	IS	-4500	5.5	90	300	IS	5500	8.5	100	300	IS	-4500
12	90	250	TEM	600	8.51	20	250	TEM	600	5.51	0	300	TEM	600	8.51	10	300	TEM	600
12.1	0	250	GS1	40	12	20	250	GS1	40	7.5	0	300	GS1	40	10.5	10	300	GS1	40
14	0	250	GS2	60				GS2	60				GS2	60				GS2	60
Injection volume: 15µL			CAD	High	Injection volume: 2µL			CAD	High	Injection volume: 1µL			CAD	High	Injection volume: 2µL			CAD	High
			EP	10				EP	-10				EP	10				EP	-10

Nr. crt.	Analyte	Molecular mass	Transition (precursor>product ion,collision energy,eV)	Relative retention time
1	Roxadustat	352	353>277.9(25) 353>250.1(35) 353>222.3(45) 353>195.0(55) 353>129.1(60) 392>291.1(-24)	1.26
2	Daprodustat	393	392>122.1(-48) 392>223.0(-48) 392>179.1(-38)	1.76
3	Daprodustat metabolite	425	424>323.1(-28) 424>137.9(-48) 424>255(-46)	0.57
4	Vadadustat	306	304.8>260.9(-18) 304.8>203.9(-30) 304.8>167.9(-38)	1.19
5	Molidustat glucuronide	490	491>260, 40 491>287, 30 491>261, 40 491>207, 65	0.56
6	SI-Mefruside	382	380.9>188.9(-38)	-
7	SI-Dezoximetasone	376	421>355(-24)	-

Table 2. Chromatographic conditions, MS conditions and MRM transitions in the same order as chromatograms in Figure 1

Validation

The methods were investigated for matrix effects, limit of identification, carry-over, extraction recovery (except molidustat glucuronide) and robustness.

- Matrix effects: 10 blank urine samples were analyzed and monitored for interferences.
- Limit of identification: 10 blank urine, spiked at 0.5 ng/mL, 1 ng/mL and 2 ng/mL were analyzed and compliance with TD2015IDCR criteria was verified.
- Carry-over was evaluated with the consecutive injection of a sample fortified at 10 ng/mL and two blank samples.
- Recovery for Daprodustat, Daprodustat metabolite, Roxadustat and Vadadustat: Two sets of 10 blank urines were analysed. The first set was fortified with a mixture of standard at a concentration level of 2 ng/mL before extraction, the second set was fortified after extraction, before organic phase evaporation. The internal standard was added before evaporation. Recovery was calculated as the ratio of the response factors of samples spiked before extraction and the response factors of samples spiked after extraction.
- Robustness was evaluated by modifying column temperature, source temperature, flow, injection volume, extraction pH, hydrolysis temperature and time, evaporation temperature, elution volume.

Results and Discussion

The low levels of the HIFs to be determined from a complex matrix as urine require to concentrate the sample and eliminate most of the interferences. The method is specific for the analytes, with no interfering signals on the target compounds signals. The LOIs and recoveries are presented in Table 3 and are compliant with WADA TD2019MRPL and ISL 10.0 documents.

Analyte	Daprodustat	Daprodustat metabolite	Roxadustat	Vadadustat	Molidustat glucuronide
LOI (ng/mL)	0,5	1	0,5	1	2
Recovery (%)	86	95	88	33	ND

Table 3. LOIs and recoveries

Carry-over was not observed at a concentration of 10 ng/mL. The recoveries are presented in Table 3. Recoveries ranged from 33% for Vadadustat to 95% for Daprodustat metabolite. All methods are robust for investigated parameters, for all analytes.

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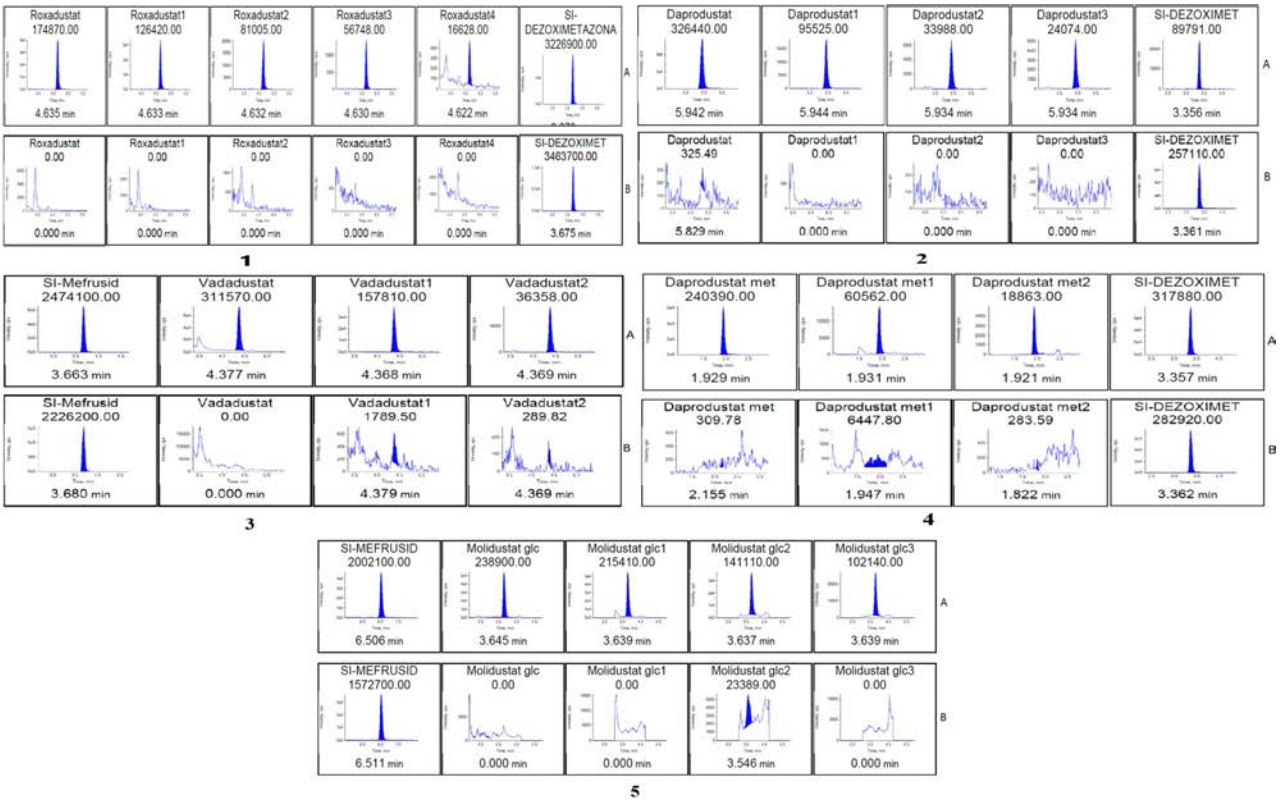

Figure 1. Blank urine spiked at LOI (A) and negative urine (B) for Roxadustat (1), Daprodustat (2), Vadadustat (3), Daprodustat Bishydroxylated Metabolite (4) and Molidustat Glucuronide (5)

Conclusions

In this article we present a method for the identification of HIF activating agents in urine. Due to the different structure of the analytes, different sample preparation protocols were used: LLE for Roxadustat, Daprodustat and it's bishydroxylated metabolite, SPE on XAD-2 for Vadadustat and SPE on weak cation exchange for Molidustat glucuronide. The specificity and the LOI show that the method can be used to identify HIF activating agents and their metabolites in doping control samples. Methods are robust for small variation of column temperature, source temperature, flow, injection volume, extraction pH, hydrolysis temperature and time (where applied), evaporation temperature, elution volume. In the future, the method for identification for Molidustat glucuronide could be improved to lower the LOI.

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

Impact of various purity grades of acetonitrile and different mass spectrometry ionization methods on limits of detection of doping substances by means of the “Dilute-and-Shoot” approach

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Abstract

Mass spectrometry has become the most frequently employed technique in doping control analysis used for the detection of prohibited substances in complex matrixes. The most important parameter of the identification of doping substances is a signal to noise (S/N) ratio that shall be greater than three to one (TD2015IDCR). The noise is usually derived from matrix and reagents interferences. The aim of this work was to find an optimal ionization mode and the best source of acetonitrile to increase the S/N ratio of analytes of interests. This poster describes a comparison of the use of different purity grades of acetonitrile and different types of ionization methods such as electrospray ionization (ESI) and UniSpray™ ionization source, both in “negative” and “positive” ion modes by using the “Dilute-and-Shoot” approach and analysis with the UPLC-MS/MS Xevo TQ-S system (Waters).

Introduction

Mass spectrometry has become the most frequently employed technique in doping control analysis used for the detection of prohibited substances in complex matrices [1]. The most important parameter of the identification of doping substances is a signal to noise (S/N) ratio that shall be greater than three to one (TD2015IDCR) [2]. The noise is usually derived from matrix and reagents interferences.

Experimental

Sample pre-treatment

Blank urine (BU) and quality control (QC) samples were prepared according to a dilute-and-shoot approach (DaS). Briefly, for DaS, 200 µL of urine was spiked with internal standards (ethyl-β-D-glucuronide-D_5, meldonium-D_3 and ampehtamine-D_{11}) and diluted with 800 µL of water. Following a short vortexing, the samples were centrifuged (10 min/14,000 rpm). Finally, the supernatant was transferred to a 96-well plate.

Instrumentation

Liquid chromatography

Analysis was performed on a UPLC™ Acquity chromatograph (Waters, Milford Massachusetts, USA) equipped with HSST3 column (1.8 µm, 2.1 × 100 mm). The mobile phase consisted of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B) and the LC gradient was employed at the constant flow rate of 300 µL/min at 45°C.

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The concentration of acetonitrile was gradually increased in a linear manner: from 0% to 60% within the first 5 minutes, from 60% to 100% in 1 minute. Finally, the column was reequilibrated for 1.5 minutes with the mobile phase of initial composition. Samples were stored at 5°C in the autosampler prior to analysis and the injection volume was fixed at 10 µL.

Mass spectrometry

Multiple reaction monitoring (MRM) of the studied substances was conducted using an Xevo™ TQ-S (Waters, Milford, MA, USA) mass spectrometer equipped with an electrospray ionization (ESI) source and a new atmospheric pressure ionization source, commercialized as UniSpray™. Analytes were investigated both in the „negative" and „positive" mode.

The desolvation gas flow was set at 800 L/h at 500°C and the source temperature was 150°C. The applied capillary voltage was 3.0 kV. The cone and collision gas flows were set at 150 L/h and 0.20 mL/min, respectively.

All data were acquired and processed using MassLynx™ software version 4.1 (Waters).

Results and Discussion

The results obtained from the Initial Testing Procedure (ITP) of quality control (QC) samples are shown in Table 1**.** BU and QC samples were analyzed two times in a routine doping control analysis both in ESI and UniSpray™ mode: at the beginning of a batch and after 30 athletes' urine samples. The "ratio" (UniSpray™/ESI) of intensities of 8/33 and 17/131 compounds analyzed in negative and positive ionization mode were below 1, respectively.

Acetonitrile of ≥99.9% purity purchased from the Fisher Chemical (Hampton, New Hampshire, USA) company was applied as the organic solvent in chromatographic separation of substances tested. Moreover, any matrix effect was not observed.

Furthermore, the impact of acetonitriles purchased from the Merck Millipore „hypergrade for LC-MS" (Darmstadt, Germany), Fisher Chemical and J.T.Baker ≥99.5% on the intensities of substances analyzed only in an ESI mode was determined. The results from this experiment showed no significant changes in intensities of the substances tested, only for acetonitriles purchased from Merck Millipore and Fisher Chemical companies. Moreover, no matrix effects for any grade of acetonitrile was observed (data not present).

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COMPOUND	IONIZATION MODE	TRACED MRMs CONE (V), COLLISION (eV)	CONCENTRATION [ng/ml] (MRM%)	INTENSITY UniSpray/ INTENSITY ESI (RATIO)
Diclofenamide	NEGATIVE	302.90>77.71(45;35)	100 (50%)	5.52
Probenecide	NEGATIVE	283.94>139.75(45;25)	20 (10%)	5.41
Methazolamide	NEGATIVE	235.00>77.83(30;15)	100 (50%)	3.86
Azosemide	NEGATIVE	368.99>325.85(50;20)	20 (10%)	2.54
Clopamide	NEGATIVE	344.06>77.88(45;35)	100 (50%)	2.42
Ethacyrnic acid	NEGATIVE	300.96>242.97(40;15)	20 (10%)	2.35
Ethyl glucuronide	NEGATIVE	221.04>84.97(14;14)	5000 (-)	2.28
Ethyl glucuronide-D5 (ISTD)	NEGATIVE	226.07>74.90(14;14)	5000 (-)	2
Chlorothalidone	NEGATIVE	336.96>189.89(45;20)	20 (10%)	1.77
Acetazolamide	NEGATIVE	220.98>82.90(30;20)	20 (10%)	1.6
Metolazone	NEGATIVE	364.04>256.98(55;25)	100 (50%)	1.55
ACB	NEGATIVE	283.80>77.93(50;25)	50 (25%)	1.48
Indapamide	NEGATIVE	364.03>188.80(45;30)	20 (10%)	1.48
Hydroflumethiazide	NEGATIVE	329.98>302.82(50;20)	20 (10%)	1.43
Furosemide	NEGATIVE	328.98>285.00(40;15)	20 (10%)	1.39
Piretanide	NEGATIVE	361.05>79.72(40;25)	20 (10%)	1.39
Daprodustat	NEGATIVE	392.18>290.89(30;30)	1 (50%)	1.37
Methyclothiazide	NEGATIVE	357.95>322.00(30;15)	20 (10%)	1.27
Polythiazide	NEGATIVE	437.90>398.00(30;21)	20 (10%)	1.23
Cyclothiazide	NEGATIVE	388.00>268.86(50;30)	20 (10%)	1.22
Furosemide glucuronide	NEGATIVE	505.00>328.94(30;15)	20 (10%)	1.15
Cyclopenthiazide	NEGATIVE	378.00>204.86(50;30)	20 (10%)	1.02
Daprodustat Bishydroxylated Met	NEGATIVE	424.17 322.84(50;20)	1 (50%)	1.01
Trichloromethiazide	NEGATIVE	377.83>241.90(50;25)	20 (10%)	1
Bendroflumethiazide	NEGATIVE	419.98>289.07(50;25)	100 (50%)	1
Hydrochlorothiazyde	NEGATIVE	295.94>268.69(50;20)	20 (10%)	0.91
Chlorothiazide	NEGATIVE	293.97>178.86(50;45)	100 (50%)	0.82
ATFB	NEGATIVE	318.00>214.05(35;25)	10 (5%)	0.81
Bumetanide	NEGATIVE	363.14>79.70(55;25)	50 (25%)	0.77
Epitizide	NEGATIVE	424.00>204.90(45;34)	50 (25%)	0.66
Brinzolamide	NEGATIVE	382.06>77.79(50;35)	50 (25%)	0.63
Relcovaptan	NEGATIVE	618.09>200.88(30;15)	50 (25%)	0.46
Ethoxyzolamide	NEGATIVE	257.00>177.79(30;15)	50 (25%)	0.21
Fentanyl	POSITIVE	337.23>188.18(30;24)	1 (50%)	4.36
Norfenfluramine	POSITIVE	204.10>109.07(35;34)	25 (25%)	4.09
Mesocarb MT (4-OH-mesocarb)	POSITIVE	339.20>193.10(21;15)	10 (10%)	4
Mapenterol	POSITIVE	325.13>236.87(30;15)	5 (25%)	4
Pipradrol	POSITIVE	268.17>130.10(4;30)	10	3.99
Fencamfamine	POSITIVE	216.45>66.84(30;20)	10 (10%)	3.84
Phenmetrazine	POSITIVE	178.10>114.79(35;30)	50 (50%)	3.84
Selegiline	POSITIVE	188.14>91.00(42;20)	10 (10%)	3.81
Bemitil	POSITIVE	179.06>150.96(10;15)	10 (50%RL)	3.81
Buphedrone/	POSITIVE	178.25>160.13(20;10)	50/(50%)	3.71
MDPV	POSITIVE	276.16>126.15(30;25)	10 (10%)	3.71
Torasemide	POSITIVE	349.13>264.22(25;20)	20 (10%)	3.68
Mabuterol	POSITIVE	311.11>202.02(10;30)	10 (50%)	3.64
Prolintane	POSITIVE	218.19>91.07(6;24)	10 (10%)	3.49
Furfenorex	POSITIVE	230.40>80.81(25;20)	5 (5%)	3.42
Fenbutrazate	POSITIVE	368.22>90.97(10;42)	10 (10%)	3.29
Methylone	POSITIVE	208.00>159.82(25;20)	10 (10%)	3.16
Pethidine	POSITIVE	248.16>220.17(20;22)	10 (20%)	3.05
Norsufentanil	POSITIVE	277.19>96.01(8;24)	1 (50%)	3
Clenpenterol	POSITIVE	291.10>272.98(30;10)	5 (25%)	3
Cocaine MT (Benzoyloecgonine)	POSITIVE	290.20>167.97(30;20)	25 (25%)	2.97
Ethamivan	POSITIVE	224.13>151.06(8;18)	10 (10%)	2.9
Pentedrone	POSITIVE	192.10>90.84(20;20)	50 (25%)	2.88
Ecgonine Methyl Ester	POSITIVE	200.13>182.04(30;15)	25 (25%)	2.83
Mesocarb	POSITIVE	323.00>90.78(21;30)	10 (10%)	2.78
N,N-dimethylamphetamine	POSITIVE	164.04>91.10(4;20)	25 (25%)	2.77
Cocaine	POSITIVE	304.15>182.12(5;16)	25 (25%)	2.76
Procaterol	POSITIVE	291.17>273.06(10;10)	10 (50%)	2.76
Cyclazodone	POSITIVE	217.30>146.00(30;15)	10 (10%)	2.74
Molidustat glucuronide	POSITIVE	491.16>259.90(10;30)	1 (50%)	2.68
Mephedrone	POSITIVE	178.25>160.13(20;10)	50 (50%)	2.61
Butylone	POSITIVE	222.11>174.06(10;15)	10 (10%)	2.61
Methamphetamine	POSITIVE	150.09>91.01(24;18)	10 (10%)	2.52
Tulobuterol	POSITIVE	228.12>154.00(10;15)	5 (25%)	2.5
Pholedrine	POSITIVE	166.12>135.08(34;10)	10 10%)	2.47
p-FB-Fentanyl	POSITIVE	369.23>188.21(30;25)	25 (50%)	2.41
Diphenhydramine	POSITIVE	256.17>167.17(30;15)	25(-)	2.38
Dorzolamide	POSITIVE	325.06>135.02(30;30)	50 (25%)	2.37
Methylphenidate	POSITIVE	234.20>83.92(25;20)	5 (5%)	2.29
Methedrone	POSITIVE	194.12>161.03(5;20)	25 (25%)	2.27
Strycluine	POSITIVE	335.18>184.08(90;36)	10 (10%)	2.21

Table 1. The mean ratio (UniSpray™/ESI) of the substances tested. Values above „1" mean an increase of signal intensities in the UniSpray™ mode *(to be continued)*

COMPOUND	IONIZATION MODE	TRACED MRMs CONE (V), COLLISION (eV)	CONCENTRATION [ng/ml] (MRM%)	INTENSITY UniSpray/ INTENSITY ESI (RATIO)
Ritodrine	POSITIVE	288.16>120.95 (10;20)	5 (25%)	2.05
Cimaterol	POSITIVE	220.15> 143.03 (30;25)	10 (50%)	2.02
Oxethazine	POSITIVE	468.24>145.02 (10;25)	5 (5%)	2.02
Amiloride	POSITIVE	230.06>116.02 (25;30)	20 (10%)	1.93
Sufentanyl	POSITIVE	387.21>238.18 (56;20)	1 (50%)	1.93
Normmethylfentanyl	POSITIVE	247.35>98.14 (30;20)	1 (50%)	1.84
2-ethylamino-1-phenylbutane	POSITIVE	178.16>91.01 (12;16)	25 (25%)	1.83
Quinethazone	POSITIVE	290.04>272.92 (30;15)	50 (25%)	1.83
Cimbuterol	POSITIVE	234.16>160.05 (30;15)	5 (25%)	1.82
Oxilofrine	POSITIVE	164.10>133.10 (10;15)	25 (25%)	1.81
Etilefrine ISF	POSITIVE	164.10>91.10 (10;20)	25 (25%)	1.77
Norcarfentanil	POSITIVE	291.17>259.16 (40;10)	1 (50%)	1.73
Mozavaptan	POSITIVE	428.23>119.03 (10;40)	50 (25%)	1.73
Carboxyfinasteride	POSITIVE	403.30>335.12 (55;30)	20	1.72
Brombuterol	POSITIVE	366.98>292.79 (30;20)	5 (25%)	1.7
Meldonium	POSITIVE	147.04>58.03 (30;10)	10 (50%)	1.68
Ritalinic acid	POSITIVE	220.09>83.96 (25;20)	25 (25%)	1.68
Alfentanyl	POSITIVE	417.26>268.15 (68;18)	1 (50%)	1.67
Heptaminol	POSITIVE	146.09>69.01 (30;15)	25 (25%)	1.64
Dextromoramide	POSITIVE	393.26>306.20 (52;24)	5 (10%)	1.64
Clenproperol	POSITIVE	244.97>132.01 (50;20)	10 (50%)	1.63
Meldonium-D3 (ISTD)	POSITIVE	150.13>62.09 (30;15)	500 (-)	1.52
Trimetazidine	POSITIVE	267.19>181.09 (4;12)	10 (50%)	1.52
Triamterene	POSITIVE	254.10>237.07 (45;30)	20 (10%)	1.51
Ortetamine	POSITIVE	150.06>105.05 (10;15)	25 (25%)	1.5
Sibutramine	POSITIVE	280.26>138.94 (25;15)	25 (25%)	1.48
Norfentanyl	POSITIVE	233.17>83.99 (5;16)	1 (50%)	1.42
Fluticasone propionate MT	POSITIVE	453.10>293.03 (20;20)	30 (100%)	1.37
Vilanterol	POSITIVE	485.94>158.84 (10;35)	5 (25%)	1.31
Higenamine	POSITIVE	272.13>107.05 (10;20)	10 (50%)	1.29
Propylhexedrine	POSITIVE	156.20>68.81 (40;15)	50 (50%)	1.28
Brinzolamide	POSITIVE	384.07>135.92 (30;40)	50 (25%)	1.22
Eplerenone	POSITIVE	415.21>162.95 (30;20)	20 (10%)	1.21
Oxilofrine Sulphate ISF	POSITIVE	243.96>164.03 (50;15)	50 (50%)	1.21
Efaproxiral (RSR13)	POSITIVE	342.00>122.00 (35;28)	5	1.2
Amphetamine-D11 (ISTD)	POSITIVE	147.11>98.05 (15;15)	100 (-)	1.18
Benzylpiperazine	POSITIVE	177.14>91.08 (30;18)	50 (50%)	1.18
OH-amphetamine	POSITIVE	135.00>107.04 (30;10)	25 (25%)	1.15
Pentedrone MT	POSITIVE	180.10>90.73 (15;25)	50 (25%)	1.13
Fencamine	POSITIVE	385.54>236.20 (45;22)	10 (10%)	1.11
Mephentermine	POSITIVE	164.14>133.10 (28;10)	10 (10%)	1.1
Octopamine Sulphate ISF	POSITIVE	215.93>136.00 (30;10)	10 (1%)	1.1
Pirbuterol	POSITIVE	241.16>185.02 (30;15)	5 (25%)	1.07
Benzthiazide	POSITIVE	431.93>90.95 (30;25)	20 (10%)	1.02
Fenoterol	POSITIVE	304.15>107.02 (12;28)	5 (25%)	0.94
Acetazolamide	POSITIVE	222.92>180.93 (30;15)	20 (10%)	0.92
Xipamide	POSITIVE	355.05>122.01 (30;26)	20 (10%)	0.9
Conivaptan	POSITIVE	499.21>180.97 (45;40)	20 (10%)	0.89
Dobutamine	POSITIVE	302.09>90.96 (20;40)	25 (25%)	0.8
6β-OH-Eplerenone	POSITIVE	431.21>353.31 (30;20)	20 (10%)	0.7
Olodaterol	POSITIVE	387.19>162.97 (10;20)	5 (25%)	0.61
Mebutizide	POSITIVE	382.07>297.86 (10;10)	50 (25%)	0.5
Benzylhydrochlorothiazide	POSITIVE	388.02>104.98 (50;15)	20 (10%)	0.46
Althiazide	POSITIVE	384.00>262.13 (20;20)	50 (25%)	0.45
Buthiazide/ Butizide 2	POSITIVE	354.00>337.00 (35;18)	20 (10%)	0.38
Tolvaptan	POSITIVE	449.20>118.97 (30;40)	20 (10%)	0.37
Reproterol	POSITIVE	390.18>221.02 (50;25)	10 (50%)	0.3
Canrenone	POSITIVE	341.21>107.10 (10;30)	20 (10%)	0.23
Ethoxyzolamide	POSITIVE	259.02>177.87 (50;15)	50 (25%)	0.2
Lixivaptan	POSITIVE	474.14>137.02 (10;40)	50 (25%)	0.15
FG-4592	POSITIVE	281.03>205.90 (25;20)	1 (50%)	0.14

Table 1. *(continued)* The mean ratio (UniSpray™/ESI) of the substances tested. Values above „1" mean an increase of signal intensities in the UniSpray™ mode

In another experiment, the impact of ESI and UniSpray™ mode in the confirmatory analysis of amiloride was compared (Figure 1). A significant increase of intensities for three MRMs of amiloride in the UniSpray™ mode was observed.

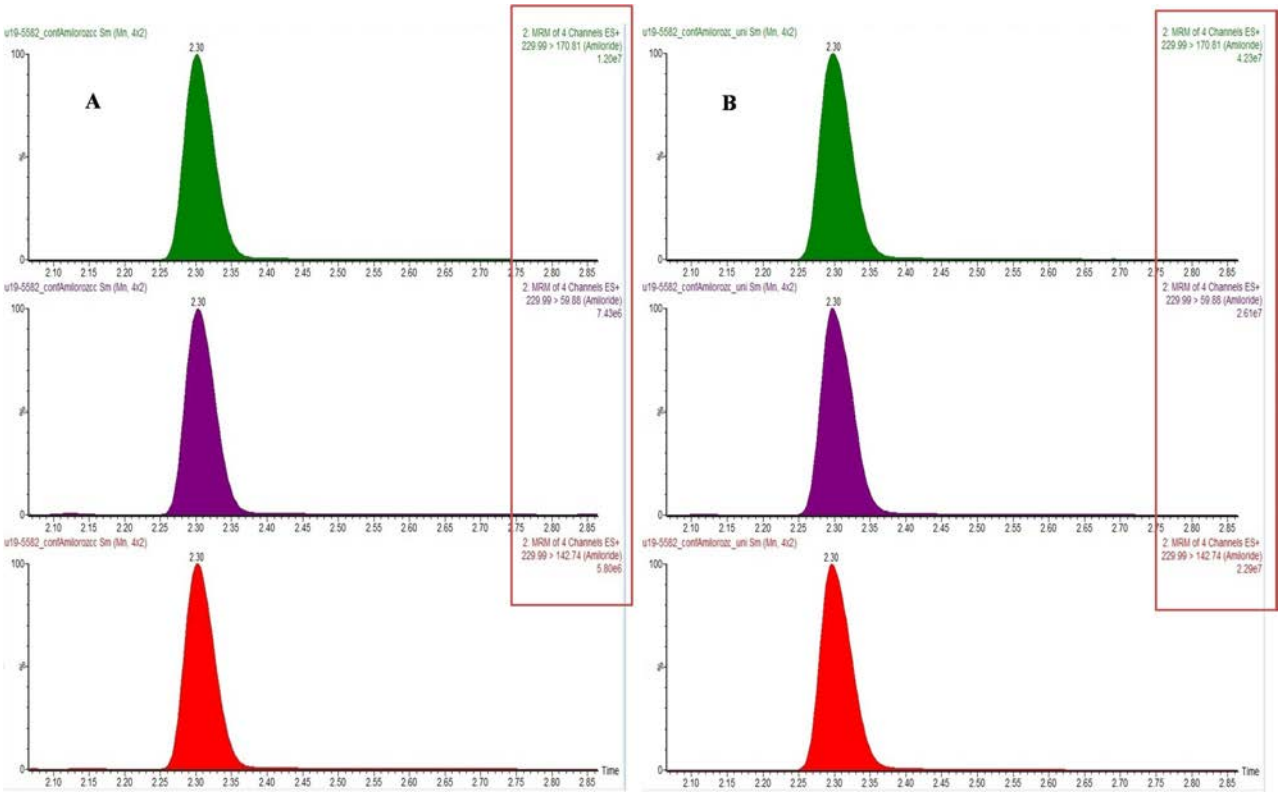

Figure 1. Chromatograms of athlete sample method obtained from the confirmatory analysis of amiloride. Sample prepared with the DaS method and analyzed in an ESI(+) **(A)** and UniSpray™ **(B)** ion modes. Amiloride was traced at the cone voltage (CV) set at 25 V with the following selected precursor ion–product ion transitions at their respective collision energies (CE): m/z 229.99 > 59.88, CE 20 eV; m/z 229.99 > 142.74, CE 25 eV; m/z 229.99 > 170.81, CE 20 eV.

Conclusions

The results from this study show that the UniSpray™ mode is an alternative type of ionization method for doping control purposes. Moreover, this analytical tool may be useful in the identification of low concentrations of diuretics or anabolic agents. To improve the intensities of compounds with the „ratio" below 1, the optimum set-up of source for these compounds (e.g., capillary: „X" and „Z" position and determine collision energies) is necessary.

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UHPLC-HRMS multi-screening method for doping control analyses in urine samples

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Abstract

Doping analysis is prone to continuous evolution. Laboratories have to manage a high number of samples and regularly improve the analytical performance in daily routine operations. Moreover, there is a need to expand the scope of the established methods by including new compounds periodically. Therefore, even more powerful assays than those already employed are necessary. In the recent years High Resolution Mass Spectrometry (HRMS) has widened the scope of multi-screening methods in routine analysis. The capability of working with accurate mass and high resolution full-scan data allows the development of sensitive and selective methods that can be easily updated to new analytes, and the ability to perform retrospective analysis.

In this study, Ultra-High Performance Liquid Chromatography (UHPLC) coupled to HRMS was performed in order to develop a multi-target approach assay for different compounds such as stimulants, diuretics, hypoxia inducible factor (HIF) stabilizers, metabolic modulators, masking agents and β-agonists as stated in the World Anti-Doping Agency (WADA) List of Prohibited Substances. The best UHPLC-HRMS conditions to evaluate 115 different compounds in the same assay (92 stimulants, 13 diuretics, 6 HIF agents, 2 metabolic modulators, 1 masking agent and 1 β-agonist) were established. Several acquisition methods such as full scan, targeted-Single Ion Monitoring (t-SIM), and full scan with in-source collision-induced dissociation (CID) were combined in positive and negative ionization modes for the determination of all the studied compounds. Moreover, an All-Ions Fragmentation (AIF) scan was also included to provide additional ions for identification.

Under optimum conditions, the method was validated with regards to limits of detection, selectivity, matrix effects, and intra-day precision. Method validation was performed at or below the minimum required performance levels specified by WADA technical documents for all the analytes, hence showing the potential of the optimized methodology for these compounds in doping control analyses.

Introduction

For as long as sporting events have existed, attempts to achieve a competitive edge, especially with the use of performance-enhancing drugs, have been made by athletes of all kinds of sports. Because of this, a list of prohibited substances in and out-of-competition and banned doping methods has been constantly updated by the WADA [1].

The accurate and precise determination of these doping agents is necessary. Laboratories have to manage an increasing number of samples, analytes and continuously lower the required analytical performance level in routine daily operations [2-4]. Therefore, in this work a fast, sensitive and selective

method based on the combination of an easy dilute-and-shoot approach and HRMS is proposed for the multi-target screening of a large number of analytes in the context of doping control.

Experimental

Chemicals and Reagents

All chemicals and reagents were of analytical standard quality or equivalent purity. Diphenylamine and Piretanide Impurity A were used as internal standards (ISTD).

Sample Preparation

The sample preparation consisted of a dilute-and-shoot procedure: 500 µL of urine were centrifuged for 5 minutes at 12,000 rpm. Then, the sample was diluted in a 1:5 ratio with a solution of acetonitrile:H_2O 1:99 with 1% formic acid containing the internal standards diphenylamine and piretanide impurity A at 20 ng/mL and 100 ng/mL, respectively.

Liquid Chromatography

LC separation was carried out in a Vanquish Flex chromatograph (Thermo Fisher Scientific). The LC system was equipped with an UPLC BEH column (C18, 2.1 x 100 mm, 1.7 µm particle size) from Waters (Milford, Massachusetts, USA). The column temperature was set to 45°C. The organic solvent (buffer A) consisted of a mixture of 0.1% of formic acid in methanol with 1mM ammonium formate, and the aqueous phase (buffer B) was water acidified with 0.1% of formic acid and 1mM ammonium formate. The gradient was as follows: initial conditions at 100% B, 97% B at 2.5 min, 97% B at 5.5 min, 10% B at 5.8 min, and 3% B at 8 min. Total run time was 11 min. The flow rate was set to 0.3 mL/min and the injection volume was 10 µL.

Mass Spectrometry

A Q-Exactive (Thermo Fisher Scientific) with electrospray source operating in positive and negative ionization modes was employed. The parameters of the ion source were the following: spray voltage 3 kV, capillary temperature 320°C, auxiliary gas temperature 300°C, S-lens 60%, sheath gas 50 AU and auxiliary gas 50 AU. Acquisition data was performed as stated in Table 1. TraceFinder v 4.1 software (Thermo Fisher Scientific) was used for LC-HRMS control and data acquisition.

1) Full MS: - Runtime: 0-8 min - Resolution: 35.000 - AGC target: 3e6 - Maximum IT: 100 ms - Scan range: 67-750 *m/z*	**2) Targeted-SIM (t-SIM)** - Runtime: 4,7-8 min - Resolution: 70.000 - AGC target: 2e5 - Maximum IT: 120 ms - MSX (multiplexed ions): 10 - Isolation window: 3 *m/z* - Isolation offset: 1 *m/z* - Scan range: 270-500 *m/z*
3) Full MS with CID (Collision-Induced Dissociation) - Runtime: 4-5,3 min - Resolution: 17.500 - AGC target: 1e6 - Maximum IT: 20 ms - Scan range: 100-500 *m/z* - In-Source CID: 100 eV	**4) AIF (All Ion Fragmentation):** - Runtime: 0-8 min - Resolution: 17.500 - AGC target: 3e6 - Maximum IT: 100 ms - NCE: 50 eV - Scan range: 50-750 *m/z*

Table 1. Optimized parameters used in each of the combined acquisition methods

Results and Discussion

The multi-screening method was effectively tested and validated according to WADA requirements. The successfully optimized acquisition methods and some examples in spiked urines are shown in Figure 1.

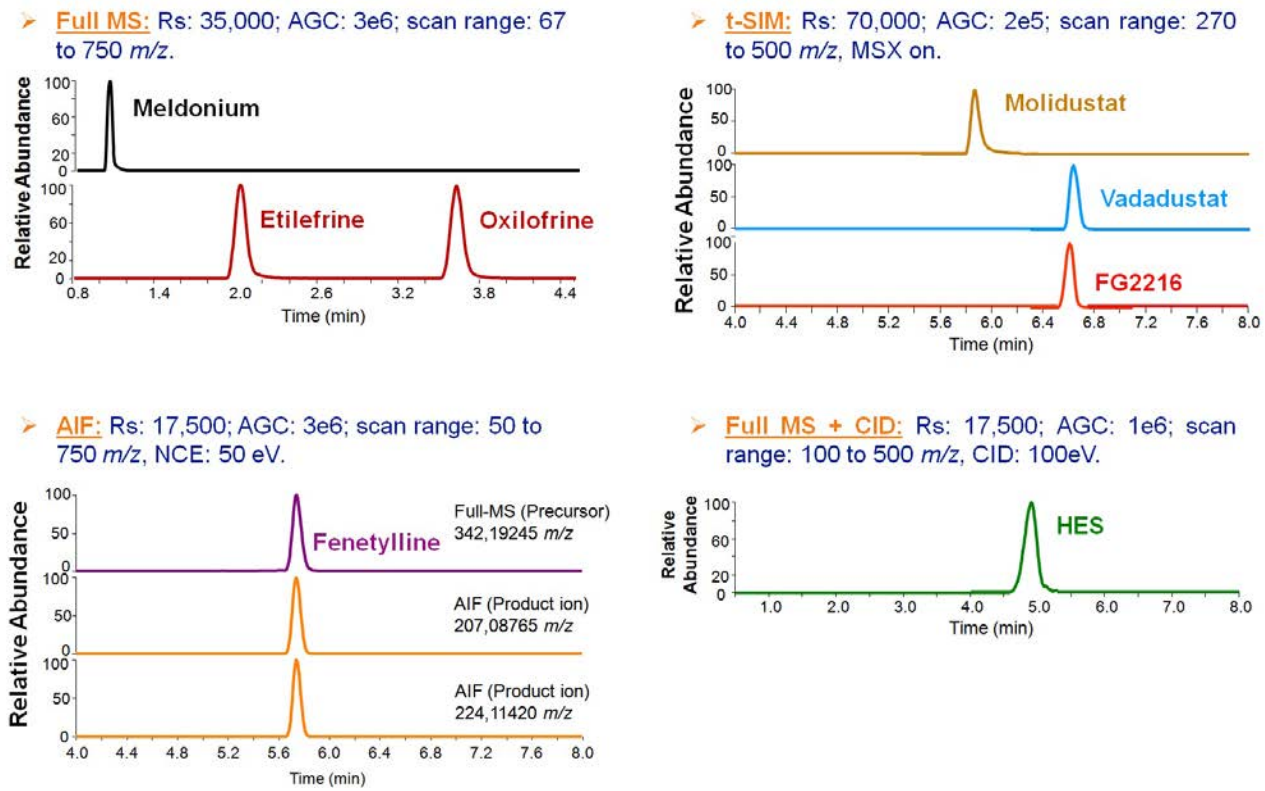

Figure 1. Description of the different acquisition methods and chromatogram examples obtained from spiked blank urines. Meldonium, etilefrine, oxilofrine and fenetylline were all spiked at 100 ng/mL; HES was spiked at 500 µg/mL and molidustat, vadadustat and FG-2216 were spiked at 20 ng/mL.

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Satisfactory sensitivity was obtained with the full-scan for most of the analytes, except for the HIF stabilizers for which a targeted-SIM acquisition, both in positive and negative ionization, was required. In addition, multiplexing and extraction windows were also necessary to optimize scan time and ensure adequate sensitivity and selectivity. This acquisition mode allowed achieving half of the very low required MRPL for HIF stabilizers, established at 2 ng/mL by WADA. Information about these compounds can be found in Table 2.

HIF stabilizer	*m/z*	Polarity	RT (min)
FG2216	279,0178	-	6,60
Vadadustat	307,0480	+	6,64
Molidustat	315,1312	+	5,79
FG4592	353,1132	+	6,74
GSK1278863	394,1973	+	6,98
GSK2391220	424,1725	-	6,49
BAY1163348	489,1488	-	5,38

Table 2. Inclusion list parameters used in targeted-SIM for the detection of the HIF stabilizers

In-source fragmentation enabled the detection of polymeric compounds such as HES or dextran [4]. Furthermore, the additional acquisition mode with AIF helped the identification of target compounds, avoiding unnecessary reanalysis of samples.

Method validation results are summarized in Table 3. Due to the use of accurate mass, artificially high S/N ratios were obtained for some compounds. In those cases, Low control concentration, matrix effects and their variability were taken into account to estimate the limit of detection (LOD), ensuring that all compounds were detectable in all samples analyzed. All of the compounds had an intra-assay precision (%RSD) better than 15% except for the HIF stabilizers due to the very low concentration tested. Nevertheless, all HIF stabilizers were detected in n=6 different matrix samples spiked at 1 ng/mL (½ of MRPL). Obtained LODs are compliant with MRPL stablished by WADA (always < 50% MRPL) for all compounds. Minimum interfering peaks were observed at the retention time of the analytes. Due to the high sensitivity the detection of the compounds was always possible at the required sensitivity even for those analytes with higher background, such as the low mass stimulants.

Group	# of compounds	% of compounds with Matrix Effect<30%	% of compounds with LOD ≤ ½ MRPL	Low Control RSD (%)	High Control RSD (%)
S2.1.2 HIF Activating Agents	6	67	100	6-20	8-48
S3. β-2 agonists	1	100	100	5	14
S4. Hormone and Metabolic Modulators	2	100	100	3-5	4-6
S5. Diuretics and Masking Agents	14	65	100	4-12	2-9
S6. Stimulants	92	71	100	1-4	2-7

Table 3. Summary of the validation results of the proposed method

Conclusions

A comprehensive and open initial testing procedure by UHPLC–HRMS has been developed and validated for the qualitative determination of 115 analytes in human urine. All detection limits were compliant with

WADA's requirements. A high degree of specificity and sensitivity was achieved by the use of high resolution mass spectrometry. The application of the HRMS has been shown as a promising tool for doping control purposes since it allows a non-target analysis and the possibility of retrospective analysis of the samples. This Initial Testing Procedure can be easily expanded to other conjugated and non-conjugated compounds, such as β-blockers, phase II AAS metabolites, other β2-agonists and additional diuretics without having to modify the established acquisiton parameters.

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de la Cal A, Espinosa P, Blázquez I, Fernández B, García L, de Pedraza L, Carreras D, Muñoz G

Solving problems with HILIC columns: Compounds with difficult chromatographic behavior

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Abstract

Hydrophilic interaction chromatography (HILIC) is gaining popularity for its ability to retain and separate highly polar, hydrophilic and charged compounds, an area where common reverse-phased liquid chromatography (RPLC) methods fail.
The aim of this work is to develop a screening method for the simultaneous determination of polar and charged substances. This kind of substances are very difficult to analyze in the currently multi-analyte procedures used in antidoping laboratories, which usually are based on RPLC, due to these substances eluting with the solvent front and suffering from inefficient electrospray ionization, high ion suppression effects and asymmetrical peak shapes.
Here we present a fast and sensitive method based on dilut-and-shoot and hydrophilic interaction liquid chromatography (HILIC) specifically developed for the detection of charged substances such as meldonium, highly polar substances such as the catecholamine octopamine and sulfo-conjugated and glucuronide-conjugated derivatives of doping substances (octopamine, molidustat, ethanol).
Advantages of the method were the low sample volume, only 10 µL, and the high dilution, 1/50 (V/V), for minimizing matrix effects. Detection of analytes was performed by a triple quadrupole mass spectrometer under positive and negative electrospray ionization conditions and multiple reaction monitoring acquisition mode (MRM). In all the cases, the limit of detection was below the 50% of the minimum required performance level of WADA.

Introduction

The main goal of the antidoping laboratories is the development of screening methods able to analyze as many substances as possible among those included in the WADA prohibited list. For this purpose, RPLC coupled to mass-spectrometry has commonly been the method of choice because of its versatility for the separation of a great number of substances belonging to different families, including apolar compounds as well as analytes of moderate polarity.
The objective of this work is to develop a screening method for the simultaneous determination of substances which are difficult to analyze in procedures currently adopted for the laboratories due to their low chromatographic performance and/or low ionization efficiency. Examples are represented by meldonium, octopamine and sulfo-conjugated, molidustat glucuronide and ethylglucuronide.
Although at present multi-target HILIC approachs have already been presented [1-2], this novel developed method enables for the first time the simultaneous detection of the five compounds above in a single method.

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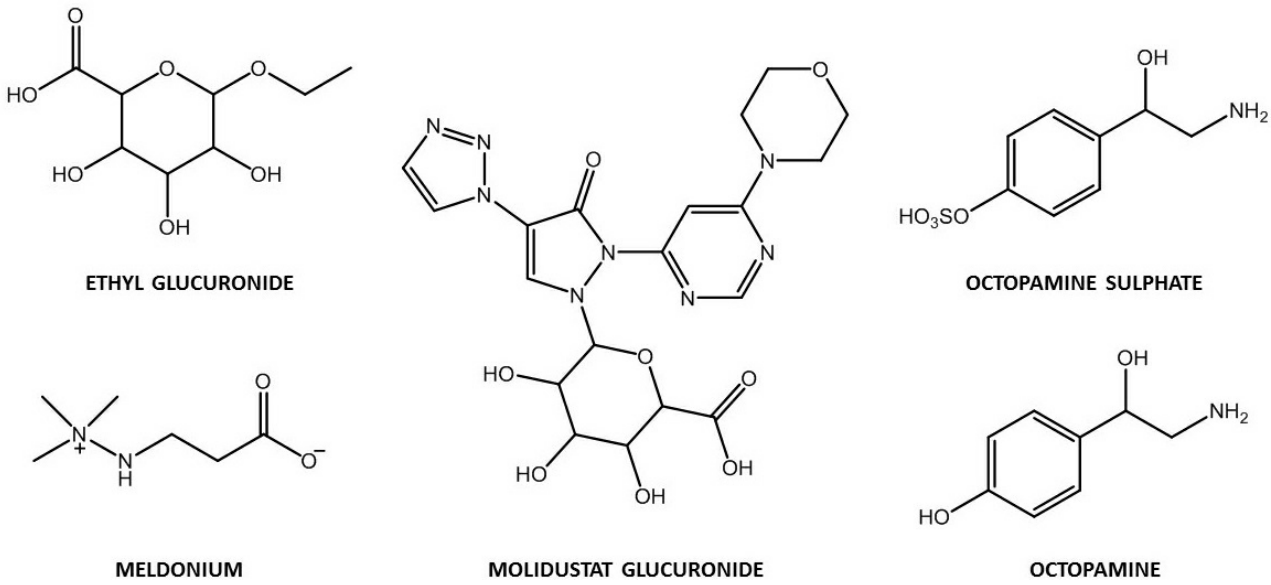

Figure 1. Chemical structures of the targeted compounds

Experimental

Sample treatment:

Samples were prepared according to a dilute-and-shoot approach. 10 µL urine was spiked with d_3-salbutamol and d_5-ethylglucuronide as internal standards and was diluted to 500 µL with acetonitrile/ 5mM ammonium acetate buffer with 0.1% acetic acid [90/10 (v/v)]. The sample was mixed and injected into the LC-MS/MS system.

Instrumental Analysis

Chromatographic separation was performed by an HPLC Agilent 1260 infinity, equipped with a Poroshell 120 HILIC column (2.1 x 50 mm, 2.7 µm particle size) from Agilent. The mobile phase consisted of 5mM ammonium acetate with 0.1% acetic acid (A) and acetonitrile (B) and a gradient elution was employed at a constant flow rate of 300 µL/min at 30°C

Gradient elution started with 90% B maintained for 1 min followed by linear gradient to 70% B in 5 min. The organic modifier was increased to initial conditions at 7.1 min and the final equilibration time was 4 min, resulting in an overall run time of 11 min. The sample injection volume was fixed at 5 µL.

Detection was achieved using a Sciex 5500Qtrap mass spectrometer equipped with an electrospray ionization source working in both positive and negative polarities. The ion source temperature was operated at 550°C, the applied capillary voltage was set at +5500 V in positive mode and at -4500 V in negative mode.

The mass spectrometer was operated in multiple reaction monitoring (MRM) schedule as acquisition mode with a detection window of 60 s and dwell scan time 0.7 s. Three ion transitions per analyte and one ion transition per each internal standard were used to identify of each analyte. The employed traced MRMs are listed in Table 1.

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Compound	MW (Da)	Ionization mode	Q1 (m/z)	Q3 (m/z)	CE (eV)	RT (min)	LOD (ng/mL)
d3-Salbutamol (ISTD)	242	Positive	243	169	19	5	-----
Octopamine	153	Positive	154	91	27	5,1	50
				107	33		
				119	17		
Octopamine sulphate	233	Positive	234	136	19	0,9	500
				91	41		
				119	29		
Meldonium	146	Positive	147	58	33	8,5	10
				59	21		
				42	87		
Molidustat glucuronide	490	Positive	491	260	41	1,6	1
				287	27		
				261	39		
d5-Ethyl glucuronide (ISTD)	227	Negative	226	85	-22	1,2	-----
Ethyl glucuronide	222	Negative	221	75	-20	1,2	500
				85	-22		
				113	-20		

Table 1. Target substances, MS parameters, LOD and Retention times (RT)

Results and Discussion

Instrumental parameters were optimized by infusion of the standard solution of each analyte. The full-scan spectra were performed to identity and to select the most abundant precursor ion. Ethanol was ionized in negative mode and the rest were detected as protonated ions. MS/MS experiments were then carried out to select the diagnostic ion transitions to develop the MRM acquisitions method (Table1). Different mobile phase compositions, gradient programs, flow rates, column temperatures, source parameters and sample treatments were evaluated. The conditions listed above were the result of the optimization of these parameters to obtain the best sensibility, peak shape, selectivity and chromatographic retention.

Method validation of the analytical approach was carried out according to ISO/IEC 17025 and WADA guidelines considering the parameters: limit of detection (LOD), selectivity, specificity, intra-essay precision, carryover, matrix effects and robustness.

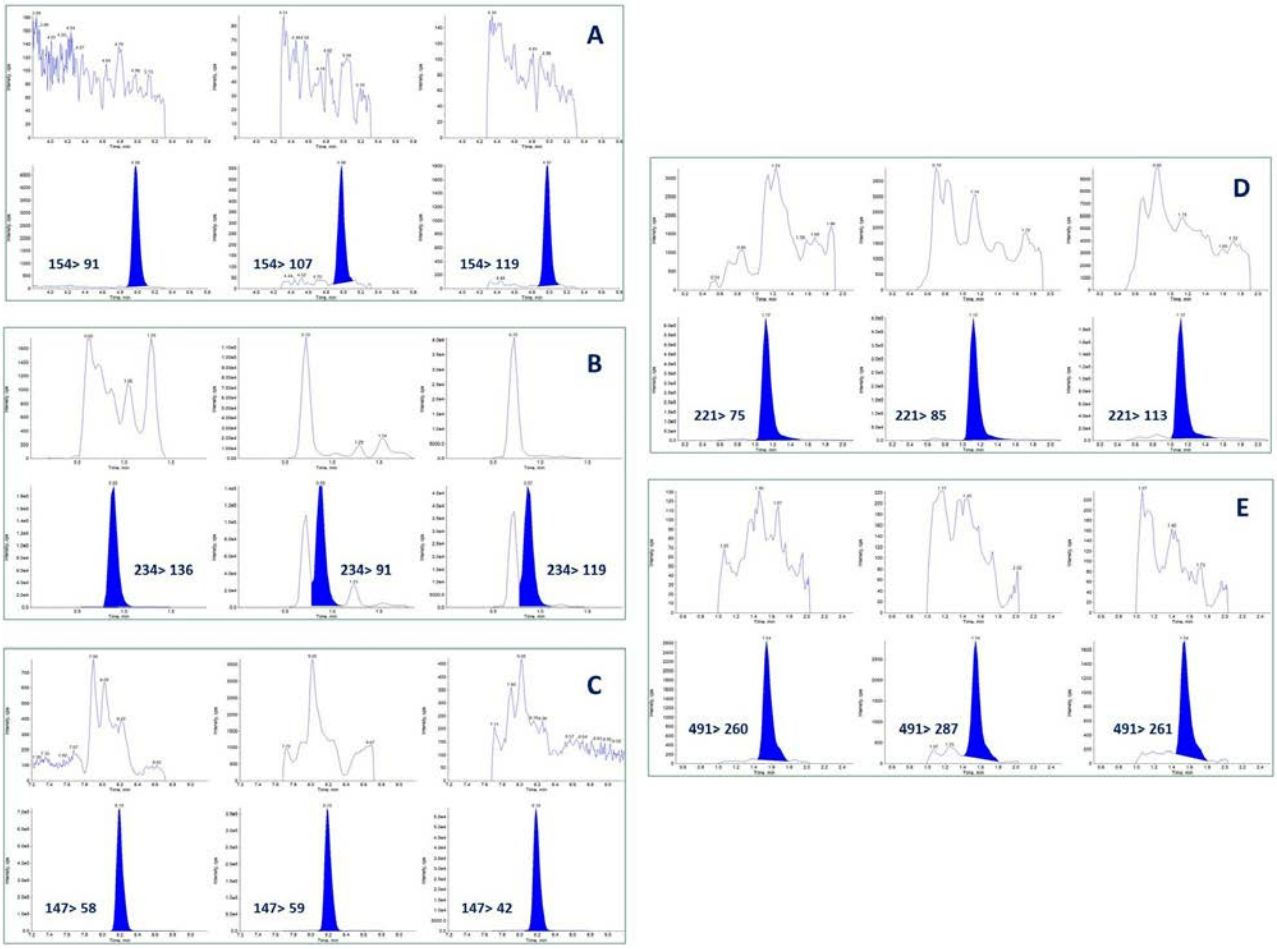

Figure 2. Chromatograms of a blank urine sample (up) and a quality control sample spiked at the indicated concentration (down): (A) Octopamine 1000 ng/mL, (B) Octopamine sulphate 100 ng/mL, (C) Meldonium 200 ng/mL, (D) Ethylglucuronide 5 µg/mL , (E) Molidustat glucuronide 2 ng/mL

The method was proven specific (no significant interferences were detected at the retention times of the analytes under investigation), sensitive (in some case limits of detection significantly lower than the 50% of the MRPL, see Table 1) and reproducible (retention times and relatives abundances). Carryover signals were not detected in blank urine samples that were injected after fortified urine samples.

The performance and the applicability of the method in real samples were evaluated by the analysis of samples containing ethanol, meldonium, octopamine, and molidustat. All the analytes were clearly detected and were distinguishable from matrix interferences. They could be identified by their characteristic fragment ions and retention times, satisfying the criteria for compounds identification established in the WADA technical document TD2015IDCR.

So far, it has been used to detect and confirm meldonium in 12 routine doping control samples in a concentration range between 10 and 614 ng/mL, resulting in 10 adverse analytical findings (AAF).

The labeled IS (EtG-d5) also enables a reliable semi-quantification of ethylglucuronide in urine samples after alcohol consumption.

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Conclusions

It was demonstrated that this newly developed method manages to solve the problems arisen from the analysis of five highly polar and ionizable compounds with difficult chromatographic behavior on common RPLC procedures. The method has been tested to be specific and sensitive with limits of detection below the 50% of the minimum required performance level (MRPL) established by WADA. A fast and simple protocol (“dilute-and-shoot”), minimal sample volume (10 µL) and large dilution (1:50) are very useful tools for doping control analysis.

The applicability of the method has been successfully demonstrated by the analysis of interlaboratory samples (supplied by WADA and WAADS) and doping control routine samples.

Additional experiments are currently in progress to include other analytes and to evaluate other types of HILIC stationary phases.

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Fernández-Álvarez M[1], Cuervo D[1], Baltazar-Martins JG[2], Plata MDM[3], Muñoz-Guerra JA[3], Lara B[2], Del Coso J[4], Muñoz G[1], Carreras D[1]

Determination of tramadol and its metabolites in plasma by using liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q-TOF/MS)

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Exercise Physiology Laboratory, Camilo José Cela University, Madrid, Spain[2];
Doping Control, Spanish Agency for Health Protection in Sport, Madrid, Spain[3];
Exercise Physiology Laboratory, Rey Juan Carlos University, Madrid, Spain[4]

Abstract

Tramadol is a commonly prescribed analgesic which is not included on the WADA List of Prohibited Substances; instead, this compound is placed on the WADA Monitoring Program, where it has remained since 2012. The outcomes of this program have revealed that tramadol is frequently used in cycling and other endurance sports. Since March 2019, the International Cycling Union (UCI) has implemented a new in-competition ban on the use of this narcotic across all its disciplines.

As a part of a project which aims for the evaluation of the performance-enhancing advantages of tramadol consumption, a simple procedure based on liquid–liquid extraction (LLE) using *tert*-butyl methyl ether (TBME) followed by LC-Q-TOF detection has been validated for the quantification of tramadol and its main metabolites (*O*-desmethyltramadol (M1) and *N*-desmethyltramadol (M2)) in plasma. Calibration curves were linear over the concentration ranges of 10–400 ng/mL (parent compound, PC), 2.5–100 ng/mL (M1) and 1.25–50 ng/mL (M2), leading to limits of quantification (LOQ) of 19 ng/mL, 5.2 ng/mL and 2.7 ng/mL, respectively. The accuracy, intra-day and inter-day precision were evaluated at three levels of concentration (low, medium and high) and the attained values were similar to those ones found in the literature for the target compounds.

This approach was applied to the analysis of samples obtained from seven healthy volunteers after oral administration of tramadol (1–2 mg/kg) in three alternate-day intakes. The highest plasma concentrations were observed 1–3 h after consumption. Additionally, other metabolites have been tentatively identified based on their accurate mass and MS/MS fragmentation.

Introduction

There is an ongoing controversy about the performance enhancement associated with tramadol use in sport; meanwhile, it seems clear that the side effects attributed to its consumption (nausea, somnolence, deficits in attention and concentration, etc.)[1] might increase the risk of accidents during competition and training. In fact, since March 2019, the UCI has executed a new in-competition prohibition on the use of tramadol [2].

The goal of this study is the description and validation of a simple and rapid methodology for the quantification of tramadol PC, M1 and M2 in plasma via accurate mass quadrupole time-of-flight LC-MS.

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This protocol has been applied to investigate the pharmacokinetics of the target compounds after oral administration of tramadol to seven athletic volunteers.

Experimental

Tramadol, *O*-desmethyltramadol and *N*-desmethyltramadol were purchased from LGC, TRC and Cerilliant, respectively. ^{13}C-d$_3$-Tramadol, d$_6$-*O*-desmethyltramadol, d$_3$-*N*-desmethyltramadol (ISTDs) were acquired from Cerilliant. Methanol and acetonitrile were obtained from Fisher Chemical, TBME from VWR, formic acid from Scharlau and tris base from Sigma-Aldrich.

Calibration standards and quality control (QC) samples were prepared by spiking blank human plasma (Biowest) with the appropriate methanolic solution (PC, M1 and M2) obtained by dilution of stock and commercial solutions.

Figure 1 depicts sample collection after oral administration of tramadol, approved by the Research Ethics Committee of Camilo José Cela University. Blood samples, drawn into EDTA tubes, were centrifuged and plasma separated.

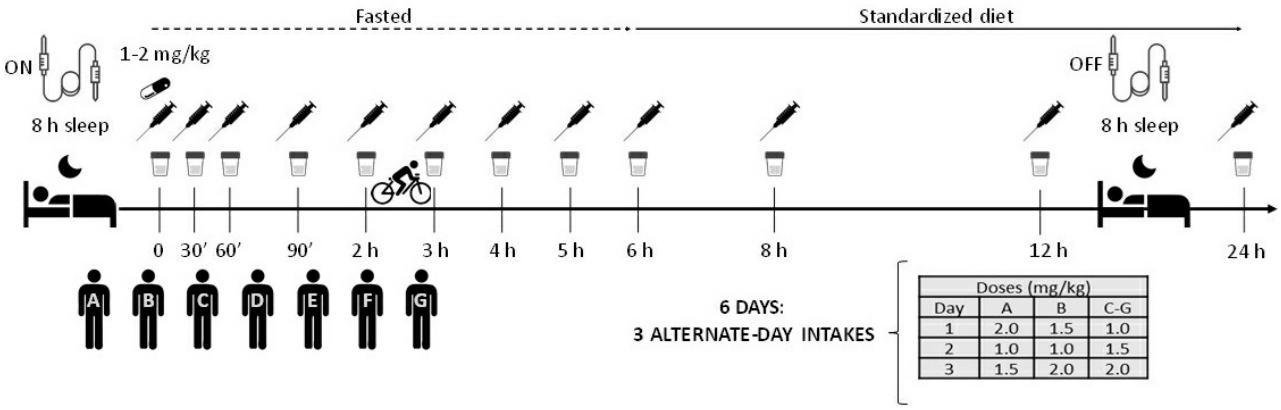


Figure 1. Description of the sample collection after oral administration of Tramadol to seven healthy athletic volunteers

Sample preparation was adapted from Campanero *et al* [3]. Briefly, 10 µL of ISTD solution (5 µg/mL) and 1 mL of tris buffer (pH 11, 10 mM) were added to 250 µL of sample in a glass tube. Once vortex-mixed, 3 mL of TBME was added and the tube was capped and shaken (10 s). After centrifugation and freezing (-30°C), the organic layer was removed, evaporated to dryness, reconstituted in 500 µL of water (0.2% formic acid) and transferred to a vial.

Samples were analysed in positive mode in a 1290 Infinity HPLC coupled to a 6550 iFunnel Q-TOF mass analyser equipped with an electrospray ionization source with Agilent Jet Stream technology (Agilent Technologies). Compounds were separated employing an Agilent Poroshell 120 EC-C18 column (2.1 x 50 mm, 2.7 µm). The mobile phase (0.4 mL/min) consisted of water (A) and acetonitrile (B), both containing 0.2% of formic acid. The gradient was: 0-1 min, 1% B; 1–7 min, to 60% B; 7–7.1 min, to 100% B; 7.1–9.1 min, 100% B; 9.1–9.2 min, to 1% B; 9.2–11.0 min, 1% B. The injection volume was 10 µL. The capillary and fragmentor voltages were 4000 V and 150 V, respectively. Data were acquired in full scan or target MS/MS mode.

Results and Discussion

The suitability of the proposed methodology for the quantification of tramadol PC, M1 and M2 in plasma was assessed in terms of linearity, limits of quantification (LOQs), accuracy, combined standard uncertainty (u_c) and precision. The outcomes of the validation, summarized in Table 1, were obtained from the results of ten experiments carried out on ten different days by two qualified operators.

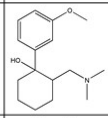
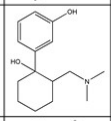
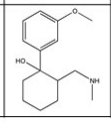
Compound	Structure	$[M+H]^+$	t_R (min)	Linearity		LOQ (ng/mL)	Level	Concentration (ng/mL)	Accuracy (%)	u_c (%)	Precision (CV, %)	
				Concentration range (ng/mL)	r						Intra-day (n=3)	Inter-day (n=10)
Tramadol PC		264.1958	3.72	10-400	0.99974	19	Low	60	100	10.3	1.6	8.2
							Medium	200	107		1.1	5.9
							High	360	98		1.3	6.4
Tramadol M1 (*O*-desmethyltramadol)		250.1802	3.05	2.5-100	0.99970	5.2	Low	15	95	11.6	1.4	6.6
							Medium	50	106		1.5	8.6
							High	90	100		1.3	8.0
Tramadol M2 (*N*-desmethyltramadol)		250.1802	3.76	1.25-50	0.99970	2.7	Low	7.5	96	8.4	1.7	4.4
							Medium	25	104		1.4	6.7
							High	45	100		1.8	5.3

Table 1. Linearity, limits of quantification (LOQs), accuracy, combined standard uncertainty (u_c) and precision of the validated method

The validated method was applied to investigate plasma concentrations of the target compounds in a pharmacokinetic study performed with seven healthy volunteers (A-G) after oral administration of tramadol (1–2 mg/kg). Figure 2 shows concentration-time profiles in plasma. The median and interquartile range (IQR) of dose adjusted plasma concentrations for tramadol PC, M1 and M2 were 81.0 and 23.7–112, 22.4 and 10.7–30.1, and 5.1 and 1.6–9.2 ng/mL per mg/kg, respectively. The median metabolic ratios to M1 and M2 were 0.33 (IQR, 0.23–0.44) and 0.08 (0.04–0.12), respectively. Maximum plasma levels were reached within 1 and 3 h after administration with concentrations ranging between 111–484 ng/mL (PC), 25–100 ng/mL (M1) and 3.6–37 ng/mL (M2). Moreover, contrary to the results obtained in urine [4], concentrations in this matrix were close or below the LOQs in samples collected at 24 h.

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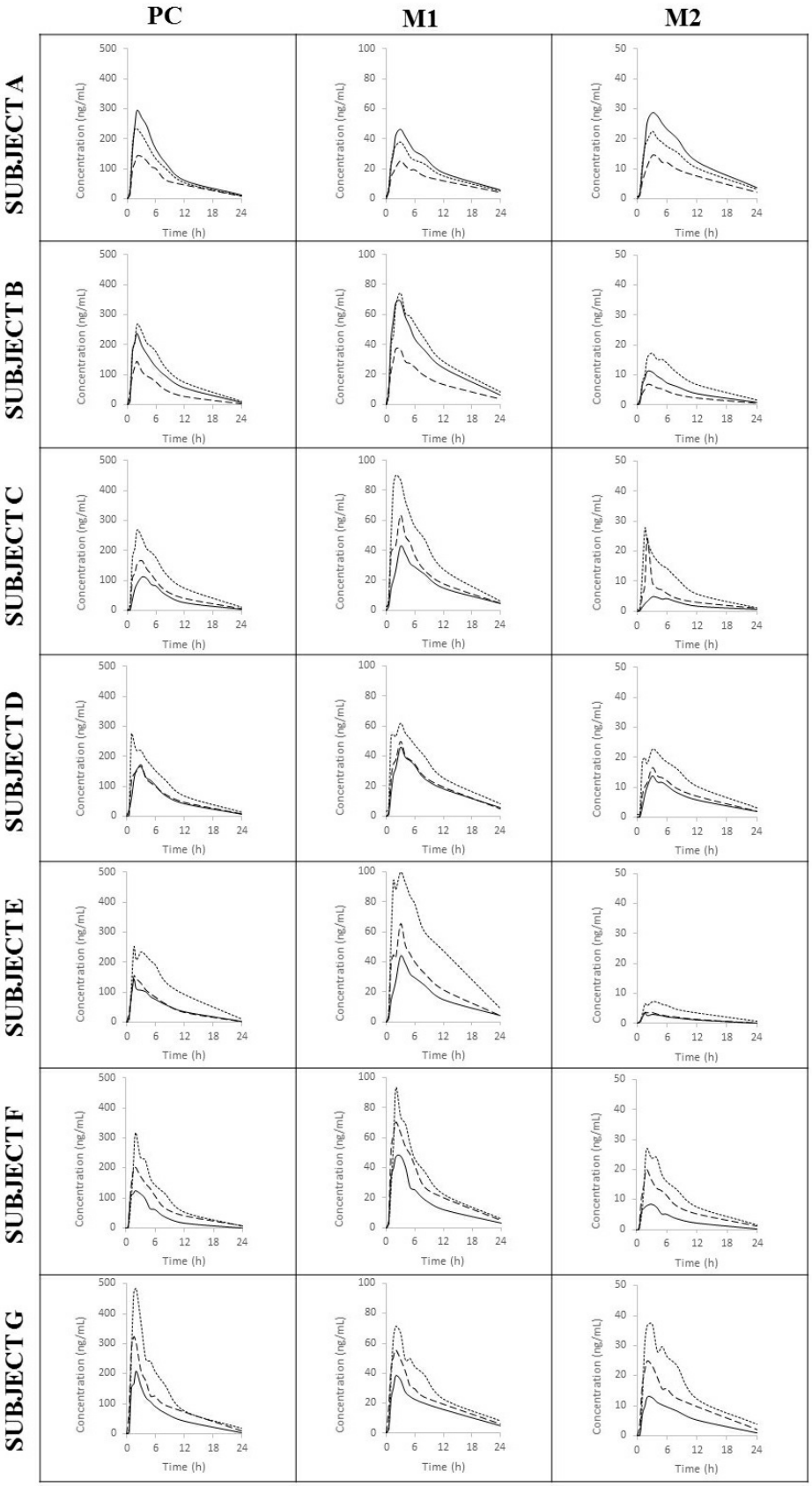

Figure 2. Concentration curves in plasma for subjects A-G: day one (solid line), day two (dashed line), day three (dotted line). See the corresponding doses for each day in Figure 1.

The metabolism of tramadol was further investigated through the tentative identification of other biotransformation products, mainly on the basis of their accurate masses, examination of experimental Product Ion spectra obtained at different collision energies (10, 20, 40 eV) and research published by other authors [5,6]. The proposed metabolites with their accurate masses, molecular formulae and retention times are listed in Table 2. Detection of *N,O*-didesmethyltramadol (named M5 in bibliography) is remarkable because this compound is, besides M1, the only known active tramadol metabolite [7]. For M5, plasma peaks at 1.5-4 h were observed.

Metabolite	Accurate mass	Molecular formula	t_R (min)
N,O-didesmethyltramadol	236.1645	$C_{14}H_{21}NO_2$	3.10
Hydroxytramadol	280.1907	$C_{16}H_{25}NO_3$	3.05
Hydroxy-*O*-desmethyltramadol	266.1751	$C_{15}H_{23}NO_3$	2.26

Table 2. Additional tramadol metabolites identified in the administration study

Conclusions

Quantification of tramadol PC, M1 and M2 in plasma using QTOF detection led to acceptable results in terms of linearity, sensitivity, accuracy and precision. The proposed methodology was suitable to perform pharmacokinetic investigations.

Unlike urine, concentrations in plasma one day after administration were barely quantifiable, which suggests that plasma could be more useful as matrix for tramadol analyses in an in-competition doping control context.

Finally, despite the lack of the corresponding reference materials, it was possible to explore deeper into tramadol biotransformation by the tentative identification of other metabolites and thanks to the high resolution and exact mass capabilities of the LC-Q-TOF/MS system.

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Cuervo D[1], Fernández-Álvarez M[2], Baltazar-Martins JG[3], Plata MDM[1], Muñoz-Guerra JA[1], Lara B[3], Del Coso J[4], Muñoz G[2], Carreras D[2]

Determination of tramadol and its metabolites in urine via accurate-mass quadrupole time-of-flight (Q-TOF) LC/MS

Doping Control, Spanish Agency for Health Protection in Sport, Madrid, Spain[1];
Madrid Anti-Doping Laboratory, Madrid, Spain[2];
Exercise Physiology Laboratory, Camilo José Cela University, Madrid, Spain[3];
Exercise Physiology Laboratory, Rey Juan Carlos University, Madrid, Spain[4]

Abstract

The analgesic drug tramadol has been banned by the International Cycling Union (UCI) in 2019, remaining as a monitored substance (i. e. not prohibited) by the World Anti-Doping Agency (WADA). While there is a current debate about the performance enhancement associated with its consumption, several studies have clearly shown the spread of its use in certain sports (especially cycling) and its link with secondary effects that can cause a risk of falling.

As part of a project designed to shed light on the question of the compliance of the criteria substantiating the inclusion of tramadol in the WADA List of Prohibited Substances, a method for the quantification of this drug and its two main metabolites in urine and plasma has been developed in our laboratory. By using a straightforward sample preparation and accurate-mass quadrupole time-of-flight (Q-TOF) LC-MS detection, the wide range of concentrations that can be found in these matrixes after the intake of the substance could be covered and fully validated.

The methodology used for the quantification of tramadol, *O*-desmethyltramadol and *N*-desmethyl-tramadol in urine and the validation results are presented in this poster. The protocol was applied to the analysis of the samples collected during the first administration studies (1-2 mg/kg) to cyclist volunteers. Concentration peaks were observed at 4 hours after administration for all volunteers and doses. Additionally, by using the high resolution and accurate mass capabilities of the HPLC-QTOF system, more than 15 secondary metabolites could be detected in the samples from the excretion study.

Introduction

The analgesic drug tramadol, commonly prescribed to treat moderate to moderately severe pain, has been banned by the International Cycling Union (UCI) since 2019, remaining as a monitored substance (i.e. not prohibited) [1] by the World Anti-Doping Agency (WADA). Nevertheless, several studies have clearly shown the spread of its use in certain sports (especially cycling) [2] and its link with an increased risk of falling [3].

A method for the quantification of this drug and its two main metabolites in urine, based on accurate-mass quadrupole time-of-flight (Q-TOF) LC-MS detection, has been developed and fully validated in our laboratory. The protocol was subsequently applied to the analysis of the samples collected during an administration study carried out with several cyclist volunteers.

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Experimental

All solvents and reagents used were of analytical grade. Commercial solid standards: *O*-desmethyltramadol was purchased from TRC (North York, Canada). From there, standard solutions were prepared in methanol at a concentration of 1 mg/mL. Commercial standards in solution: tramadol (1.0 mg/mL) was from LGC (Luckenwalde, Germany). *N*-desmethyltramadol (1.0 mg/mL), ^{13}C-d_3-tramadol (1.0 mg/mL), d_6-*O*-desmethyltramadol (0.1 mg/mL) and d_3-*N*-desmethyltramadol (0.1 mg/mL) were from Cerilliant (Round Rock, TX, USA). From there, working solutions of the analytes and ISTD were prepared in methanol at adequate concentrations. Calibration standards and quality control (QC) samples were prepared by spiking blank human urine with the corresponding volume of each appropriate working solution.

Sample preparation: a dilute-and-shoot protocol was used. Two different dilutions (for low and high concentration levels) were performed: for regular samples, a 50-fold dilution was carried out by adding 4890 µL of water (0.2% formic acid) and 10 µL of ISTD solution to 100 µL of the sample; for samples with high tramadol content, a 500-fold dilution was applied by adding 4980 µL of water (0.2% formic acid) and 10 µL of ISTD solution to 10 µL of the sample. In both cases, the diluted sample was shaken on vortex for 30 s and 500 µL were transferred to HPLC vials.

All samples were analysed by a LC-MS (Q-TOF) system from Agilent Technologies (Palo Alto, CA, USA). The instrument consisted of a 1290 Infinity HPLC coupled to a 6550 iFunnel QTOF mass analyser equipped with a dual AJS (Agilent Jet Stream) ESI (electrospray ionization) source. The target compounds were separated employing an Agilent Poroshell 120 EC-C18 column (50 mm length, 2.1 mm internal diameter, 2.7 µm film thickness). Full scan acquisition was employed during validation and pharmacokinetic experiments. Target MS/MS data (Product ion mode) was also acquired in order to tentatively identify other tramadol metabolites found in urine.

Results and Discussion

A split quantification curve for high and low tramadol content samples was validated. 5 calibration curves and 5 QC sets were prepared from 6 independent working solutions. 10 experiments were performed in 10 days with calibration and QC samples prepared from different working solutions in 6 different urine samples for each experiment. The validation results are shown in Table 1. Correlation coefficients were >0.994 for all experiments and analytes, covering concentration ranges from 50-10000 ng/mL (tramadol PC), 40-4000 ng/mL (tramadol M1) and 40-2000 ng/mL (tramadol M2).

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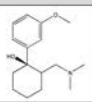
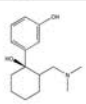
Compound	Structure	[M+H]$^+$	t_R (min)	Dilution	Linearity				Quality controls					
					Conc. range (ng/mL)	Conc. levels	r (for all 10 exp.)	LOQ (ng/mL)	Level	Conc. (ng/ml)	Accuracy (%)	u_c (%, average)	Intra-day prec. (n=3, CV, %))	Inter-day prec. (n=10, CV, %))
Tramadol PC		264.1958	3.72	1:50	10-500	5	>0.999	43.2	Low	40	108	10.5	1.9	8.9
									High	400	100		1.6	5.9
				1:500	500-10000	6	>0.996		Low	900	101		5.0	8.0
									Medium	4000	97		4.3	7.1
									High	9000	100		3.7	6.2
O-desmethyltramadol (Tramadol M1)		250.1802	3.05	1:50	5-1000	8	>0.999	37.6	Low	50	92	10.6	-	6.9
									Medium	400	94		-	7.7
									High	900	97		.	6.4
				1:500	200-4000	6	>0.994		Low	360	100		5.2	7.4
									Medium	1600	95		3.4	5.8
									High	3600	98		4.3	7.5
N-desmethyltramadol (Tramadol M2)		250.1802	3.76	1:50	5-1000	8	>0.999	39.6	Low	50	99	10.1	-	6.7
									Medium	400	97		-	6.7
									High	900	104		-	6.0
				1:500	100-2000	6	>0.996		Low	180	95		8.4	6.3
									Medium	800	95		4.1	6.0
									High	1800	98		4.4	9.3

Table 1. Validation results of tramadol PC, *O*-desmethyltramadol (tramadol M1) and *N*-desmethyltramadol (tramadol M2)

The methodology was successfully applied to an administration study carried out with cyclist volunteers in order to obtain the excretion curves of tramadol and its metabolites. The administration protocol (approbed by the Research Ethics Committee of Camilo José Cela University, Spain) was as follows: after an overnight fasting, single doses of tramadol (1, 1.5 or 2 mg/kg) were orally administered to 7 healthy volunteers in 3 alternate-day intakes. Urine samples were collected prior intake and during 24 h after drug administration. The protocol for high concentrations was applied to quantitate tramadol and its metabolites. As an example, excretion curves for one volunteer are shown in Figure 1.

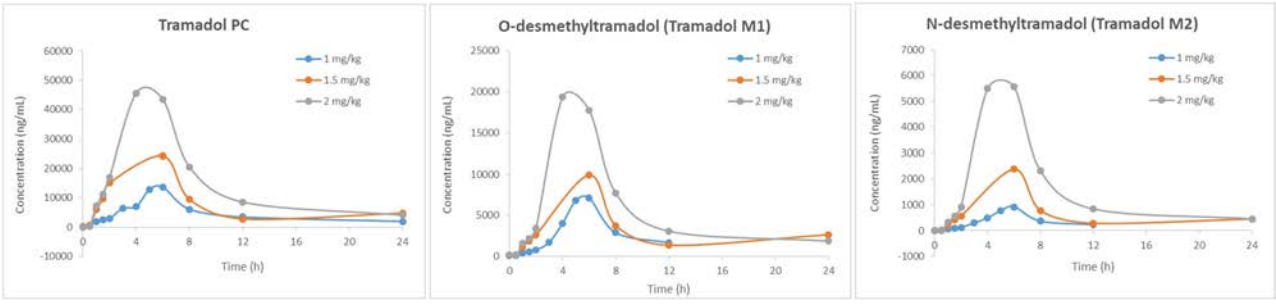


Figure 1. Excretion curves for one volunteer of tramadol and its two main metabolites

The highest concentration peaks of tramadol and its metabolites were found at 4-6 hours after administration for all subjects and doses. In all cases: conc. tramadol PC > tramadol M1 > tramadol M2, in agreement with previously published works [4]. Interestingly, concentrations higher than 1000 ng/mL (up to 8800 ng/mL) for tramadol PC were found in the urine samples collected after 24 hours of administration.

Additionally, by using the high resolution and accurate mass capabilities of the HPLC-QTOF system, more than 15 secondary non-conjugated metabolites could be detected in the samples from the excretion study (see Table 2). *O,N*-didesmethyltramadol was found to be the most important alternative metabolite in terms of intensity of signal and curve of detectability over time.

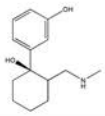
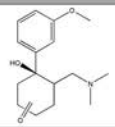
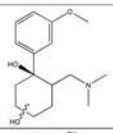
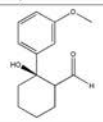
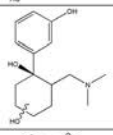
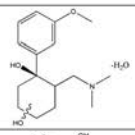
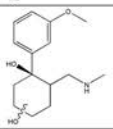
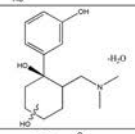
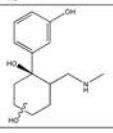
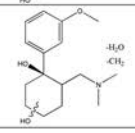
Metabolite	Formula	Structure	Isomers found	Metabolite	Formula	Structure	Isomers found
O,N-didesmethyltramadol	$C_{14}H_{21}NO_2$		1	Oxotramadol	$C_{16}H_{23}NO_3$		2
Hydroxytramadol	$C_{16}H_{25}NO_3$		3	2-formyl-1-(3-methoxyfenylcyclohexanol	$C_{14}H_{18}O_3$		1
Hydroxy-O-desmethyltramadol	$C_{15}H_{23}NO_3$		4	Hydroxydehydrotramadol	$C_{16}H_{23}NO_2$		1
Hydroxy-N-desmethyltramadol	$C_{15}H_{23}NO_3$		2	Hydroxydehydro-O-desmethyl tramadol	$C_{15}H_{21}NO_2$		1
Hydroxy-O,N-didesmethyltramadol	$C_{14}H_{21}NO_3$		2	Hydroxydehydrodesmethyl tramadol	$C_{15}H_{21}NO_2$		1

Table 2. Alternative metabolites found in the administration study

Conclusions

Quantification of tramadol and its two main metabolites in urine by using high-resolution LC-MS (QTOF) can be achieved through two protocols and a split quantification curve for high and low tramadol content samples. Correlation coefficients were >0.994 for all experiments and analytes, covering concentration ranges from 50-10000 ng/mL (tramadol PC), 40-4000 ng/mL (*O*-desmethyltramadol) and 40-2000 ng/mL (*N*-desmethyltramadol).

The methodology was successfully applied to an administration study carried out with athlete volunteers in order to obtain the excretion curves of tramadol and its metabolites. Interestingly, concentrations above 1000 ng/mL for tramadol PC were found in every urine sample collected after 24 hours of administration, which exceeds substantially the reporting value established for tramadol in the WADA Monitoring Program (50 ng/mL).

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Gotzmann A

"Operation Aderlass": Legislation as a cornerstone of anti-doping

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Abstract

Major doping cases have hit international sports repeatedly in the past. The credibility of sport and the commitment to fairness and equal opportunities are have come under scrutiny. Doping not only harms competitors in competition, but also the sport organizers, sponsors and spectators who spend their assets trusting in fair sport. Therefore, there is considerable interest in protecting sports from this existence-threatening risks. The sport community has already taken necessary and appropriate measures in order to protect athletes as well as sporting competitions from doping. The founding of the World Anti-Doping Agency (WADA) in 1999, followed by the National Anti-Doping Organizations (NADOs) and their gradual development are the authoritative basis for prosecution and punishment of doping violations in sport. The new segment of intelligence and investigations will contribute to this goal as well. These measures would remain unsatisfactory by comparison to the criminal background in light of the dimension that doping and doping networks have taken, however. The German legislator therefore established an anti-doping law in late 2015. The anti-doping legislation makes athletes' self-doping punishable. Backers risk being arrested for the crime along with the athletes. The work of NADA Germany was strengthened through the law, *e.g.* by enabling legislation for data transmission of public prosecutors in line with data protection standards for collecting, processing and using personal data of athletes. *"Operation Aderlass"* and the criminal structures detected in the Erfurt area have shown that Germany has set out on the right path by establishing its anti-doping act. The possibilities of detecting doping have grown far today. House searches like in Erfurt, where several blood bags and various material for transportation, as well as freezers, were found, are possible. NADA Germany will continue to cooperate closely with the specialized offices of public prosecutors in Munich, Freiburg and Zweibrücken to solve doping networks. This is more than necessary in order to make sure that clean athletes get their right for clean competition.
Conclusion:
"Operation Aderlass" demonstrates the importance of collaboration between a NADO and public prosecutors for fair sport. The anti-doping legislation in Germany is an important cornerstone to detect and investigate doping networks. The collaboration facilitated between law enforcement authorities and NADA is a major cornerstone to protect clean athletes. Every athlete has a right to a clean competition.

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Rossi F[1], Saugy M[2]

Dried Blood Spots: Alternative matrix to deter and detect Tramadol abuse in road cycling competitions

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REDS, Lausannne University, Lausanne, Switzerland[2]

Abstract

Tramadol is an opioid agent designed to treat chronic and acute pain. However, this drug can be misused to overcome pain arising during a prolonged physical effort while promoting numbness and confusion and decreasing attention. Tramadol has been for years in the WADA monitoring program, which shows a high prevalence of use, especially in cycling. However, despite several attempts (during 7 years), it has never been introduced in the prohibited list. It is why, in 2019, UCI decided to ban the use of tramadol via a medical rule, and to implement the rule in some road cycling competitions. Being a medical rule, it was then clear that antidoping samples could not be used to control the application of this medical rule. It was then decided to implement an alternative matrix, the dried blood spots (DBS), and to perform the analyses in a clinical laboratory, which was already experienced tin the analysis of tramadol in DBS. Thus, a method has been developed for sample preparation followed by a liquid-chromatography mass spectrometry (LC-MS/MS) analysis for the detection of tramadol and its two main metabolites. This method intends to bring the easy to use and minimal sample preparation associated with the use of DBS as matrix, with performances comparable to traditional methodologies. Thanks to this method, it was possible to detect up to 20 h the intake of therapeutic doses of tramadol by the level of tramadol in blood. Between 1 March 2019 and the last race of the season in mid-October, over 700 samples were collected and none was found positive. At first glance, the UCI tramadol ban policy had a remarkable deterrent effect in the selected races. However, in order to have a more precise evaluation on the overall impact it is necessary to wait for the 2019 WADA statistics on the monitoring program.

Published as:
Salamin, O, Garcia, A, González-Ruiz, V, et al. Is pain temporary and glory forever? Detection of tramadol using dried blood spot in cycling competitions. *Drug Test Anal*. 2020; 12: 1649-1657. https://doi.org/10.1002/dta.2923

Knoop A[1], Planitz P[2], Wuest B[2], Thevis M[1]

Evaluation of cobalt in doping control samples: Discrimination of different cobalt sources

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Agilent Technologies Sales & Services GmbH und Co. KG, Waldbronn, Germany[2]

Abstract

As a HIF activating agent, cobalt is part of the WADA Prohibited List. In its inorganic form, Co^{2+}-salts are highly potent agents to stimulate erythropoiesis leading to increased aerobic capacity. Apart from its performance-enhancing properties, intake or exposure to elevated amounts of inorganic cobalt can cause critical adverse effects. Taking all this into account, routine testing for cobalt in urine by means of ICP-MS analysis was established in 2015. Within these measurements, total cobalt concentrations are determined, notwithstanding its chemical origin. By contrast, essential vitamin B_{12}, which is composed of organically complexed cobalt resulting in a completely different mechanism of action, is classified as legitimate nutritional supplement. Herein, elevated urinary cobalt concentrations in doping control samples were diversified by follow-up experiments. Owing to the analytical needs to discriminate different cobalt sources, an excretion study comparing the intake of $CoCl_2$ and vitamin B_{12} was carried out. Subsequently, a HPLC-ICP-MS coupling was developed for the simultaneous and specific detection of both, free Co^{2+} and organic cyanocobalamin, for the first time.

Published as:
Knoop, A, Planitz, P, Wüst, B, Thevis, M. Analysis of cobalt for human sports drug testing purposes using ICP- and LC-ICP-MS. *Drug Test Anal*. 2020; 12: 1666-1672. https://doi.org/10.1002/dta.2962

Paßreiter A, Thomas A, Walpurgis K, Thevis M

First steps towards uncovering gene doping with CRISPR/Cas9

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Abstract

The discovery of the **C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats/**C**RISPR **as**sociated (CRISPR/Cas) system as a programmable, RNA-guided endonuclease, has revolutionised the utilisation of gene technology. Since it enables the precise modification of any desired DNA sequence and surpasses all hitherto existing alternatives for gene editing in efficiency and robustness as well as flexibility and simplicity, it is one of the most frequently utilised tools for editing mammalian genomes these days. But those advantages also facilitate the illicit use of the CRISPR/Cas system in order to achieve a performance-enhancing effect in sporting competitions. Since 2003, this abuse is classified as *gene doping* and is listed in the *Prohibited List* of the *World Anti-Doping Agency* (WADA), which is why there is a pressing need for an adequate analytical method to detect the misuse of the CRISPR/Cas system by athletes. Hence, the initial aim of this study accounts for the identification of the exogenous protein Cas9 from the bacteria *Streptococcus pyogenes* (SpCas9), which currently represents the predominantly used endonuclease in genome editing, in plasma samples by means of developing a bottom-up analytical approach via immunoaffinity purification, tryptic digestion, and subsequent detection by HPLC-HRMS/MS. The target analyte was spiked to plasma samples as commercially available recombinant SpCas9 protein. A limit of detection was accomplished at 25 ng/mL utilising two highly specific tryptic peptides and the achieved working range of the method was tested between 25 and 1000 ng/mL. Due to the lack of available post-administration samples, proof of concept data are subject of future studies.

Published as:
A. Paßreiter, A. Thomas, N. Grogna, P. Delahaut, M. Thevis. First Steps toward Uncovering Gene Doping with CRISPR/Cas by Identifying SpCas9 in Plasma via HPLC–HRMS/MS. *Analytical Chemistry* 2020 92 (24), 16322-16328, DOI: 10.1021/acs.analchem.0c04445

Coll S[1], Monfort N[1], Alechaga &[1], Matabosch X[1], Pérez-Mañà C[2], Ventura R[1]

Evaluation of the reporting level to detect betamethasone misuse in sports: additional studies after intranasal and intramuscular administrations

Fundació IMIM (Neuroscience department), Catalonian Antidoping Laboratory, Barcelona, Spain[1];
Human Pharmacology and Clinical Neurosciences Research Group, Hospital del Mar Medical Research Institute (IMIM), Barcelona, Spain[2]

Abstract

Betamethasone (BET) is a glucocorticoid used in sports by a wide range of administration routes. World Anti-Doping Agency (WADA)-accredited laboratories use a reporting level of 30 ng/mL to distinguish systemic administrations (oral, intravenous, intramuscular or rectal routes) from non-systemic administrations (e.g., topical and intranasal routes). The aim of the present work was to study the metabolic profile of BET in urine following intranasal and intramuscular administrations, and to evaluate the current reporting level.

BET was administered to healthy volunteers by intranasal (320 µg/day for 3 days, n = 4 males and 4 females) and intramuscular (single dose of 12 mg, n = 4 males and 4 females) administration route. Urine samples collected before and after administration were analysed using a liquid chromatography-tandem mass spectrometry method.

The current reporting level used to detect BET misuse in sports was evaluated by assessing the urinary profiles of BET and its metabolites obtained after intranasal and intramuscular administrations, together with the urinary profiles obtained after topical, oral and low-dose intramuscular administrations performed in previous clinical studies.

Published as:
Coll S, Monfort N, Alechaga É, Matabosch X, Pérez-Mañá C, Ventura R. Elimination profiles of betamethasone after different administration routes: evaluation of the reporting level and washout periods to ensure safe therapeutic administrations. *Drug Test Anal*. 2020; 1-12. https://doi.org/10.1002/dta.2928

Walpurgis K[1], Rubio A[1], Wagener F[1], Krug O[1], Knoop A[1], Görgens C[1], Guddat S[1], Thevis M[1,2]

Elimination profiles of microdosed ostarine mimicking contaminated products ingestion

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

Abstract

Ostarine is an aryl-propionamide-based selective androgen receptor modulator (SARM) with tissue-selective anabolic effects. Its misuse in sports is prohibited since 2008, and the number of adverse analytical findings (AAFs) in routine doping controls has continuously increased for almost a decade. Although no SARM drug candidate has obtained clinical approval yet, ostarine products are available online for research purposes or as a means to support performance enhancement.

The possibility of nutritional supplement contamination with minute amounts of the selective androgen receptor modulator (SARM) ostarine has become a major concern for athletes and result managing authorities. In case of an adverse analytical finding (AAF), affected athletes need to provide conclusive information, demonstrating that the test result originates from a contamination scenario rather than doping.

The aim of this research project was to study the elimination profiles of microdosed ostarine and characterize the time-dependent urinary excretion of the drug and selected metabolites. Single- and multi-dose administration studies with 1, 10, and 50 µg of ostarine were conducted, and collected urine samples were analyzed by LC-MS/MS following solid-phase extraction or enzymatic hydrolysis combined with liquid-liquid extraction.

In the post-administration samples, both the maximum urine concentrations/abundance ratios and detection times of ostarine and its phase-I and -II metabolites were found to correlate with the administered drug dose. With regards to the observed maximum levels of ostarine, the time points of peak urinary concentrations/abundance ratios, and detection windows, a high inter-individual variation was observed. However, the study demonstrated that a single oral dose of as little as 1 µg can be detected for up to nine (five) days by monitoring ostarine (glucuronide), and hydroxylated metabolites (epecially M1a) appear to offer a considerably shorter detection window (see Figure 1).

The obtained data on ostarine (metabolite) detection times and urinary concentrations following different administration schemes support the interpretation of AAFs, in particular when scenarios of proven supplement contamination are discussed, and supplement administration protocols exist.

Published as:

Walpurgis, K, Rubio, A, Wagener, F, et al. Elimination profiles of microdosed ostarine mimicking contaminated products ingestion. *Drug Test Anal*. 2020; 12:1570-1580. doi.org/10.1002/dta.2933

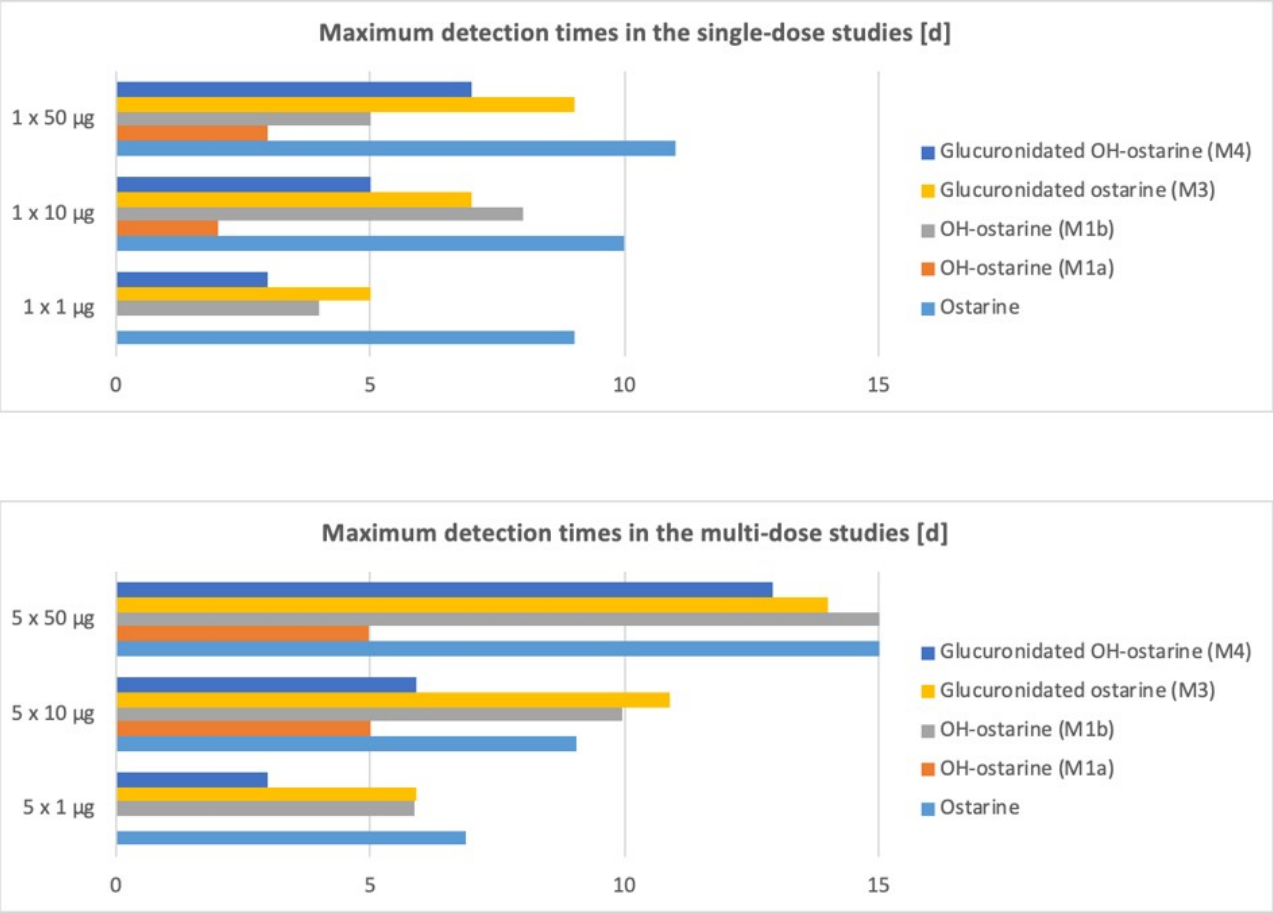

Figure 1. Summary of the maximum detection times observed in the single- and multi-dose studies

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Garzinsky A[1], Thomas A[1], Krug O[1,2], Thevis M[1,2]

Exhaled breath in doping controls: characterization of SensAbues® sampling devices using an aerosol test generator

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

Abstract

Currently, only blood and urine samples are routinely used for sports drug testing, but the interest in alternative matrices that can potentially be advantageous with regard to the invasiveness, intrusiveness, and duration of the sampling procedure, the risk for sample manipulation, the analyte stability, and the effort and costs for sample transportation and storage continuously increased over the last years. Recent studies demonstrated that exhaled breath (EB) represents a promising complementary matrix for doping control purposes. In this follow-up study, a system was developed, which can be used to simulate EB aerosol containing doping agents, as the artificial EB samples used for earlier method developments and validations had to be prepared by spiking the analytes directly onto the electrostatic filter of the sampling device. SensAbues® sampling devices were further characterized in terms of the retention efficacy by measuring the amounts of substance breakthrough (via the exit of the collection device). The analyte stability was addressed by analyzing multiple preparations of drug-containing artificial EB samples stored for 0-28 days at different temperatures (-20°C, 4°C, and RT). A total of 26 model compounds including stimulants, opioids, synthetic cannabinoids, beta-blockers and metabolic modulators were analyzed using LC-MS/MS and different analytical characteristics were determined. In addition, first-generation SensAbues® cartridges were compared to second-generation devices regarding limit of detection and recovery. All model compounds were detected via LC-MS/MS after generating artificial EB samples using the aerosol generator and no detectable amounts passed through the device at the conditions used for ventilation. When the membrane and cartridge housing of artificial EB samples were extracted separately, the ratios "$Extraction_{Membrane}/Extraction_{Cartridge}$" varied from 57-98% depending on the analyte, indicating that the analytes adsorb to both the electrostatic membrane and the container housing. Independent from the storage temperature, all model substances were detectable after a storage period of 28 days. The recoveries for all model compounds were higher than 49%, and limits of detection ranged from 1 pg to 500 pg per filter for second generation sampling devices. First generation sampling devices provided recoveries less than 49% and higher limits of detection for 7 analytes each. EB is a promising alternative test matrix for doping controls, however, further authentic post-administrations are required to provide evidence that relevant drugs or diagnostic metabolites are excreted/exhaled into the collected matrix.

Published as:
Garzinsky A, Thomas A, Krug O, Thevis M. (2020) Probing for the presence of doping agents in exhaled breath using chromatographic-mass spectrometric approaches. *Rapid Commun. Mass Spectrom.* doi:10.1002/rcm.8939

Mongongu C[1], Ericsson M[1], Madi Moussa E[1], Audran M[1], Coudoré F[2], Marchand A[1], Buisson C[1]

Quantitative determination of IGF-I in dried blood using Volumetric Absorptive Microsampling and Top-down analysis with LC-HRMS

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Laboratoire de Neuropharmacologie, INSERM UMRS 1178, Université Paris-Saclay, Châtenay-Malabry, France[2]

Abstract

Insulin-like Growth Factor I (IGF-I) is used as a biomarker to detect recombinant human Growth Hormone (rhGH) doping by athletes. For now, the only mass spectrometry assay approved by the World Anti-Doping Agency (WADA) to quantify IGF-1 in serum is a bottom-up approach based on the quantification of two peptides derived from IGF-I. However the sample preparation is time consuming and variability in the concentration obtained for the two peptides might occur. Alternatively quantification of intact IGF-I (top-down approach) was recently made possible with the increased sensitivity of high resolution mass spectrometry.

In addition to serum, dried blood spot (DBS) is also explored as a possible matrix for intact IGF-I quantification, but some limitations were previously identified, namely the inconsistent blood volume for each spot and a hematocrit (HCT) effect. Some new devices like Volumetric Absorptive MicroSampling (VAMS, Neoteryx) were developed to remove these disadvantages since they allow collection of an accurate fixed blood volume on a polymer support and limit the HCT effect. An evolution has also been made to ease the collection of a small volume of capillary blood for DBS: new devices such as TAP® (Seventh Sense Biosystems) are now also proposed as an alternative to finger pricks to obtain painless blood sampling from the upper arm. All these improvements open the door to a new way to perform doping controls for IGF-I quantification. The aim of this project was to develop and validate a fast and accurate protocol for the quantification of IGF-I based on a top-down analysis of the intact protein in 20 µL of capillary dried blood collected on VAMS.

The developed method uses single point calibration and ^{15}N-labelled IGF-I standard. The sample preparation consists of a desorption step, dissociation of IGF-I from its protein complex and protein precipitation using acidified acetonitrile followed by a mixed-mode, anion-exchange microelution solid phase extraction in 96-well format. The SPE eluate was subsequently diluted and directly analyzed by LC-HRMS (see Figure 1A for an example of a chromatogram). This quantitative method was validated in accordance with WADA International Standard for Laboratories requirements with studies of linearity, limit of quantification, precision, accuracy, specificity, recovery. The quantitative performance was found fit-to purpose with a lower limit of quantification of 25 ng/mL. Additionally, the effect of increasing HCT was also evaluated and no significant concentration-dependent HCT bias was observed for physiological HCT levels of 30-50%. The extraction using microelution SPE plate allowed fast sample clean-up facilitating high-throughput procedure before mass spectrometry analysis.

This quantification method for IGF-I performed on dried capillary blood obtained from a TAP® device was compared to WADA's recommended bottom-up method for serum IGF-I quantification. Our newly validated method was highly correlated to the bottom-up approach on serum approved by WADA (see

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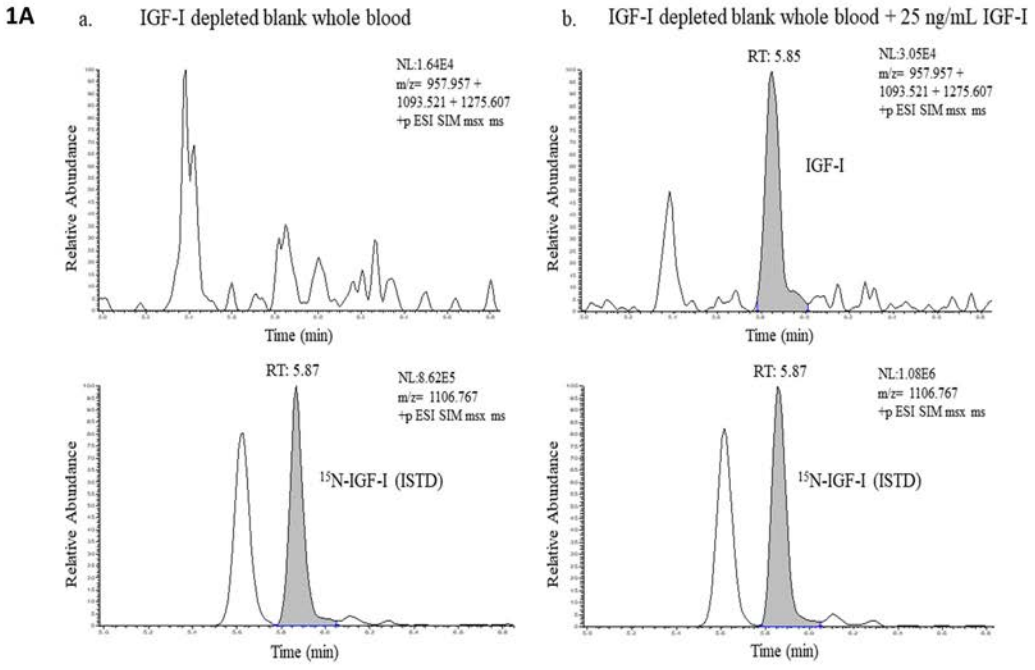
Figure 1B), but with a systematic bias (-46%) linked to the difference in matrix.

Our results support the potential use of dried capillary blood for intact IGF-I quantification as an alternative to serum. This approach would facilitate athletes' testing and may be a suitable approach to longitudinal monitoring of IGF-I.

For more details, please refer to: Mongongu C, Moussa EM, Semence F, Roulland I, Ericsson M, Coudoré F, Marchand A, Buisson C. Use of capillary dried blood for quantification of intact IGF-I by LC-HRMS for antidoping analysis. *Bioanalysis*. 2020 Jun;12(11):737-752.



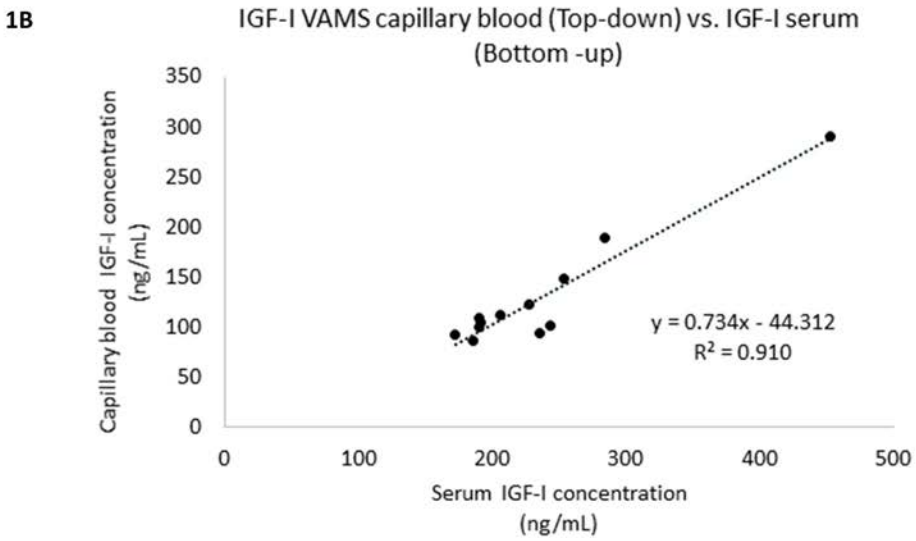


Figure 1. Extracted ion chromatograms of the IGF-I of (a) a VAMS IGF-I depleted blank whole blood and (b) a VAMS IGF-I depleted blank whole blood supplemented at 25 ng/mL IGF-I (1A) and linear regression analysis for comparison of IGF-I measurement in VAMS samples (Top-down) to the IGF-I measurements in serum samples (Bottom-up) (1B)

Miller G, Madrigal K, Lamm J, Husk J, Eichner D

Detecting growth hormone abuse in dried capillary blood spots: results from a large-scale field trial and a controlled administration study

Sports Medicine Research and Testing Laboratory, Salt Lake City, USA

Abstract

In recent years there has been a strong push in both the medical and anti-doping communities to explore the capabilities of analytical testing of dried blood. Major benefits sought by this testing include the presumed ease of collection and sample shipping, which lead to significantly lower collection costs and the possibility to increase the number of tests and frequency of testing. Many analytes have been explored in the anti-doping arena for dried blood spot (DBS) testing, however issues of reliable dried blood spot sample collection in the field remain a challenge. With the recent invention of “self-collection” devices, the hurdle of field collection may be resolved. Important to the relevance of dried blood testing is the reliable analysis of compounds only detectable in blood, namely human growth hormone (hGH). While a method for hGH testing using the Isoforms method has previously been published by our colleagues in the Barcelona laboratory, the large scale testing of hGH in an athlete population has not yet been performed. With that in mind, a non-drug field trial was recently conducted which utilized self-collection devices that automatically generate DBS from capillary blood. In this field trial, samples were collected from over 200 athletes and staff in total. These DBS samples were subsequently tested for hGH using the Isoforms method. Following analysis of the data from this non-drug field study, a further study was designed wherein a single, 2 mg dose of somatropin was self-administered to 25 male participants. DBS and matching serum samples were collected prior to the administration, as well as 4-hours, 12-hours (DBS only), and 24-hours after the injection. All samples, serum and DBS, were analyzed by the Isoforms method and compared to current decision limits that apply to serum. Data obtained during the field study and the controlled administration will be presented here.

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Dib J, Thevis M, Thomas A

Fully-automated "offline" dried blood spot (DBS) extraction and its implementation into doping control analysis

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Abstract

Dried blood spots (DBS) are a sample matrix, which can be collected under simple and minimally-invasive conditions. DBS specimens are broadly applicable in different fields of analytical disciplines, with the most common application being neonatal testing. Furthermore, these specimens are used in preclinical drug development, forensic toxicology and, increasingly, in doping control analysis. To minimize laborious and time consuming sample preparation and to increase sample-throughput of DBS, an automated sample preparation process is desirable. Using an automated offline sample preparation increases the possibilities for sample analysis. In contrast to online sample preparation/extraction methods, samples can be subjected to multiple analytical devices, if necessary, or can be re-injected. A simple DBS sample preparation method was developed, using a multi-purpose sampler (MPS) with different units to customize sample preparation after flow-through extraction of the DBS using a dried blood spot autosampler (DBSA). For sample analysis, liquid chromatography coupled to high-resolution Orbitrap mass spectrometry was used. This multi-target method covered a variety of substances from the WADA Prohibited List (anabolic agents, stimulants, beta-2 agonists etc.).

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Lange T, Thomas A, Walpurgis K, Thevis M

Fully automated dried blood spot sample preparation enables the detection of lower molecular mass peptide and non-peptide doping agents by means of LC-HRMS

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Abstract

The added value of dried blood spot (DBS) samples complementing the information obtained from commonly routine doping control matrices is continuously increasing in sports drug testing. In this project, a robotic-assisted non-destructive hematocrit measurement from dried blood spots by near-infrared spectroscopy followed by a fully automated sample preparation including strong cation exchange solid-phase extraction and evaporation enabled the detection of 46 lower molecular mass (< 2 kDa) peptide and non-peptide drugs and drug candidates by means of LC-HRMS. The target analytes included, amongst others, agonists of the gonadotropin-releasing hormone receptor, the ghrelin receptor, the human growth hormone receptor, and the antidiuretic hormone receptor. Furthermore, several glycine derivatives of growth hormone–releasing peptides (GHRPs), arguably designed to undermine current anti-doping testing approaches, were implemented to the presented detection method. The initial testing assay was validated according to the World Anti-Doping Agency guidelines with estimated LODs between 0.5 and 20 ng/mL. As a proof of concept, authentic post-administration specimens containing GHRP-2 and GHRP-6 were successfully analyzed. Furthermore, DBS obtained from a sampling device operating with microneedles for blood collection from the upper arm were analyzed and the matrix was cross-validated for selected parameters. The introduction of the hematocrit measurement method can be of great value for doping analysis as it allows for quantitative DBS applications by managing the well-recognized “hematocrit effect”.

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Solheim SA[1,2,3], Stillesby Levernæs MC[1], Mørkeberg J[3], Juul A[4], Upners EN[4], Nordsborg NB[2], Dehnes Y[1]

Dried Blood Spot analysis of testosterone esters is applicable in doping analysis

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Abstract

Misuse of testosterone, including testosterone esters, is widespread in both elite and recreational sports. Direct detection of testosterone esters in doping control samples is, however, not covered by the general screening analyses. The time-consuming and costly IRMS-analysis is necessary for detection of the excreted exogenous testosterone in urine. Detection of the esters in blood samples collected in currently used blood tubes is hampered by the continued hydrolysis by esterases present in the blood [1]. The dried blood spot (DBS) methodology appears as an attractive supplementary matrix in doping control for direct detection of testosterone esters due to inactivation of the esterases in dried blood [2], as well as the easy and cost-reduced collection and transportation compared with traditional matrices. We developed the method further for detection of four testosterone esters (testosterone decanoate, isocaproate, phenylpropionate and propionate) in DBS, which we applied to DBS-samples collected in a human administration study to demonstrate the applicability in doping control. In the administration study, DBS samples from men receiving two intramuscular injections of Sustanon® 250 (n = 9) or placebo (n = 10), were collected in Denmark and shipped to the doping control laboratory in Norway for analysis, to mimic a doping control scenario. Further, we evaluated the stability of testosterone esters in DBS stored at room temperature, by refrigeration and by freeze-storage.

The developed nanoLC-HRMS/MS method was specific and had limits of detection between 50 and 200 pg/mL for the four esters. Following testosterone ester administrations, the individual esters were detectable up to 14 days, which, despite the smaller sample volume in DBS, is comparable to the detection periods previously reported in plasma [1]. Regarding analyte stability, the testosterone esters appeared stable for at least three days in DBS stored at room temperature and highly stable (> 18 months) in DBS when stored in frozen conditions. This suggest that the samples can be shipped at room temperature and stored in frozen conditions until analysis or for long-term storage. In summary, DBS analysis of testosterone esters appears applicable in doping analysis and DBS could be the favored sample matrix for testosterone ester analysis.

Manuscript in preparation: Solheim SA, Levernæs MCS, Mørkeberg J, et al. Detectability of testosterone esters in Dried Blood Spots after intramuscular injections.

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Padilha M, Cavalcanti G, Gomes M, Carneiro G, Oliveira F, Pereira H

Can Graviola juice result an AAF for higenamine?

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Abstract

Graviola (*Annona muricate* L.) is a tropical fruit of the Annonaceae family that is grown in Asia, South America and many tropical islands (Moraes *et al.*, 2016). In Brazil *A. muricate* has a kind of commercial interest, mainly due to the nutritional properties of the fruits, which are used *in natura* or to made juices and ice creams. More than 200 chemical compounds have been identified and isolated from this plant, the most important are the alkaloids, phenols and acetogenins (Coria-Téllez *et al.*, 2018). Amongst the compounds isolated from *Anonna* is the higenamine, as described by Rinaldi in 2007.

According to the technical document (TDMRPL-2019), unmodified higenamine shall not be reported at levels below than 10 ng/mL, which means 50% of the MRPL for beta-2-agonists. In 2019 LBCD reported two AAFs for higenamine. The athletes' staff claimed the presence of higenamine in their samples could come from graviola juice consumption.

Thus, the aim of this work is verify the concentration of unmodified higenamine in urine after ingestion of graviola juice or fruit and evaluate if its level could be greater than the reporting limit established by WADA.

The first excretion study was performed after ingestion of 200 grams of fruit pulp. This study was conducted with 12 volunteers. The second study was done with 5 volunteers after ingestion of 500 mL of graviola juice.

The initial analysis was done by GC-MS/MS and LC-HRMS screenings. In the GC protocol an aliquot of 2 mL of urine was hydrolyzed with β-glucuronidase from *E.coli* and the aglycone counterparts were extracted by TBME. The final extract was derivatized with MSTFA/NH_4I/2-mercaptoethanol and analyzed by GC-MS/MS. The LC protocol, two urine aliquots were used, 10 µL of one aliquot is diluted and stored. In the second aliquot internal standard is added, followed by enzymatic hydrolysis and SPE extraction. Both aliquots are combined and analyzed by LC-HRMS. The samples which arisen a presumptive adverse analytical finding (> 10 ng/mL) were submitted to the confirmation procedure. In this case the hydrolysis step was not carried out.

The first excretion study showed two volunteers with higenamine concentrations greater than 10 ng/mL in the screening analysis. Their maximum concentrations reached were estimated at 66 ng/mL after 11h of fruit intake for one of volunteer and at 21 ng/mL after 13h of fruit ingestion for one of them. These presumptive adverse analytical findings samples were further analyzed by the confirmation procedure. On this procedure no unmodified higenamine was detected on the suspect samples arisen from initial procedures. Higenamine was not observed in the urine of the volunteers who took the fruit juice.

All higenamine presents in the Graviola pulp seems to be excreted as phase II metabolite after fruit ingestion. According to our data, the consumption of Graviola (pulp) or Graviola juice is not able to generate an AAF for higenamine.

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de Wilde L, Roels K, van Eenoo P, Deventer K

Update on the use of 2D-LC-MS/MS in doping analysis

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Abstract

Previous research on the use of turbulent flow online SPE LC-MS/MS for the detection and confirmation of diuretics and masking agents has shown that workload, matrix effect and retention time shift is reduced by applying this technique. The aim was to develop a method, preferably using the same columns and mobile phases, for the detection and confirmation of stimulants, compliant with WADA's criteria. Because many compounds are in the stimulants category and putting all transitions in one method would be detrimental for the quality of the peaks, the method is divided in three sub methods. The first sub method includes the most frequently detected stimulants, the second sub method contains transitions for the other, less frequently detected stimulants. The third sub method was optimized for the detection and confirmation of cocaine and its metabolites (benzoylecgonine and methylecgonine) due to loading problems for methylecgonine. The confirmation of several routine samples previously declared positive and EQAS samples with the developed method shows that turbulent flow online SPE-LC-MS/MS can be used for the detection and confirmation of several classes of WADA's prohibited list.

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de La Torre X, Colamonici C, Molaioni F, Jardines D, Iannone M, Botrè F

Intentional or accidental clostebol detection. An Italian Story

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Abstract

Clostebol (4-chloro-testosterone) is a weak anabolic androgenic steroid included in the S1. Section of the WADA list of prohibited substances and methods. The detection of its abuse its human sports has been increasing in the last years according to WADA statistics due to the use of more sensitive instrumentation by the WADA accredited laboratories. Clostebol formulations for therapeutic use are sold in a reduced number of countries only. Its detection in Italy is quite frequent (> 40% of the cases worldwide) since it's present in a popular preparation for topic use (Trofodermin®). Its therapeutic use is mainly for the treatment of skin injuries, often associated to neomycin.

Although clostebol is prohibited independently of the administration route used, in many occasions the athletes involved have claimed its accidental contact, often trough contact with another individual. The results management authorities often ask the compatibility of the findings reported by the laboratory with the athletes' declarations during the hearing process.

In this work, we present an investigation intended to discriminate the transdermal application of clostebol from an oral application based on a potential different metabolism linked to the administration route, as happens for other steroids.

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Görgens C[1], Walker K[2], Boeser C[2], Wijeratne N[2], Martins C[2], Guddat S[1], Thevis M[1,3]

Paperspray mass spectrometry – A potential novel technique for the detection of polar compounds in sports drug testing? A pilot study

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Abstract

High performance liquid chromatography coupled to mass spectrometry (LC-MS) is widely used in sports drug testing, enabling high-throughput initial testing procedures. With the development of increasingly powerful instrumentation, a tendency towards simplified assays has been observed in doping control, where sample pretreatment has been reduced to a minimum, leading eventually to so called “dilute-and-inject” assays. However, extraordinary hydrophilic compounds still represent a challenging task in LC-MS due to their divergent chromatographic behavior.

In this pilot study, paperspray mass spectrometry was investigated as a novel and complementary technique for the initial testing of highly polar compounds that are considered as prohibited in sports. The technique utilizes a strategy that allows the ionization of analytes from complex sample matrices spotted on a paper strip. By minimizing sample preparation and omitting chromatographic separation, mass spectrometric results can be obtained in less than two minutes. All experiments were carried out using the Thermo Scientific™ VeriSpray™ ion source on a TSQ Altis™ triple quadrupole mass spectrometer. As model compounds meldonium, metformin, bemitil, p-hydroxyamphetamine, oxilofrine, octopamine and its sulfoconjugate, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), myo-inositol trispyrophosphate (ITPP), ethyl glucuronide (ETG), ethyl sulfate (ETS), tramadol, and cyano-cobalamine were chosen.

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Kraiem S[1], Al-Mahamed H[1], Al-Menhali A[1], Al-Thani N[1], Helaleh M[1], Samsam W[1], Al-Jaber MY[1], Bouabdallah S[2], Touil S[2], Georgakopoulos C[1], Beotra A[1], Mohamed Ali V[1,3], Al Maadheed M[1,3]

Analytical strategy for the detection of ecdysterone and its metabolites *in vivo* in uPA(+/+)-SCID mice with humanised liver, human urine samples and estimation of the prevalence of its use in anti-doping samples

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Abstract

Ecdysteroids are of interest as potenial sport performance enhancers, due to their anabolic effects. The aims of the current study were to outline an analytical strategy for the detection of the abundant and most commonly studied ecdysteroid ecdysterone (20-hydroxyecdysone, 20-OHE), and its metabolites 1) following admistration of pure 20-OHE to uPA(+/+)-SCID mice with humanised liver, 2) in a human volunteer after ingestion of two supplements, each containing concentrations of 20-OHE unlikely to produce anabolic effects and the other expected to be anabolic and, 3) to estimate the prevalence of the use of 20-OHE amongst elite athletes.

Sixteen supplements that were readily available from online suppliers were tested and quantitated for the presence of 20-OHE. The urine samples were collected from uPA(+/+)-SCID mice with humanised liver before and after administration of 20-OHE by oral gavage. A healthy volunteer ingested supplements containing low/median (0.0088 mg/capsule, recommended dose 0.017.6 mg) and high (2.3 mg/capsule, recommended dose 4.6 mg) levels of 20-OHE, Desire X and Turkesterone, with a 45-day washout in between. The urine samples from both SCID mice and human volunteers were analysed for 20-OHE and its metabolite(s) by gas and liquid chromatography coupled to mass spectrometry. Prevalence of 20-OHE was determined in anti-doping samples (n=1000) that were destined for discard.

Of the 16 supplements tested, only five showed detectable levels of 20-OHE, with concentrations ranging from 0.0004 – 2.3 mg per capsule. The urine of uPA(+/+)-SCID urine showed the presence of 20-OHE and its metabolite, 14 deoxyecdysterone, within 24 hours after ingestion. In humans, both the parent and the metabolite were detectable within 2 to 5 hours after ingestion. However, the metabolite was detectable for longer than the parent. After ingestion of a low dose (Desire X), the parent and metabolite were detectable for 70 hours and 48 hours, while following the higher dose (Turkesterone) it was 96 hours and 48 hours, respectively. The analysis of urines from the elite athletes showed four positive cases for 20-OHE, suggesting a prevalence of use of 0.4%.

In conclusion, dietary supplements claiming to contain 20-OHE, as per label, showed highly variable concentrations, ranging from undectable to 2.3 mg. 20-OHE and its metabolite could be detected in urines, of both uPA(+/+)-SCID mice and humans, after ingestion of pure ecdysterone or when formulated in a supplement, respectively. There was a difference in the detection window for both the parent and

the metabolite depending on dose of ingestion. Interestingly, at anabolic doses, both the parent and/or the metabolite were likely to be detectable for longer, upto 4 days after a single dose. The prevalence of its use by elite athletes was relatively low, compared to other anabolic steroids. However, this needs to be confirmed in other populations, as well as for other related ecdysteroids.

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Longitudinal steroid profiling in healthy women: impact of transdermal testosterone administration

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Abstract

The implementation of the 'urinary steroidal module' of the Athlete Biological Passport (ABP) has positively improved the detection capability of testosterone (T) doping in sport. However, this tool suffers from some limitations due to several confounding factors such as enzyme polymorphism, bacterial contamination and ethanol consumption that may alter the specificity of the model. Moreover, in females, the low circulating testosterone level combined with the lack of negative T feedback hamper a sensitive identification of urinary fluctuations caused by T administration. Therefore, new strategies have recently been explored to improve detection of T doping, such as measurement of steroid hormones in blood. In the present work, we investigated the effect of transdermal testosterone treatment on the blood and urinary steroidal profiles in healthy women.

Seven healthy women volunteers with regular menstrual cycles were recruited in a three-phase clinical trial. Each phase corresponded to one menstrual cycle. The first cycle was the control phase, followed by the treatment phase during which testosterone gel was administered daily. The third phase corresponded to the post-treatment phase. Endogenous steroids were quantified in serum samples using a validated UHPLC-MS/MS method (Ponzetto, Mehl *et al.* 2016), whereas LH, FSH and SHBG were measured by immunological assays. Urine samples of two volunteers were also analyzed by a routine GC-MS method for the steroid profile according to the criteria of the WADA technical document in force.

Among the quantified steroids in serum, testosterone and DHT were the most affected by the 28-day T treatment. The treatment did not influence the menstrual status of the volunteers, i.e. naturally cycling hormones such as progesterone, LH and FSH were unaffected by the treatment. SHBG slightly increased during treatment phase, but not significantly. Longitudinal monitoring of T and DHT concentrations using intra-individual thresholds showed that a high percentage of samples collected during treatment phase exceeded individual limits set for the expected fluctuation. Blood steroid profile of the two volunteers was then compared with the urinary steroid profile.

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Zanitzer K[1], Erceg D[2], Forsdahl G[3], Gmeiner G[1]

Comparison of the ratios of sulpho-conjugated and glucuro-conjugated phase II metabolites of endogenous steroids in urine samples after administration of testosterone preparations via UHPLC-HRMS

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Clinical Trials Unit, Childrens Hospital Srebrnjak, Zagreb, Croatia[2];
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Abstract

Intra-muscular injection of testosterone esters is a widely practised way of performance enhancement in sports. In doping control, the steroidal profile of athletes is monitored via the analysis of urine samples to detect abnormalities caused by administration of anabolic androgenic steroids (AAS). The aim of this project was to develop a complementary method that uses liquid chromatography and high resolution mass spectrometry to gain information about the glucuronide- and sulphate-conjugated phase II main metabolites of testosterone and their ratio changes after administration as testosterone esters. The method was validated according to the WADA criteria of the International Standard for Laboratories 9.01 (ISL) and applied on human samples. In a prior clinical study, 6 subjects had received a single injection of either Sustanon® – a mixture of 30 mg testosterone propionate, 60 mg testosterone phenylpropionate, 60 mg testosterone isocaproate and 100 mg testosterone decanoate – or Nebido® – 1000 mg of testosterone undecanoate. Urine samples were taken up to 60 days after the application. The samples were prepared via solid phase extraction. The results show the change of the concentrations and the ratios of glucuronides to sulphates over the sampling period. Due to the simultaneous detection of the steroid sulphates and glucuronides, an amplification effect of the alterations in post-administration samples was detectable. Though, the amplification seems to be dependent on the natural T/E ratio of the subject.

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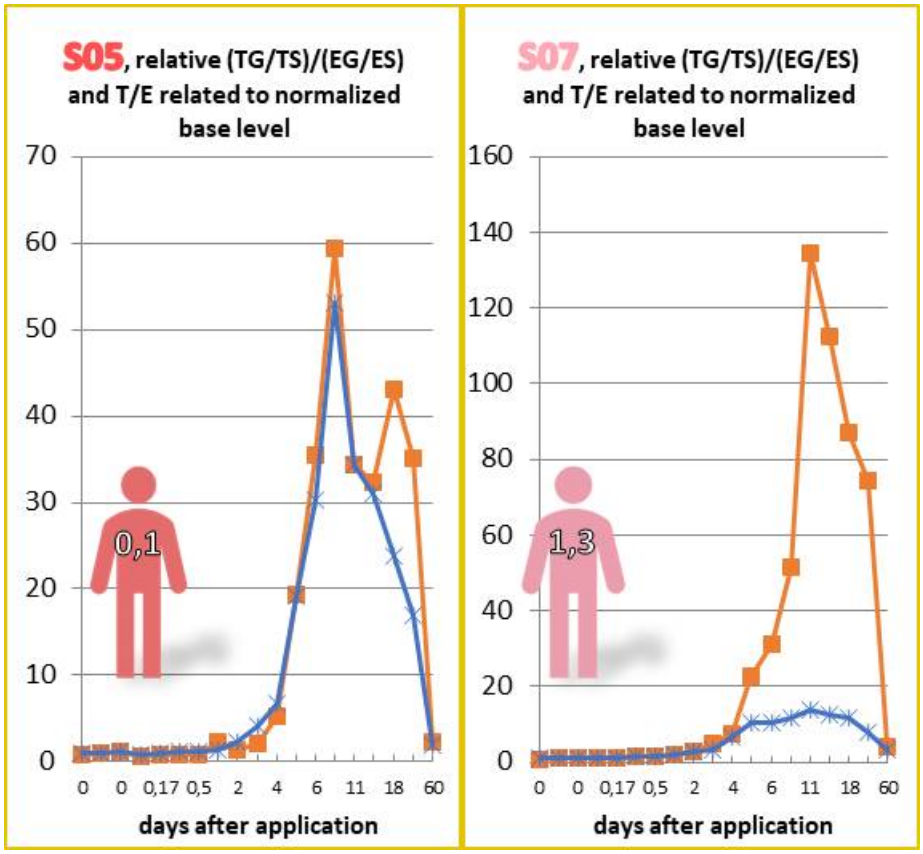

Figure 1. Combined ratio (ES/EG)/(TS/TG) (orange squares) in comparison with the T/E values (blue crosses) in urine of subject S05 and S07 over all sample points after application of Nebido® showing the relative change referring to normalized mean initial (ES/EG)/(TS/TG) and T/E with reference to the base T/E value of the subjects depicted in the human icon

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de Wilde L, Roels K, van Renterghem P, van Eenoo P, Deventer K

Steroid profiling in urine of intact glucuronidated and sulfated steroids using liquid chromatography-mass spectrometry

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Abstract

Detection of endogenous anabolic androgenic steroids (EAAS) misuse is a major challenge in doping control analysis. Currently, a number of endogenous steroids, which constitute the steroid profile, are quantified using gas chromatography (GC). With this methodology, only the sum of the free and glucuronidated steroids is measured together. A dilute-and-shoot LC-MS method, which is compliant with the quality requirements for measuring EAAS established by the World Anti-Doping Agency (WADA), was developed and validated containing glucuronidated and sulfated steroids in order to gain some extra information and to expand the existing steroid profile. The developed method is, to the best of our knowledge, the first method to combine both steroid glucuronides and sulfates, which is compliant with the quality standards of the technical document on EAAS, established by WADA. The first advantage of this new steroid profile is the reduced sample preparation time, as it is a direct injection method of diluted urine. A second advantage is the ability of the used gradient to separate 5α-androstane-3α,17β-diol-3-glucuronide (5ααβdiol3G), 5α-androstane-3α,17β-diol-17-glucuronide (5ααβdiol17G), 5β-androstane-3α,17β-diol-3-glucuronide (5βαβdiol3G) and 5β-androstane-3α,17β-diol-17-glucuronide (5βαβdiol17G) allowing to gain specific information on these isomers, which cannot be accomplished in GC-MS screening due to hydrolysis. This steroid profile also contains free testosterone, 5α-androstane-3,17-dione and 5β-androstane-3,17-dione as markers of degradation. In total, 17 compounds and 10 isotopically labelled internal standards are included in this method.

This work has been published in Journal of Chromatography A:
L. De Wilde, K. Roels, P. Van Renterghem, P. Van Eenoo, K. Deventer. Steroid profiling in urine of intact glucuronidated and sulfated steroids using liquid chromatography-mass spectrometry. *J Chromatogr A* 1624 (2020) 461231. doi:10.1016/j.chroma.2020.461231.

Albertsdóttir AD[1], van Gansbeke W[1], Coppieters G[1], Balgimbekova K[2], Polet M[1], van Eenoo P[1]

Searching for new long term metabolites of metenolone and drostanolone by gas chromatography-mass spectrometry with a focus on non-hydrolysed sulphates

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The Athletes' Anti-Doping Laboratory, Almaty, Kazakhstan[2]

Abstract

In sport doping control, anabolic androgenic steroids (AAS) are responsible for the majority of adverse analytical findings (AAF), indicating their popularity among athletes. The search for improved detection methodologies and metabolites with longer detection times remains an important task of which the impact is highlighted by the high number of positive cases after reanalysis of samples that were stored for several years, e.g. samples of previous Olympic Games. Over the last decade, the potential of sulphated metabolites as long term markers for AAS abuse has been illustrated. In 2019, the compatibility of gas chromatography mass spectrometry (GC-MS) with non-hydrolysed sulphated steroids was demonstrated and this approach allows incorporating these compounds in a broad GC-MS initial testing procedure at a later stage. However, research is first needed to pinpoint which non-hydrolysed sulphated metabolites are beneficial (i.e., detection window extension) for inclusion in an initial testing procedure.

In this work, a search for new long term metabolites of two popular AAS, metenolone and drostanolone, was undertaken through two excretion studies each. The excretion samples were analysed by gas chromatography chemical ionization triple quadrupole mass spectrometry (GC-CI-MS/MS) after application of 3 separate sample preparation methodologies (i.e., hydrolysis with *E.coli* and *H. pomatia.* and non-hydrolysed sulphated steroids).

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* This year's Manfred Donike Award for the best oral presentation went to Adalheidur Dóra Albertsdóttir from the Belgian Anti-Doping Laboratory in Ghent for her study on new long-term metabolites of AAS. For metenolone in particular, a sulfated metabolite was identified that significantly expands the detection window by including the commonly omitted sulfoconjugated analyte whilst maintaining routinely available instrumentation. Her research results are of high practical value, as they can be directly implemented in routine analysis by all laboratories.

Putz M, Piper T, Thevis M

Metabolite identification studies of trenbolone

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Abstract

Trenbolone is a synthetic anabolic steroid, which has been misused for performance enhancement in sports. The detection of trenbolone doping in routine sports drug testing programs is complex as methods utilizing gas chromatography/mass spectrometry are limited due to unspecific derivatization products and by-products while liquid chromatography/mass spectrometry-based assays occasionally suffer from high limits-of-detection. The number of previously reported metabolites in human urine is small, and most analytical methods rely on targeting epitrenbolone, trenbolone glucuronide and epitrenbolone glucuronide. In order to probe for the presence of additional trenbolone metabolites and to re-investigate the metabolism, an elimination study was conducted. One single dose of 10 mg of 5-fold deuterated trenbolone was administered to a healthy male volunteer and urine samples were collected for 30 days. For sample processing, published protocols were combined considering unconjugated, glucuronic acid, sulfo- and alkaline-labile conjugated steroid metabolites, and the sample preparation strategy consisted of solid phase extractions, liquid-liquid extractions, metabolite de-conjugation, HPLC fractionation, and derivatization. Analytical methods included gas chromatography/thermal conversion/ hydrogen isotope ratio mass spectrometry combined with single quadrupole mass spectrometry as well as liquid chromatography/high accuracy/high resolution mass spectrometry of the hydrolyzed and non-hydrolyzed samples. Twenty deuterium-labelled metabolites were identified including glucuronic acid, sulfo- and cysteine conjugates and characterized by parallel reaction monitoring experiments yielding corresponding product ion mass spectra. Main metabolites were identified as trenbolone-diol derivatives and potential trenbolone-diketone derivatives excreted in their glucuronic acid and sulfo-conjugated form with detection windows of 5 and 6 days, respectively. Further characterization was conducted with pseudo MS^3 experiments of the intact conjugates and by comparison of their mass spectra with reference material. For 4,9,11-estratriene-3,17-dione, reference standards are commercially available, and the trenbolone-diol derivative was synthesized in-house.

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Putz M, Piper T and Thevis M (2020) Identification of Trenbolone Metabolites Using Hydrogen Isotope Ratio Mass Spectrometry and Liquid Chromatography/High Accuracy/High Resolution Mass Spectrometry for Doping Control Analysis. *Front. Chem.* **8**:435. doi: 10.3389/fchem.2020.00435

Piper T[1], Heimbach S[2], Adamczewski M[2], Thevis M[1]

Investigations on the potential impact of different doping agents on the steroid profile by means of a steroidogenesis assay

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Abstract

The steroid profile, i.e. the urinary concentrations and concentration ratios of selected steroids, is used in sports drug testing to detect the misuse of endogenous steroids such as testosterone. Since several years, not only population-based thresholds are applied but individual reference ranges are derived from multiple test results of the same athlete, creating the steroidal module of the so-called Athlete Biological Passport (ABP). In order to maintain a high probative force of the passport over several years, samples collected or analyzed under suboptimal conditions should not be implemented in the longitudinal review. This applies to biologically affected or degraded samples and also to samples that have to be excluded due to the presence of other substances potentially (or evidently) altering the steroid profile.

While for several traditional doping agents their possible impact on the steroid profile is well recognized, for many less frequently detected or new substances this information is rarely accessible or available. Also substances that are not on the Prohibited List (i.e. medications, nutritional supplements) may alter the urinary steroid profile. Here, an *in vitro*-based method supporting the determination of a possible impact of less well investigated substances on the steroid biogenesis would be beneficial.

A subset of 19 different doping agents comprising anabolic steroids such as stanozolol, selective androgen receptor modulators (SARMs) like Andarine, selective estrogen receptor modulators (SERMs) such as clomifene, and the growth hormone secretagogue ibutamoren were investigated, employing an androgen receptor activation test, an androgen receptor binding assay, an aromatase assay, and a steroidogenesis assay.

In addition to the well-established liquid chromatography/mass spectrometry based analytical approach, measurements concerning the steroidogenesis assay were complemented for a subset of steroidal analytes by gas chromatography/mass spectrometry.

While the preliminary data collected suggest the possibility to estimate if or not a substance will have an impact on the overall steroid metabolism, predicting which parameter in particular may be influenced remains difficult.

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Polet M, de Wilde L, van Renterghem P, van Eenoo P

A fast GC-C-IRMS method for the detection of epiandrosterone sulfate in urine

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Abstract

In doping control, to confirm the exogenous origin of exogenously administered anabolic androgenic steroids (AAS), a gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) analysis is performed. Recently published work suggests epiandrosterone sulfate (EpiAS) as a promising IRMS target compound for the detection of AAS, capable of prolonging the detection window. However, EpiAS is only excreted in urine in its sulfoconjugated form while all other IRMS target compounds are excreted glucuronidated, meaning that EpiAS cannot be incorporated in the existing IRMS methods. A separate extensive sample preparation needs to be performed on this compound with a different hydrolysis and extraction procedure and a different liquid chromatography (LC) clean-up.

The current work presents a new, fast and easy to implement EpiAS IRMS method. The approach was based on the direct GC analysis of non-hydrolysed EpiAS, making the solid phase extraction, hydrolysis and acetylation step redundant. Sample preparation consisted of a simple liquid liquid extraction, followed by LC fraction collection.

A population study was performed to check the compliance with the criteria drafted by the World Anti-Doping Agency (WADA). To verify the applicability of the developed approach, the method was applied to the samples of four administration studies (i.e., dehydroepiandrosterone (DHEA), testosterone gel (T gel), androstenedione (ADION) and intramuscular testosterone undecanoate. In contrast to previously published data, the strength of EpiAS as target compound and the prolongation of the detection window in comparison with the conventional IRMS target compounds was less pronounced.

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Hülsemann F[1], Fußhöller G[1], Lehn C[2], Thevis M[1]

Excretion of 19-norandrosterone after consumption of boar meat

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Abstract

As reported in the literature and with regard to the TD2019NA, the consumption of the offal of intact, non-castrated pigs could lead to the excretion of 19-norandrosterone in urine. GC/C/IRMS analysis might not be able to differentiate between endogenous or such a 'pseudo-endogenous' origin of urinary 19-norandrosterone compared to an endogenous reference. In some cases the $\delta^{13}C$ values fulfill the criteria of an adverse analytical finding due to differing food sources of boar and consumer. However, consumption of offal (liver, kidneys, testicles) of boars is not very common in Germany and, thus, the occurrence of such an analytical finding after consumption of boar's offal is comparably unlikely.
On the other hand, the consumption of wild boar meat has increased in Germany within the last years. About 20,000 tons of wild boar meat are consumed per year. In order to assess the probability of the occurrence of urinary 19-norandrosterone after consumption of wild boar meat, urine samples of twelve subjects were tested. IRMS analysis was performed if urinary 19-norandrosterone was detected by GC/MS as well as on bulk material of German boars.
In nearly half of the urine samples, traces of 19-norandrosterone was detected after consumption of 200 to 400 g of boar meat. The highest urinary concentration was 2.9 ng/mL, and urinary 19-norandrosterone was predominantly detected from one to nine hours after the meal. $\delta^{13}C$ values of urinary 19-norandrosterone ranged from -18.9 to -23.3 ‰, leading to at least one AAF when applying the decision criteria of the TD2019NA. IRMS analysis of defatted dry mass of boars from Germany showed that $\delta^{13}C$ values for wild boar's steroids are unpredictable and may vary from -13 to -25‰.

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Piper T[1], Rubio A[2], Thevis M[1]

Investigations on carbon isotope ratios of potential novel long term metabolites of testosterone and testosterone prohormone misuse - 5α- and 5β-androstane-3α,17α-diol

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Abstract

Testosterone (T) and T-prohormone administration still constitutes a major problem in sports drug testing. The detection of its misuse remains complicated as T is an endogenous steroid, i.e. produced and excreted into urine by every athlete. Doping control laboratories employ gas chromatography/mass spectrometry (GC/MS)-based techniques to screen for elevated urinary T concentrations on a routine basis. As soon as elevated concentrations or concentration ratios like T over epitestosterone (E) are detected, samples are forwarded to isotope ratio mass spectrometry (IRMS) for confirmation purposes by measuring the carbon isotope ratios (CIR) of urinary steroids.

In a recent study it could be demonstrated that especially epiandrosterone (EPIA), a minor urinary metabolite of T, enables significantly prolonged detection times after the administration of T or T prohormones using IRMS [1]. While the main metabolites of T, androsterone (A) and etiocholanolone (ETIO) are significantly influenced for less than 18 h only, the minor metabolites 5α-androstane-3α,17β-diol (5aDIOL) and 5β-androstane-3α,17β-diol (5bDIOL) allow for detection of T administrations for approx. 24 to 36 h. EPIA prolongs this detectability remarkably for up to 120 h and can therefore be called a long-term metabolite for CIR determinations.

The main drawback of EPIA is its excretion into urine solely in its sulfoconjugated form. Commonly, for both the screening procedure relying on urinary concentrations and the confirmation via IRMS, doping control laboratories employ exclusively steroids excreted as glucuronic acid conjugates. This necessitates an additional time-consuming step in sample preparation for cleavage of sulfoconjugated steroids if one aims for EPIA. A long-term metabolite excreted glucuronidated would circumvent this limitation and could be easily implemented into current doping control methods. Two promising candidates were identified with 5α-androstane-3α,17α-diol (5aEpiD) and 5β-androstane-3α,17α-diol (5bEpiD), which have already been investigated regarding their potential to detect E administrations in the past [2]. Both steroids could prolong the detection time of E considerably. This finding was corroborated by an excretion study encompassing the administration of dehydroepiandrosterone.

The already published method for the CIR analysis of 5aEpiD and 5bEpiD was significantly improved overcoming the described problem of co-elutions between both target analytes [2]. This necessitated the complete re-validation of the novel method including the investigation of a reference population encompassing n = 72 male and female individuals. Excretion study samples from administration trials encompassing 4-androstenedione, testosterone and testosterone-gel were investigated to elucidate the potential of both metabolites.

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As the results so far were not conclusive, further research on additional excretion studies is ongoing and the complete results will be published elsewhere.

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Iannella L, Botrè F, Colamonici C, Curcio D, Ciccarelli C, de La Torre X

Potential masking effect of different prednisolone based pharmaceutical formulations on IRMS confirmation analysis

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Abstract

Isotope ratio mass spectrometry allows to discriminate the exogenous drugs from the endogenously or *in situ* produced compounds through their ^{13}C content determination. Synthetic steroids normally show delta values, $\delta^{13}C$ ‰, more negative than the endogenous ones, due to have a more depleted ^{13}C isotopic composition of the natural precursors mainly used in the industrial manufacturing processes.
In this work, we focused on the IRMS confirmation analysis of the exogenous origin of prednisolone and prednisone, two synthetic glucocorticoids banned by WADA when administered “in competition” by systemic routes. We have already developed and fully validated a GC-C-IRMS method according to WADA requirements, proving its applicability in case of the administration of drugs with typical exogenous delta values. The carbon isotopic composition of 14 worldwide commercially available prednisolone and prednisone pharmaceutical preparations were determined. One prednisolone nasal formulation (Sofrasolone®) showed an uncommon delta value (-17.84 ‰), suggesting the use of raw materials different from those typically selected for the steroids pharmaceutical synthesis. Its less negative delta value, even if still distinguishable from the typical endogenous European Caucasian delta values range, overlaps the ones found in the Americas. Currently, the nasal administration route of prednisolone is not prohibited by WADA. We investigated how a potential combination of a banned oral prednisolone preparation, Sintredius®, with the non banned nasal prednisolone formulation, Sofrasolone®, could affect the IRMS analyses results. Urine samples from two volunteers (23 and 28 years old) administered with Sofrasolone® (2 puffs three hours for four times a day in a single day, as suggested in the package insert) were collected before and within the 48 hours after the nasal spray administration. Additional samples were analysed after the simultaneous intake of Sofrasolone® (2 puffs every three hours for four times a day) and Sintredius® (one single oral dose). All the urine samples were suitably pre-treated and purified to isolate prednisone, prednisolone and the endogenous reference compounds (ERCs: THS, PD and PT), before the injection in the GC-C-IRMS system and the $\delta^{13}C$ values determination. The absolute $\Delta\delta_{(ERC-TC)}$ ‰ after the nasal administration of Sofrasolone® were all above 4 ‰, the value we proposed as adequate limit to confirm the exogenous administration of prednisolone and prednisone. As expected, after the administration of Sintredius®, the prednisolone and prednisone $\delta^{13}C$ (‰) values dramatically dropped to delta values more negative that the endogenous ones. The urinary concentrations of the two target compounds rapidly decreased within the 12 hours after the oral drug administration but remained higher than 30 ng/mL. At the end of this period, the resulting delta values were more affected by the contribution of Sofrasolone®. After the last nasal Sofrasolone® nasal puff, the $\delta^{13}C$ (‰) shifted towards values less depleted and overlapping with the endogenous PD delta values, showing that the association of two different prednisolone formulations based on the same active principle but produced from different natural starting materials could mask a substance misuse.

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Piper T[1], Fußhöller G[1], Geyer H[1], Danila M[2], Toboc A[2], Thevis M[1]

Detecting the misuse of 7-oxo-DHEA by means of carbon isotope ratio mass spectrometry in doping control analysis

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Abstract

The misuse of 7-oxo-DHEA (3β-hydroxyandrost-5-ene-7,17-dione) is prohibited according to the World Anti-Doping Agency (WADA) code. Nevertheless, it is easily available as a dietary supplement and from black market sources. In two recent doping control samples, significant amounts of its main metabolite 7β-OH-DHEA were identified, necessitating further investigations.

As both 7-oxo-DHEA and 7β-OH-DHEA are endogenously produced steroids and no concentration thresholds applicable to routine doping controls exist, the development and validation of a carbon isotope ratio (CIR) mass spectrometry method has been desirable. Another metabolite of 7-oxo-DHEA, the 7α-OH-DHEA isomer was also considered and excretion studies encompassing 7-oxo-DHEA, 7-oxo-DHEA-acetate and in-house deuterated 7-oxo-DHEA were conducted and evaluated with regards to urinary CIR and potential new metabolites of 7-oxo-DHEA.

Numerous urinary metabolites were identified, some of which have not been reported before while others corroborate earlier findings on the metabolism of 7-oxo-DHEA [1]. The CIR of both 7-oxo-DHEA and 7β-OH-DHEA were significantly influenced for more than 50 h after a single oral dose of 100 mg and a novel metabolite (5α-androstane-3β,7β-diol-17-one) was found to prolong this detection time window by approximately 25 h. Applying the validated method to routine doping control specimens presenting atypically high urinary 7β-OH-DHEA levels clearly demonstrated the exogenous origin of 7-oxo-DHEA and 7β-OH-DHEA. As established for other endogenously produced steroids like testosterone, CIR allow for a clear differentiation between endo- and exogenous sources of 7-oxo-DHEA and 7β-OH-DHEA. The novel metabolites detected after administration may help to improve the detection of 7-oxo-DHEA misuse and simplifies its detection in doping control specimens.

In order to identify samples suspicious for 7-oxo-DHEA administration a batch of 1000 routine doping control samples was semi-quantified regarding its urinary 7β-OH-DHEA levels and a preliminary threshold for 7β-OH-DHEA will be proposed.

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Coppieters G, Judák P, van Haecke N, van Renterghem P, van Eenoo P, Deventer K

A high-throughput assay for the quantification of intact Insulin-like Growth Factor I in human serum using online SPE-LC-HRMS

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Abstract

Quantification of IGF-I is relevant in both doping control as a biomarker of growth hormone (GH) misuse in sports, and in the clinical field for longitudinal follow-up of patients with disorders related to the GH axis. Currently, better standardization of IGF-I measurements using mass spectrometry is in our best interest as it would enable long-term monitoring of an athletes' IGF-I levels by its addition to the Athlete Biological Passport (ABP). Here, a simplified and rapid top-down LC-HRMS method for quantification of IGF-I in human serum is presented. A ten-minute precipitation-based offline sample preparation is combined with online sample clean-up and separation on a conventional LC, resulting in a total runtime of nine minutes in between injections. The method was validated in the relevant range of 50-1000 ng/mL for the following parameters: linearity, precision, bias, limit of quantification (LOQ), carry-over, selectivity, recovery and ion suppression. As proof of concept, the presented LC-HRMS assay was compared with results from a previous inter-laboratory study on intact IGF-I quantification using four human GH administration samples. It was additionally compared with the IDS-iSYS immunoassay using 47 athlete serum samples, showing good overall agreement with a slight positive bias of 24.2 ng/mL for the LC-HRMS assay at a mean sample concentration of 234 ng/mL. Also, a discrepancy between commercially available IGF-I reference material for the calibration of quantitative assays is discussed. This is of importance if LC-MS assays for IGF-I are to be harmonized.

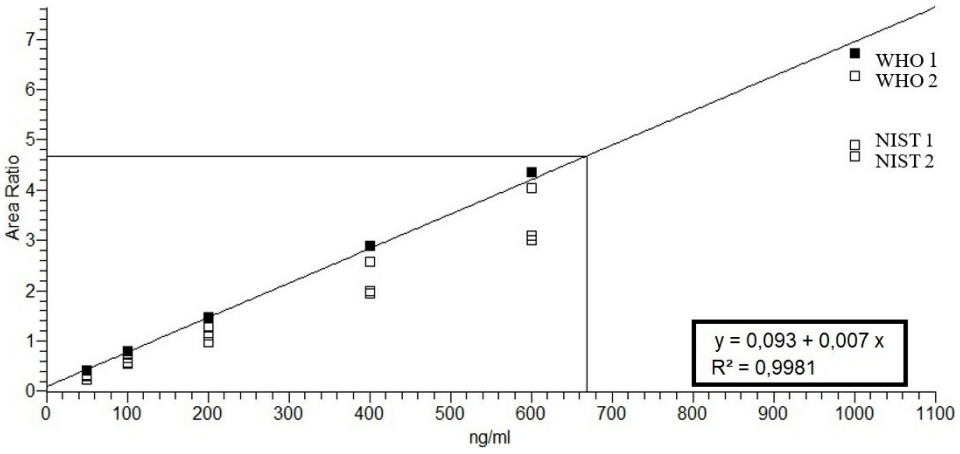

Figure 1. Two IGF-I reference standards were compared using four calibration curves spiked in rat serum. (WHO 02/254 from NISBC and SRM 2926 from NIST respectively; two different lots each)

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Renovato-Martins M[1,2], Amorim C[1], Magdalena I[1], Dudenhoeffer-Carneiro AC[1], López S[1], Nunes I[1], Pizzatti L[1], Aquino Neto F[1], Mirotti L[1], Pereira HM[1]

Monocytes as sensors of autologous blood transfusion detection

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Abstract

Autologous Blood Transfusion (ABT) is a doping alternative to obtain a quick increase in the oxygen availability. However, ABT cannot yet be detected directly by any current "anti-doping" method, which means that a comprehensive approach is needed to determine ABT detection in doping control purpose. Therefore, we elected monocytes as sensors of heme and iron metabolism which are altered after the cooling process of red blood cells.

The aim of this work was to investigate the effect of blood storage lesions on monocytes activation. The activation of monocytes was evaluated by incubating human monocytes with fresh, 21-day refrigerated blood, or mixtures of both (10 or 20% of stored blood) from healthy volunteers. Taqman Low Density Array analysis revealed a different signature of monocytes previously incubated with 10 or 20% of stored blood, which presented increased levels of mannose receptor (CD206), Fractalkine receptor (CX3CR1), ferritin, Heme-oxyganse-1 (HO-1) and Spi-C, while no differences regarding proinflammatory cytokines were found, revealing that these cells did not undergo a pro-inflammatory profile.

Furthermore, Western blotting analysis corroborated these data and showed increased expression of ferritin (3-fold-increase) and Spi-C (2-fold-increase) in monocytes incubated with 10 or 20% of stored blood, when compared to non-stored blood. The latter was presented as a promising sensor in the detection of free heme. Significant changes in free heme (Day 0: 7.18 µM; Day 21: 72.01 µM) and hemoglobin (Day 0: 7.77 µM and 31.65 µM) concentration were detected by colorimetric assay. Furthermore, cytometric analyses revealed that the storage process increased the release of microparticles from red blood cells (2.9-fold-increase). Therefore, through the identification of new targets, our study opens the way for the development of bioanalytical assays for the detection of autologous transfusion in athletes, with promising perspectives in terms of screening.

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

Urinary detection of insulin analogues: Improvements in the Ghent laboratory

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Abstract

Whereas the analysis of small peptides with MW < 2kDa is considered by now a routine procedure in the majority of anti-doping laboratories, the measurement of peptides with MW > 2 kDa is still challenging. The group of insulin and its synthetic analogues is one of the classes of peptide hormones that remains difficult to detect.

This presentation will describe the improvements that have recently been achieved in insulin analysis in the laboratory of Ghent. Results of a method development as well as results on the investigation of urinary detection windows will be discussed.

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Dehnes Y[1], Bogetveit S[1], Solheim SA[2], Sletten C[1], Mørkeberg J[2], Baastrup Nordsborg N[3]

Effect of testosterone administration on GH-biomarkers IGF-1 and PIIINP

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Abstract

The Endocrine module of the Athlete Biological Passport (ABP), is under development for detection of doping with growth hormone (GH) and its releasing factors. As part of this process, it is important to gain information about possible confounding factors. Further, to fully exploit the data in the ABP, it is important to know how doping affects the markers in the different modules. We performed a randomised, double-blinded controlled study where subjects received two injections two weeks apart of either a mix of four testosterone esters (Sustanone 250) or placebo. Serum samples were collected before and after the administrations. The aim of this study was to see if administrated testosterone affects the GH-dependent biomarkers insulin-like growth factor type 1 (IGF-1) and N-terminal propeptide of type III procollagen (PIIINP), as well as the calculated GH-2000 score (Holt RI, 2015). IGF-1 and PIIINP were analysed with validated immunoassays for the respective markers (Immunotech A15729 IGF-I IRMA and Orion UniQ^{TM} P-III-NP RIA). There was a significant difference between the placebo and Sustanone-receiving groups in the concentration levels of both IGF-1 and PIIINP after, but not before, the administrations. Between the placebo and Sustanone-groups, the largest change after administration was seen for PIIINP, however, the individual response in the GH-markers to the testosterone administration varied greatly between subjects.

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Renovato-Martins M[1,2], Dudenhoeffer-Carneiro AC[1], Du Rocher-Silva B[1], Soares F[1], Aquino Neto FR[1], Hasan M[3], Rahaman KA[3], Muresan A[3], Oh-Seung K[3], Mirotti L[1], Pereira HM[1]

Erythrocyte phenotyping of international athletes by flow cytometry

Chemistry, Chemistry Institute, Rio de Janeiro, Brazil[1];
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Doping Control Center Korea, Institute of Science and Technology, Seoul, Korea[3]

Abstract

Blood transfusion is performed by cheating athletes to rapidly increase oxygen delivery to exercising muscles and enhance their performance. Homologous blood transfusion happens when blood from a different person is transfused. The method used to detect this type of doping is based on flow cytometry, by identifying variations in blood group minor antigens present on erythrocytes surface. Different authors and anti-doping laboratories use different panels of erythrocyte antigens to allow the detection of mixed populations. In total, there are more than 300 different red blood cells antigens and the detection is based on the most abundant families, normally in a total of around 8- 10 antigens. It has been reported that number of occurrences of two individuals sharing an identical phenotype in the same population of athletes is 5 times higher than the theoretical probability. This work describes the data of athletes who were analyzed by flow cytometry for the prevalence of different erythrocytes surface antigens (C, E, Fya, Fyb, Jka, Jkb and S) in a population of 360 international athletes from all 5 continents.

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Narduzzi L[1], Buisson C[2], Morvan M[1], Marchand A[2], Audran M[2], Le Bouc Y[3], Ericsson M[2], Le Bizec B[1], Dervilly G[1]

ABP-omics: a combined use of the haematological module and the “steroidomics passport” to track GH users

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INSERM, Centre de Recherche St-Antoine, Sorbonne Université, Paris, France[3]

Abstract

The detection of micro-doses of recombinant human Growth Hormone (rhGH) is challenging, due to its rapid turnover and the population-wise variability of its bio-markers, IGF-I and P-III-NP and their score (GH-2000 score). The longitudinal tracking of the GH-2000 score (endocrine module of the ABP) is a good indicator of rhGH abuse, being able to partially track users in case of micro-dosing [1,2]. Nevertheless, some authors already pointed out the influence of rhGH on further proteins [3], which might be used as further markers. Starting from this assumption and aiming to discover new markers, in this study we explored the effect of rhGH on two physiological pathways: 1) the whole hematopoiesis and 2) the entire steroidogenic pathway (steroidomics).

14 healthy volunteers were split in two equal groups and administered with 6 micro-doses of EPO every 2/3 days (10UI/kg, group EPO), or with the same micro-doses of EPO micro-doses of GH (2UI, group EGH). Full blood EDTA, urine and plasma were collected at day 0, day 4, day 11 (end of treatment), and day 14. The hematopoietic profile was determined using the Sysmex XT 2000i (Sysmex Corporation, Kobe, Japan), following the WADA’s recommendations (TD2019BAR), collecting also the data of the platelets and the leukopoiesis in the analysis. The steroidogenic profile has been determined using the steroidomics approach developed in our laboratory [4] from 50 µL of urine. The endocrine profile has been determined following WADA recommendations (hgh-biomarkers-test-v1.0-2015-en.pdf).

The data has been analyzed using Geometric Trajectory Analysis (GTA [5]), to compare the evolution of the trends of the various parameters, instead of comparing their absolute concentrations. The results clearly showed several leukopoietic parameters (white blood cells, neutrophils, basophils, eosinophils and monocytes) affected by rhGH, while erythropoiesis and platelets were not affected (Figure 1A). The sterodomics profile was also affected by rhGH administration (Figure 1B). Many steroids were altered at day 4, but only few of them were still altered at day 11 and 14. Among those, 5-(α and β)-Androstanedione, 5β-androstane-3α-17α-diol and 17β-estradiol seemed to be the most affected ones. From an anti-doping perspective, the analysis of the GTA data through OPLS-DA of the leukopoietic and steroidal biomarkers together with endocrine biomarkers (IGF-1 and P-III-NP) allowed to correctly classify over 98% of samples with no false positives, a promising result if compared to the GH-2000 score (about 50% accuracy) and the endocrine module (about 75% accuracy) applied on the same sample-set.

In this study, we demonstrated the effect of the rhGH on several parameters of the leukopoietic and steroidal profiles. Combining this information together with the endocrine module of the ABP seems to increase sharply the odds to track rhGH users. The method still needs further validation to confirm the

findings and to understand the role of the confounding factors on such parameters. A full description of this work has been submitted to an international scientific journal and is currently under peer-review [6].

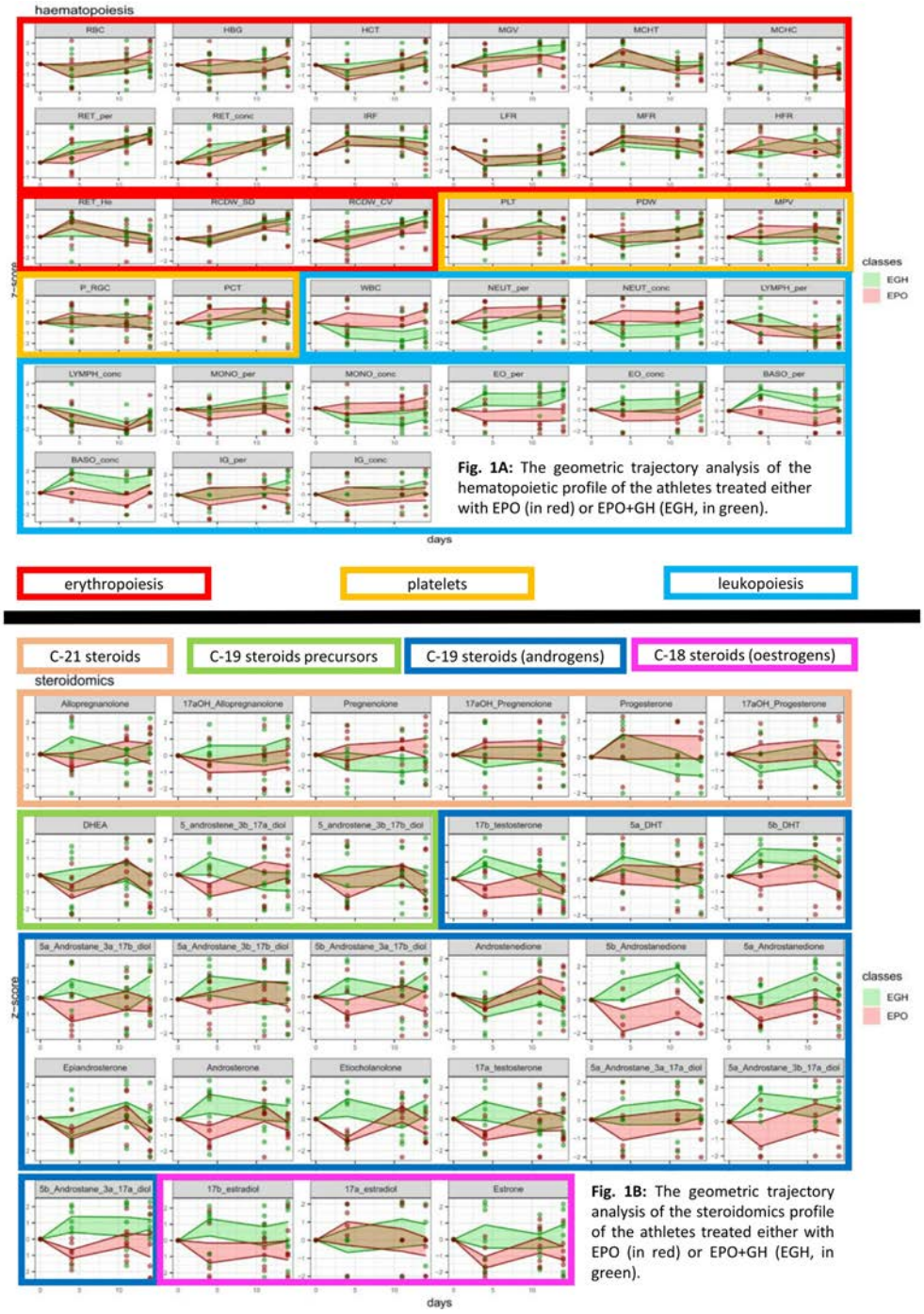

Fig. 1A: The geometric trajectory analysis of the hematopoietic profile of the athletes treated either with EPO (in red) or EPO+GH (EGH, in green).

Fig. 1B: The geometric trajectory analysis of the steroidomics profile of the athletes treated either with EPO (in red) or EPO+GH (EGH, in green).

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Voss SC, Elsaftawy W, Saghbazarian S, Al-Kaabi A, Mohamed Ali V, Georgakopoulos C, Al Maadheed M, Orie N

Testing the applicability of HRP-conjugated Anti-EPO antibodies for direct rhEPO detection – proof of concept

Doping Analysis Lab, Anti-Doping Lab Qatar, Doha, Qatar

Abstract

The method to detect recombinant human EPO is very time consuming and takes approximately 2.5 working days. In addition, the use of a secondary anti-EPO-HRP antibody increases the potential risk of cross-reactivity with untargeted proteins. Therefore, we tested 3 commercially available anti-EPO-HRP antibodies and an in-house produced anti-EPO-HRP conjugate for their potential to be applied in anti-doping analysis. One commercial antibody and the in-house conjugate showed promising results reducing the analysis time to approx. 9 hours.

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Dehnes Y[1], Miller G[2], Naud J[3], Martin L[4], Reihlen P[5], Reichel C[6]

Inter-laboratory validation of biotinylated clone AE7A5EPO-antibody for EPO detection by single blotting of urine and blood samples

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Sports Medicine Research and Testing Laboratory (SMRTL), Salt Lake City, USA[2];
Laboratoire de contrôle du dopage Montreal (INRS), Montreal, Canada[3];
Agence française de lutte contre le dopage (AFDL), Paris, France[4];
German Sport University Cologne, Institute of Biochemistry, Cologne, Germany[5];
Seibersdorf Labor GmbH Doping Control Laboratory, Seibersdorf, Austria[6]

Abstract

The EPO Working Group set out to investigate the sensitivity and specificity of an updated protocol for ESA-analysis (C. Reichel, Cologne Workshop on Dope Analysis, 2018), which employs a biotinylated primary antibody and omits the use of a secondary antibody. The aim of this project was to test if both blood and urine samples can be analyzed with single blotting without the unwanted labelling of non-ESA proteins. Six laboratories belonging to the EPO Working Group analyzed and reanalyzed blood and urine samples and compared the results with those obtained when applying their validated routine methods. The inter-laboratory validation has included different sample purification strategies (e.g. StemCell ELISA, MAIIA Purification Kit and magnetic beads) as well as different electrophoretic methods (SAR-, SDS- and IEF-PAGE).

We have found that the protocol applying a biotinylated primary antibody is a sensitive method for detection of ESAs in both urine and blood samples, and can be used with SAR-PAGE, IEF-PAGE and SDS-PAGE. The signal-to-noise ratio is higher in most of the evaluated gels, compared to currently used procedures. The data from the inter-laboratory validation, including issues that arose and how these can be dealt with, will be presented.

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Reichel C[1], Erceg D[2], Lorenc B[1], Scheiblhofer V[1], Farmer L[1], Zanitzer K[1], Geisendorfer T[1], Gmeiner G[1]

Data from a low-dosed rEPO administration study

Doping Control Laboratory, Seibersorf Labor GmbH, Seibersdorf, Austria[1];
Srebrnjak Children's Hospital, Zagreb, Croatia[2]

Abstract

Recombinant human erythropoietin (rhEPO) stimulates the production of red blood cells and is thus frequently misused in endurance sports in particular as performance enhancing drug. Direct tests to detect doping with rhEPO are mostly based on electrophoretic techniques, utilizing either differences in the net charge of EPO's isoforms (isoelectric focusing polyacrylamide gel electrophoresis - IEF-PAGE) or molecular mass (N-lauroylsarcosinate (sarcosyl) polyacrylamide gel electrophoresis - SAR-PAGE and sodium dodecyl sulfate polyacrylamide gel electrophoresis – SDS-PAGE) [1-3]. In order to escape detection, some cheating athletes switched to so-called "microdosing" of rhEPO. It designates the frequent use (e.g. daily or every second day) of very low doses of rEPO (e.g. < 10 IU/kg BW) instead of applying larger doses (e.g. 30 IU/kg BW) twice or more times per week [4].
In 2015, the detection window of SAR-PAGE for administered rhEPOs was significantly improved by performing most manual Western blot processing steps with an automatized blot processor (BlotCycler) [5-6]. Upon initiative of the author of this article and Carsten Lück of Bio-techne (USA), the key detection antibody of the method (clone AE7A5 EPO-antibody [7]) was custom-biotinylated in spring 2018, which led to a further significant increase in sensitivity.
The purpose of thep; current project was to investigate the detection window of a very low microdosed (7.5 IU/kg BW) rhEPO biosimilar (Retacrit/epoetin zeta) after intravenous and subcutaneous administration to humans and by utilizing the biotinylated EPO-antibody in a further sSAR-PAGE protocol.
With the improved protocol, microdosed Retacrit (7.5 IU/kg BW) was detectable for at least 52 hours after intravenous administration. Detection windows were approximately the same for serum and urine and doubled after subcutaneous administration (ca. 104 hours). Remarkably, the "rhEPO-smear [8] ranged from the endogenous band up to the lower edge of the NESP band and looked more like a double band up to 18-24 hours post administration (Figure 1). After that, the band inside the diffuse area disappeared, but the smear itself was still present. Previous studies applying different electrophoretic techniques and the not further optimized SAR-PAGE protocol revealed considerably shorter detection windows for rhEPO-microdoses. Since the new biotinylated antibody performed significantly more sensitive than the non-biotinylated version, it should preferably be used for the initial testing procedure (ITP) in EPO doping control.

For more details, please refer to the full article published in Drug Testing and Analysis [9].

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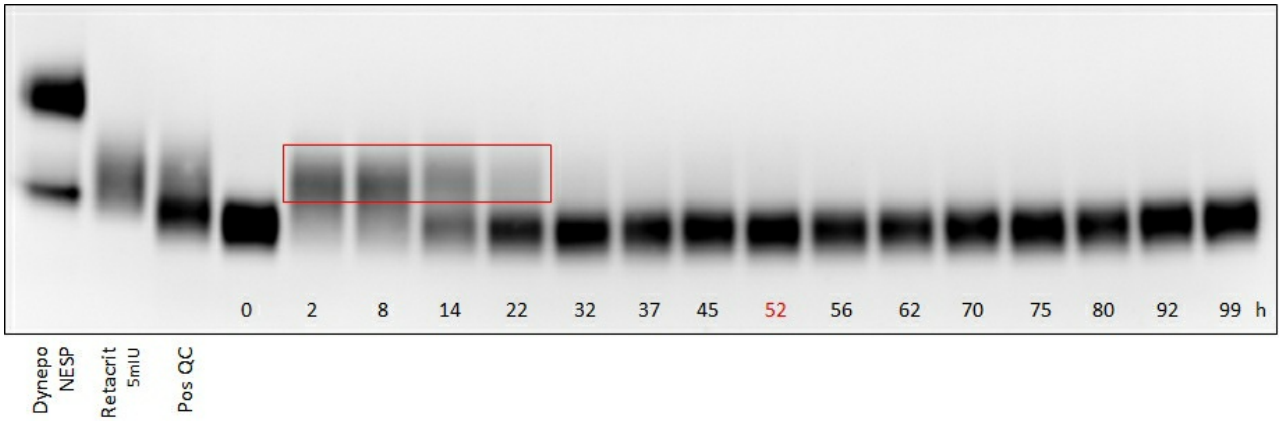

Figure 1. The improved SAR-PAGE protocol allowed the detection of microdosed Retacrit (7.5 IU/kg BW) in serum and urine for at least 52 hours after intravenous administration. The detection window doubledafter subcutaneous administration.

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Leuenberger N[1], Salamin O[1], Gottardo E[1], Schobinger C[1], Reverter-Branchat G [2], Segura J[2], Saugy M[3], Tissot J[4], Kuuranne T[1]

Detection of stimulated erythropoiesis using the transcriptomic *ALAS2* biomarker in dried blood spot samples

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Integrative Pharmacology and Systems Neuroscience Research Group, Barcelona, Spain[2];
Center of Research and Expertise in Anti-Doping sciences - REDs, Lausanne, Switzerland[3];
Transfusion interrégionale CRS, Epalinges, Switzerland[4]

Abstract

The Athlete Biological Passport (ABP) has been implemented more than 10 years ago to improve the detection of blood doping. While this tool has considerably helped to study and reduce the prevalence of blood manipulation, athletes accustomed to this monitoring tool by avoiding large hematological fluctuations and adapting their doping regimen. On the other side, the logistic related to the ABP (phlebotomy, refrigerated transport, urgent analysis) is cumbersome and thus, involves significant costs. It is therefore necessary to further improve ABP sensitivity with additional biomarkers and to discover alternative strategies for sample collection. In this study, we developed a method to quantify the RNA-based *ALAS2* biomarker in dried blood spots (DBS). The robustness of the method and the effects of storage conditions on the expression levels of *ALAS2* were assessed. The method was then applied to the monitoring of endogenously (blood withdrawal) and exogenously (injection of recombinant human erythropoietin) stimulated erythropoiesis. Quantification of *ALAS2* expression in DBS was reproducible and unaffected when DBS were stored at room temperature. Following withdrawal of one unit of blood, the *ALAS2* RNA level in DBS increased significantly for up to 15 days. Increase in the expression level of this biomarker observed in DBS samples was more pronounced than the impact on the conventional hematological parameters used in ABP. After exogenous stimulation of erythropoiesis via recombinant human erythropoietin injections, *ALAS2* gene expression in DBS increased considerably, with an average of 8-fold. Based on this study, the measurement of transcriptomic biomarkers in DBS appears as a promising and complementary approach for the improvement of the ABP sensitivity.

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Walpurgis K[1], Weigand T[1], Knoop A[1], Thomas A[1], Reichel C[2], Dellanna F[3], Thevis M[1,4]

Detection of follistatin-based inhibitors of the TGF-β signaling pathways in serum/plasma by means of LC-HRMS/MS & Western blotting

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Abstract

Cytokines of the TGF-β superfamily such as myostatin and activin A are considered as key regulators of skeletal muscle mass. *In vivo*, their activity is controlled by different binding proteins such as follistatin (FST), whose interaction with the circulating growth factors prevents activation of the activin type II receptors. FST-based protein therapeutics are therefore not only promising drug candidates for the treatment of muscular diseases but also potential performance-enhancing agents in sports.

Within this study, two complementary detection assays for FST-based inhibitors of the TGF-β signaling pathways in doping control serum and plasma samples were developed by using both monomeric FST and dimeric FST-Fc fusion proteins as model compounds. The initial testing procedure is based on immunoaffinity purification, tryptic digestion, and LC-HRMS/MS, offering high specificity by targeting tryptic signature peptides of FST. As the glycoprotein is also produced endogenously, the confirmation method employs immunoaffinity purification, SDS-PAGE, and Western blotting in order to detect the intact proteins and differentiate synthetic FST-Fc constructs from naturally occurring FST isoforms. Both assays were found to be highly specific with an estimated detection limit of 10 ng/mL.

Moreover, a commercial sandwich-ELISA was used to determine endogenous FST values. The detected FST serum levels of healthy volunteers were found below 5 ng/mL, which is in accordance with reference values from the literature and below the doping control detection methods' LOD.

The presented assays expand the range of available tests for emerging doping agents and the initial-testing procedure can readily be modified to include further protein drugs.

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Miller G, Cox H, Manandhar A, Husk J, Eichner D

Evaluation of CD71, Band3, and serum markers as evidence of recombinant EPO use: results from a non-drug longitudinal study and a blinded, placebo-controlled administration study

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Abstract

While the Athlete Biological Passport has shown to be a valuable tool in anti-doping, efforts to improve its sensitivity are constantly ongoing. Primarily, much work focuses on the analysis of additional markers which may aid in detecting ‘microdosing’ of EPO or blood transfusions. Recent work in our laboratory has focused on measuring two proteins, CD71 and Band3, which are cell-surface proteins that correlate with the immature reticulocyte fraction (IRF) and total erythrocytes, respectively. Importantly, these proteins can be reliably measured on dried blood spots, whose implementation would likely result in more frequent athlete monitoring and a more sensitive biological passport. To proceed with the analysis in an anti-doping setting, two studies were designed to assess the behavior of these proteins in an athletic population. In the first study, twenty-five patients (males and females) were longitudinally monitored for 8 weeks without drug intervention to understand the variability and precision of CD71 and Band3 measurements in dried blood spots. These measurements were compared to the variability seen in currently monitored ABP markers in whole blood, specifically Ret%, IRF% and HGB. Following these analyses, a further study (single blind, placebo-controlled, non-crossover) was designed to understand the effect of EPO use on these proteins. In this study, patients in the drug group were administered 40 IU/kg Epogen, subcutaneously, six times over the course of three weeks. Following a ten-day washout, the same patients were administered 900 IU Epogen, intravenously, eight times over the course of three weeks. Changes in CD71 and Band3 were assessed following drug intervention and compared to the changes seen in the placebo group (same injection schedule with saline instead of EPO). Additionally, the serum proteins ferritin and soluble transferrin receptor were also monitored during this study in an attempt to identify additional markers that would increase the sensitivity of the ABP. Results from these two studies will be presented here.

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Nazir J, Cowan D, Caldwell R, Walker C, Bartlett C, Brailsford A, Wolff K

A Menu Driven Laboratory Information Management System (DCLIMS) for Anti-Doping Laboratories

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Abstract

Most Anti-Doping laboratories have adopted one form of Laboratory Information Management Systems (LIMS) or another to assist with their day to day operations: from Sample Receipt and Sample Location Tracking, to Sample Analysis and Reporting. However, most laboratories are still experiencing one or two issues with their current LIMS. Although, many commercial of the shelf LIMS are available that may be configured to work for an Anti-Doping Laboratory and address some of these issues, they have inherent disadvantages. For instance, they were not specifically designed and developed with an Anti-Doping Laboratory in mind. Most commercial LIMS have a hierarchal tree view navigation system that does not suit all systems, maintenance and support can be very costly: Additional requirements include WADA specific requirements. Here, we present a low cost system (DCLIMS), which was developed in-house at the Drug Control Centre, King’s College London. DCLIMS is menu driven, very easy to operate, and above all, easily altered by a trained analyst to meet WADA’s ever-changing requirements.

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Son J[1,2], Cho Y[1,3]

Model System for Brain Doping: Identification of the Correlation between Performance Enhancing and Alterations of Neurochemical Profiling

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Abstract

Brain doping which stimulates specific brain region to increase sport performance has been reported consistently, but there is no definite provision or detection method to deal with it. We have made the following plans to establish the brain doping diagnosis system:

1) Develop a brain doping model system that transmits stimuli using transcranial direct current stimulation (tDCS) equipment.

2) To investigate neurotransmitters mediating brain doping signals, urine samples obtained through a model system are analyzed with neurotransmitters analysis method.

3) Select the markers of brain doping by observing a comprehensive pattern of neurotransmitters and establish the brain doping diagnosis method.

We developed a model system of brain doping using rats and conducted a preliminary experiment. The stimulus was delivered using the tDCS to the rat brain, and the performance enhancing before and after the stimulus were compared through three independent behavioral testing: endurance, balance, and muscle strength. At the same time, urine was collected to determine the correlation between motor capacity and neurotransmitters through the neurotransmitter detection method developed in the previous year. Preliminary experiments with the model system showed some improvement in endurance and balance, and neurotransmitters showed different patterns before and after stimulation. Based on this results, a deep study observing the changes in the motor performance according to the stimulus site and intensity, and grasping the alteration of the pattern of neurotransmitters in more detail is in progress. In conclusion, we will discover core multiple biomarkers of brain doping and suggest the direction of the diagnosis method detecting brain doping.

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Application of the Zebrafish Water Tank Model to investigate the budesonide metabolic profile

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Department of Pharmaceutics, Laboratório de Tecnologia Industrial Farmacêutica (LabTIF), Federal University of Rio de Janeiro, Rio de Janeiro, Brazil [2]

Abstract

The identification of the metabolites of a prohibited substance allows the selection of analytical targets for doping control, since most drugs undergo metabolic processes in the body. Currently, metabolism evaluation studies using the zebrafish water tank (ZWT) model have been applied for some doping agents, but, in this context, the class of glucocorticoids (GLI) were not yet evaluated. In the present work, the metabolic profile of the GLI budesonide (BUD) through ZWT was compared to the profile already described for humans. Samples collected after administration of BUD were analyzed by liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) for the identification and characterization of the metabolites. The results indicate that the main metabolic pathway of BUD by ZWT focused on the production of metabolites with the acetal fraction preserved. In this sense, ZWT model was able to produce the main target metabolite for doping control purposes, the 6β-hydroxy BUD, and other metabolites from hydroxylation, 6,7-dehydrogenation, 11-oxidation and 20-reduction reactions.
However, the 16α-hydroxy prednisolone (16α-OH-PRED), the main urinary metabolite in humans, and the other metabolites associated with acetal fraction cleavage were not observed. The lack of butyrylcholinesterase activity in the zebrafish organism may be related to the absence of the 16α-OH-PRED pathway metabolites. The investigation of the intermediate products related to choline-based esters drugs corroborates to these findings. The evaluation of BUD metabolism kinetics in the ZWT model shows considerable differences between the models, such as the preference in the ZWT model for the C11 oxidation reaction, whereas in humans the main metabolite comes from acetal cleavage. Despite the observed limitations, the results indicate that the ZWT model is applicable for the assessment of GLI metabolism, since part of the zebrafish metabolic reactions observed for BUD was similar to general GLI metabolic pathway in humans. The present work allows to consider the application and limitation of ZWT to assess the metabolism of other doping agents with similar metabolic pathways of BUD in humans.

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Prado E[1], Gomes G[1], Lima de Sá C[1], Matos R[1], Sardela V[2], Anselmo C[1], Nunes I[1], Ramalho G[1], Vanini G[3], Padilha M[1], Oliveira MA[4], Oliveira A[4], Canuto C[4], Amaral L[4], Siqueira D[4], Aquino Neto FR[3], Pereira HM[1]

Study of the metabolism of the cathinones α-PVP, methylone and 4-chloro-dimethylcathinone through the Zebrafish Water Tank Model

LBCD, Chemistry Institute, Rio de Janeiro, Brazil[1];
Chemistry, Chemistry Institute, Rio de Janeiro, Brazil[2];
Laboratório de Apoio ao Desenvolvimento Tecnológico (LADETEC), Chemistry Institute, Rio de Janeiro, Brazil[3];
ICCE/DGPTC/PCERJ, Civil Police of Rio de Janeiro State, Rio de Janeiro, Brazil[4]

Abstract

Zebrafish Water Tank (ZWT) is a *in vivo* experimental model used to investigate the metabolism of doping agents from different classes with success. ZWT is based on the orthology between humans and zebrafish metabolic enzymatic machinery already described in literature.

The term synthetic cathinone is used to describe a major category of psychoactive substances which congregates hundreds of compounds structurally related, easily synthesized from other amphetamine-like compounds. Due to the huge molecular diversity of the cathinones, knowledge about their toxicology or metabolic pathways is scarce. The objective of this work was to evaluate the metabolism of α-PVP, methylone and 4-chlorodimethylcathinone (4-CDC) through the ZWT model comparing their metabolites with the ones described in literature for humans. Aliquots of the water recipient where the fish was treated with the substance were prepared by solid phase extraction and analyzed by LC-HRMS operating in full scan, all ion fragmentation and data independent acquisition modes. The metabolites were surveyed by the exact mass of the parent ion and confirmed by MS/MS fragmentation. The metabolism of α-PVP were previously studied in human and *in vitro* experiments. All metabolites reported in the literature were detected in ZWT: *N*-dealkylated (M1, *m/z* 178.12264), (M2, *m/z* 266.17507), hydroxylated (M3, *m/z* 248.16450), (M4, *m/z* 250.18015), (M5, *m/z* 264.15942) and the β-ketone reduced (M6, *m/z* 234.18524) and (MX, *m/z* 246.14885). No metabolism study was previously reported for 4-CDC. Hence, the metabolic pathways were deduced considering the structural similarity among synthetic cathinones. As a result, the reduced metabolite (M1, *m/z* 214.09932), nor-metabolite *N*-dealkylated (M2, *m/z* 198.06802), bis nor-metabolite (M3, *m/z* 184.05236), hydroxylated (M4, *m/z* 228.07858), reduced and hydroxylated (M5, *m/z* 230.09423) were detected. Contrary to all other cathinones, no methylone metabolites were observed in ZWT. Human, urinary metabolites of methylone have already been described in human with contribution from CYP2D6, with minor contributions from CYP1A2, CYP2B6, and CYP2C19. It also reported a time-dependent loss of CYP2D6 activity when the enzyme was preincubated with methylone. The loss of activity reached a maximum rate of inactivation at high methylone concentrations, suggesting that methylone is a mechanism-based inhibitor of CYP2D6. Although no ortholog of human CYP2D6 has been found in the fish, zebrafish has been described to metabolize dextromethorphan, a substrate of human CYP2D6, to dextrorphan. This indicates that not having a human CYP ortholog in zebrafish does not necessarily mean that there will be no biotransformation of drugs. Considering different β-keto designed drugs metabolites (e.g., ethylone and *N*-ethylpentylone), it

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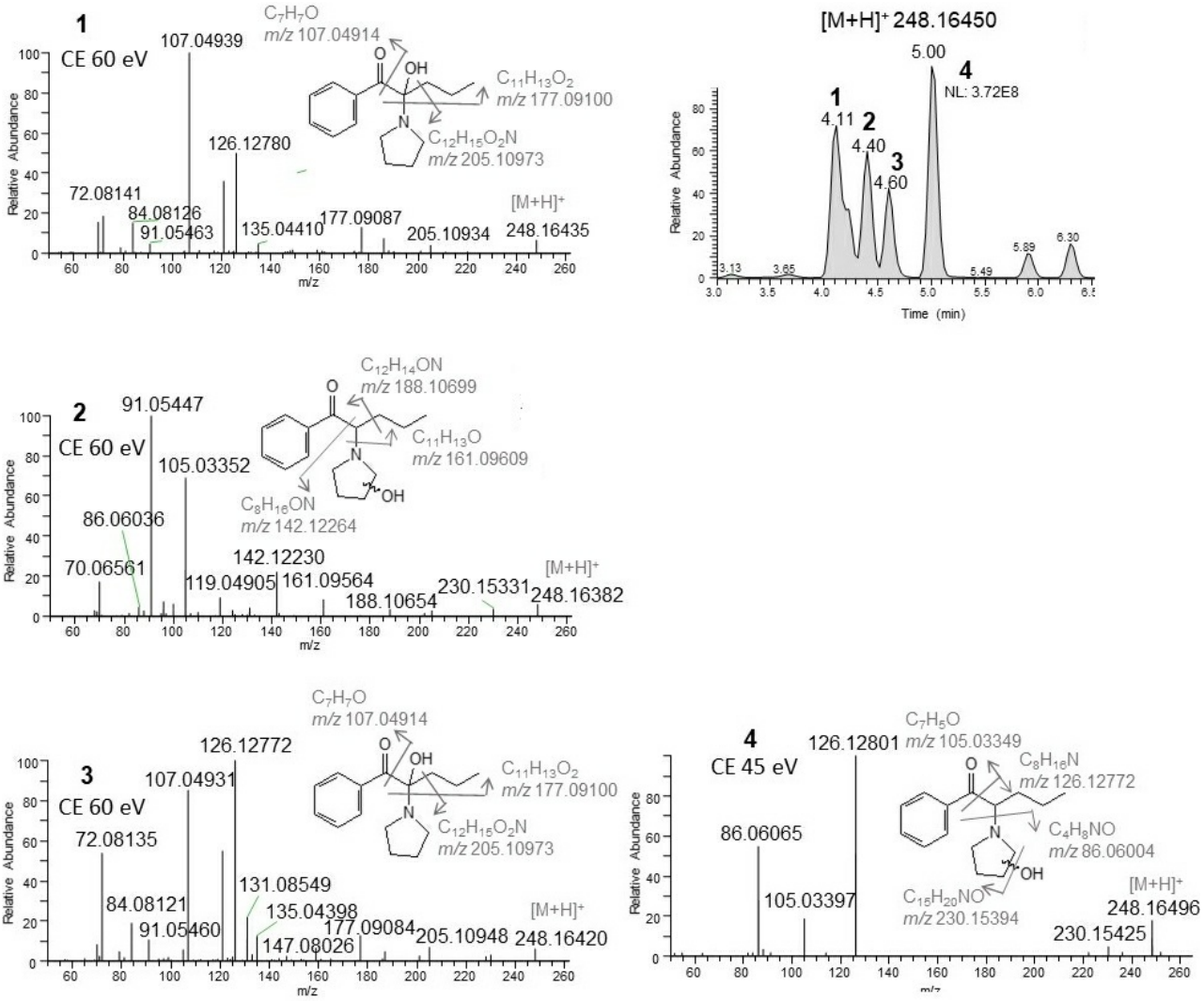
is possible that methylone exhibits a behavior on CYP isoenzymes for zebrafish, which is similar to the action of methylone on human CYP2D6. The ZWT model produced all metabolites of α-PVP described in the literature. Based on the structural similarity, it was possible to extrapolate that ZWT also mimic the human metabolic pattern for 4-CDC. The inhibitory effect of methylone in the ZWT needs further experiments to be confirmed.



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Pettersson-Bohlin K[1], Sjöö M[2], Sjöö J[2]

The sniffing dog/anti-doping dog Molly - A new dimension of doping controls

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Abstract

There are many steps in successful anti-doping testing: the planning of the sample collection, the sample collection itself, transportation to the laboratory, different analyses performed and evaluated, and finally the results are summarized and sent to the customer. Each step needs to be optimized but it all starts with the strategy of sample selection and sample collection.

The introduction of out-of-competition testing is a great success, which initiated the “Whereabout System” for top athletes. In order to simplify and improve sample collection, studies are being done on new non-invasive sampling methods, for example, saliva, exhaled breath, hair, and dried blood spots. Another part of the sample collection that can give an extra dimension is the addition of a sniffer dog. With the capability of a sniffer dog’s nose, arenas, lockers, and bags can be scanned for substances prohibited in sports. Any marking by the dog can be a reason for further investigation or initiate a sample collection. In Sweden, the 4-year-old springer spaniel Molly is trained according to the training program for customs sniffer dogs. She has been working since 2017 together with her handlers at the Swedish NADO Riksidrottsförbundet who also double as DCO. Molly’s work has so far resulted in several positive cases. When Molly is searching the area, her handlers also observe any suspicious behavior of the onlookers. She also interacts with athletes and coaches, which encourages discussions about doping and spreading awareness.

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Ecdysterone: A snapshot of use in the athlete's population

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Medicina Sperimentale, Sapienza University of Rome, Rome, Italy[3]

Abstract

Ecdysteroids are steroids mainly produced by invertebrate species (where they act mainly as moulting hormones), but they are present in large amount also in certain species of plants (where they act as protective chemical agents against predatory insects).
Studies dating back 1963 reported that several ecdysteroids present the capacity to enhance muscle growth. In the 1980s, the most active phytoecdysteroid, ecdysone, was suspected to be used by Russian Olympic athletes. In 2019, investigations on the effect of ecdysterone on human sport exercise have demonstrated the ability of this natural compound to increase sports performance. In January 2020, ecdysterone was placed on the monitoring list of the World Anti-Doping Agency, to follow its diffusion in professional sports.
Here we report the ecdysterone findings during doping control tests carried out at the Italian antidoping laboratory of Rome, with the aim to obtain a snapshot of ecdysterone use in the athlete's population.

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Continuous Quality Improvement in doping control laboratories: Implementation of ISO/IEC 17025:2017 and WADA ISL 2019, Challenges and Future Perspective

National Dope Testing Laboratory, New Delhi, India

Abstract

Effective quality management system is the foundation of a doping control laboratory. The accreditation of doping control laboratories is governed by the ISO/IEC 17025:2017 and WADA International Standard for laboratories. In recent years the doping laboratories were in the transition stage to implement the revised versions of both the said standards for the expected improvement of their quality management system. The main objective of these standards remains to set out the requirements for laboratories to demonstrate that they are technically competent, operate within an effective Quality Management System and are able to produce valid results.

Though this transition had not been facile as it demanded review and transformation implementation at structural, organizational and technical level. Moreover, the doping control laboratories need to have an harmonized approach in dealing with the technical and quality related issues which require an in-depth study on the protocols being adapted. This paper will outline not only the approach which originated within the spirit of the execution of these two revised standard in the current setup of NDTL, India, but which is then extended to a pragmatic implementation followed by challenges which were faced, the proposed solutions and future prospective for further improvement. Amongst the doping control laboratories, it's imperative that effective communication takes place to disseminate developments, and the outcome of this work is intended to prompt such discussion. The novelty of this work stems from the realization of critical factors determining a successful implementation of ISO/IEC 17025:2017 and WADA International Standard for Laboratories, Version 10.0 within the doping control laboratories.

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Indirect detection of steroids abuse in sports based on androgenic activity measurement by AR Calux® bioassay

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Doping Control, Spanish Agency for Health Protection in Sport, Madrid, Spain[3];
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BioDetection Systems BV, Amsterdam, The Netherlands[5]

Abstract

In the Anti-Doping field the detection of AAS is carried out with methodologies based on gas chromatography-mass spectrometry that are applied to extracts prepared from the athlete´s urine.
In these recent years, the great potential of the use of androgen receptors (AR) is beginning to be seen as an indirect way of detecting steroid abuse in urine. The mammalian androgen sensitive reporter gene assay (AR CALUX® bioassay), measuring compounds interacting with the AR can be used for the steroids analysis without the need to know in advance its chemical structure.
In this study, the potential of BDS´s CALUX® technology was studied as an indirect method to detect the administration of AAS evaluating its efficacy to detect the administration of a single dose of 250 mg of testosterone cypionate using a triple-blind randomized, placebo-controlled crossover trial with a population of twelve athletes among whom the UGT2B17 gene deletion was present, evenly distributed in heterozygous (*ins/del*), wild-type homozygous (*ins/ins*) and mutated homozygous (*del/del*) groups.
Urine samples were collected from each participant over a 7-month period at the minute woke up. From every volunteer were used ten samples after placebo administration (C1), ten samples after testosterone administration (C2) and between two and four samples in the clearance period, 15 to 30 days after T administration (C3). The samples were split in two equal parts of 2.5 g of urine. The preparation process included urine hydrolysis (only in a portion of each sample), liquid-liquid extraction, concentration and analysis by CALUX® bioassays.
The CALUX® bioassays comprise of humane bone cells (U2-OS), incorporating the firefly luciferase gene coupled to Responsive Elements (REs) as a reporter gene for the presence of receptor mediating compounds. Cells that are exposed to compounds that activate or inactivate the incorporated RE not only express proteins that are under normal circumstances associated to RE, but also luciferase. By addition of the appropriate substrate for luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor binding, which is benchmarked against the relevant reference compound, dihydrotestosterone (DHT). Analysis results were interpolated in a calibration curve. Logarithmic transformation of the receptor response in terms of ng equivalent of DHT was calculated for normalization of the results to assess differences between polymorphic groups.
According to our preliminary results, a significant statistical difference in terms of AAS-induced bioluminescence was observed ($p < 0.001$) in the mean of 10 determinations on consecutive days after

the administration of testosterone compared to the average of the 10 determinations after the administration of placebo. Figure 1 shows the results obtained after AR CALUX® bioassay analysis a) without and b) with HCl hydrolysis, for an individual of each polymorphism:(*ins/ins*), (*ins/del*), (*del/del*).

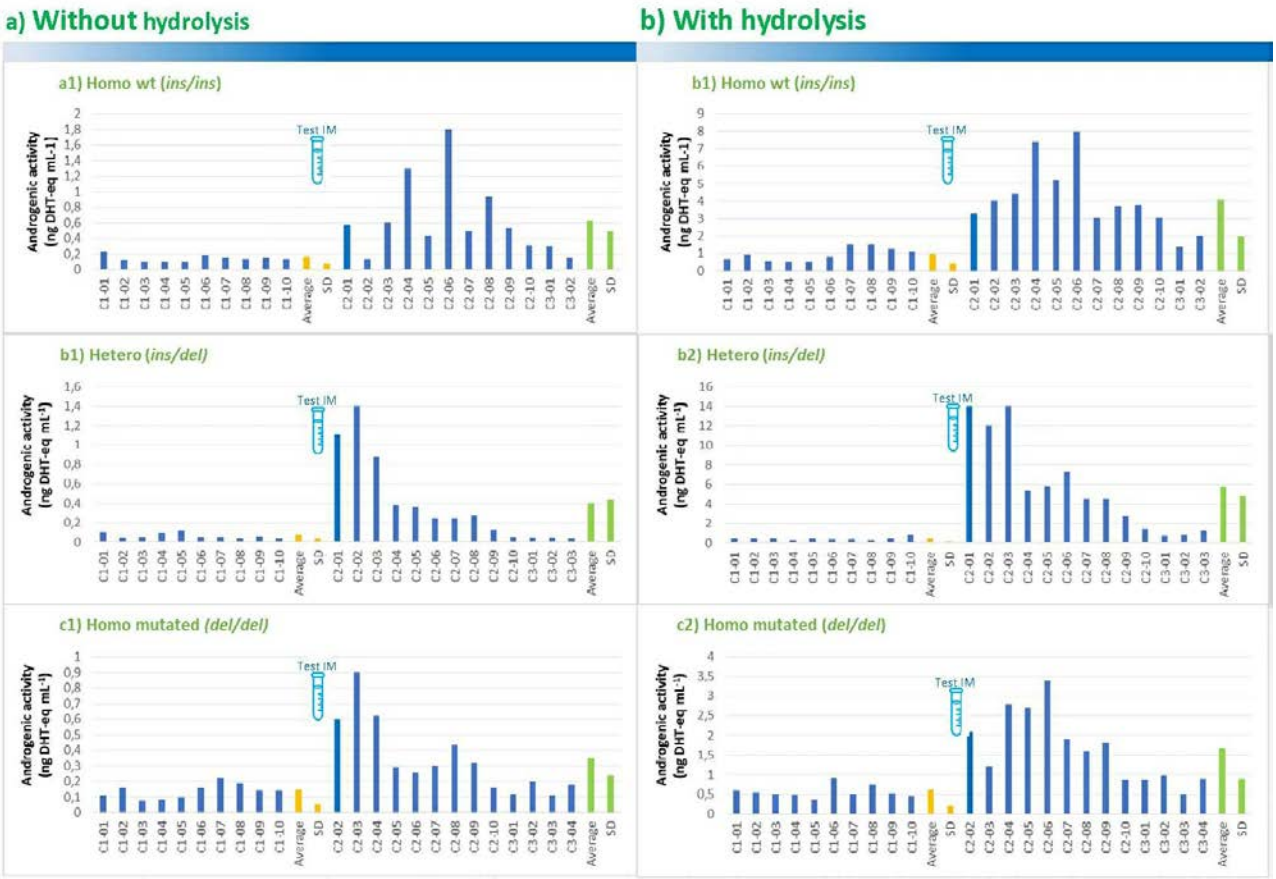

Figure 1. Androgenic activity after AR Calux® bioassay analysis a) without and b) with HCl hydrolysis for an individual of each polymorphism

The means ratio between the two periods, depending on the type of polymorphism, was: *del/del* 3.31 (CI: 95%: 2.07-5.29), *ins/del* 4.15 (CI 95 %: 3.05-5.67) and *del/del* 2.89 (CI95%: 2.42-3.46).

When the interaction is evaluated, with and without HCl hydrolysis, to assess whether the increase in DHT values that occurs after T administration is independent of the polymorphic group we observe there are significant differences without HCl (p=0,002), the analysis of comparisons between groups two-two pairs shows differences in the intensity of signal for the ins/del polymorphism (*ins/ins* vs *ins/del*, p=0.003 and *ins/del* vs *del/del*, p=0.002) but when hydrolysis is applied there are not statistically significant differences among the three polymorphism groups (p=0.250).

In spite of obtaining significant differences between genotypes without hydrolysis, the results make possible to conclude that AR CALUX® bioassay analysis allows to detect the exogenous administration of testosterone independently of the genotype for the three types of polymorphism. However, the change is significant only when an individual is compared to their own baseline values. AR CALUX® bioassay analysis appears to be capable of detect any steroid with androgenic activity (known and unknown) both in doping control and in other fields, such as analysis of nutritional supplements or leisure sports.

The extended study of this work was sent to a journal for publication.

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IAAF Doha 2019 World Championships – Sysmex XN-1000 Hematology Results

Doping Analysis Lab, Anti-Doping Lab Qatar, Doha, Qatar[1];
Anti-Doping Lab Qatar, Doha, Qatar[2]

Abstract

752 ABP samples were analyzed during the IAAF World Championships 2019 with the new Sysmex XN-1000 instrument. Results of hematological parameters are presented by discipline and gender and put into context to previously published data analyzed by the Sysmex XT-2000i.

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Dried blood spot in doping control test: evaluation of different blood collection devices

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Medicina Sperimentale, Sapienza University of Rome, Rome, Italy[2]

Abstract

Various promising applications of dried blood spot (DBS) were recently developed and published for the analysis of both small molecules and proteins in the doping control field. Indeed, the collection of capillary blood obtained by finger is minimally invasive and easily to perform; in addition, DBS provides several advantages compared to conventional venous blood sampling, including robustness, enhanced stability of the target analytes at room temperature and easier storage and transport. Nonetheless, different challenges have to be evaluated when DBS is used, including the spot size and homogeneity, blood volume, stability, extraction efficiency, multiple hematocrit effects and blood to plasma ratio.
Here we reported preliminary data obtained by evaluating the potential of different blood collection and separation devices to determine doping agents, especially those prohibited in competition.

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Reihlen P, Blobel M, Völker-Schänzer E, Schänzer W, Geyer H, Thevis M

Case reports of re-analyzed rEPO suspicious doping control urine samples from 2011-2014

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Abstract

Before the publication of the current Technical Document for EPO (TD2014EPO) in May of 2014, IEF-PAGE analysis was mandatory for reporting an *Adverse Analytical Finding* (AAF) for recombinant erythropoietin (rEPO). While the Technical Documents (TD) for EPO analysis published before 2014 did evolve in the adaptation of identification criteria and allowance of new methods, the core of a mandatory IEF-PAGE was maintained. Over the past years, the Cologne anti-doping laboratory has identified several samples of different federations to be suspicious for rEPO but the positivity criteria outlined in the TDs applicable at the time would not allow reporting as AAFs for rEPO. The TD2014EPO allows samples to be reported on the basis of separation by molecular weight alone (e.g. SDS-PAGE, SAR-PAGE). This had opened the possibility for re-analysis of long-term-stored rEPO-suspicious samples, some of which are shown in this work. The obtained data of the samples show how effective the re-analysis of doping control samples can be. It was also notable how robust and reliant molecular weight-based separation for EPO analysis performed even after years of sample storage.

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GHRH daily administration can be determined with longitudinally monitoring of IGF-I

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Abstract

GHRH are used for its property to stimulate the endogenous production of IGF-I and one can speculate that the IGF-I change should be reflected in a longitudinally passport of IGF-I. Here we have for the first time showed that longitudinally monitoring of IGF-I can be an informative biomarker for detecting administration with GHRH. We have performed a study including four healthy men *i.v.* administered with (GHRH FERRING, Somatorelin$_{(1\text{-}44)}$ (0.1 ug/kg) for five consecutive days. Blood samples were taken prior to (3 baselines), daily (day 0-4) and post (1.5 h to 24 h) the last injection. Serum IGF-I and P-III-NP were quantified by Immunotech A15729 IRMA and UniQ RIA kits, respectively. Neither of the subjects reached a GH2000 score above 9.98. When IGF-I were longitudinally monitored, all participants show values outside an individual calculated threshold (mean 3 baselines ± 3SD). Subject 1 and 4 were atypical during the treatment period and up to 24 hours and 72 hours post dose, respectively. Subject 2 and 3 were only atypical on the last active days (day 3 and day 4) and up to 24 h post dose. The increase in IGF-I were between 25 to 50%. P-III-P did not increase to the same extent and only subject 1 displayed abnormal values. The advantage with this approach is that the detection window seems to be over 12 hours as opposite to direct measurements as the peptide or any degrading metabolites can only be detected for some hours.

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Optimization of an immunopurification method for the screening of growth hormone-releasing hormones in urine doping control

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Pompeu Fabra University, Barcelona, Spain[2]

Abstract

Growth hormone-releasing hormone (GHRH) is a 44 amino acid hypothalamic peptide that stimulates the growth hormone secretion [1]. During the last years, different findings have corroborated suspicions of a growing black market with illicit synthesis of GHRH analogue hormones, which are being developed both on the basis of the intact peptide (tesamorelin) and on the 1-29 fragment (sermorelin and CJC-1295). GHRH analogues are supposed to have performance enhancing effects for athletes. Therefore, they are considered as prohibited substances according to section S2 of the World Antidoping Agency (WADA) list [2].

GHRH analogues are found at very low concentrations in urine (at the pg/mL level). For this reason, extensive enrichment and purification steps are required prior to liquid chromatography-mass spectrometry (LC-MS). Due to their peptidic nature, immunological methods are preferred [3]. Immuno-affinity purification (IP) based on magnetic beads (MBs) offers improved extraction efficiency, selectivity and reproducibility, optimum antibody orientation and minimal non-specific interaction compared to other purification strategies, and the obtained extracts are suitable for their analysis by LC-MS [4].

However, choosing the MBs surface functionalities that provide the best recoveries is not so straightforward and requires the well understanding of their properties. In order to maximize peptide recovery for the GHRH analogues during the IP step, we tested the performance of MBs with different supports, chemical affinities and binding capacities prior to the analysis of urine samples by liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) using a Quadrupole-Orbitrap instrument [5]. First, as Protein A strongly interacts with the fragment crystallizable (Fc) region of the antibody, allowing optimum orientation for the antibody-antigen interaction, we selected Protein A MBs from two different manufacturers (MagnaBind^{TM} and LOABeads^{TM} SerAscA, see characteristics in Figure 1A). However, as there is no covalent bond between the antibody and the MBs, the elution conditions (typically acidic conditions) are harsh enough to elute the antibody together with the target peptides. As an alternative to the Protein A MBs, we also investigated LOABeads^{TM} AffiActive (Figure 1A), which are functionalized with amino-reactive groups and covalently bind to the antibody. However, this strategy results in a random orientation of the antibody on the beads, fact that could potentially cause a reduction in the antibody binding efficiency.

The best recoveries for all the analytes were obtained using the LOABeads^{TM} SerAscA MBs (see Figure 1B), probably to due their high binding capacity, excellent orientation and the presence of an agarose support, which enables minimal unspecific binding of other proteins due to its high hydrophilic nature, high porosity, chemical stability and lack of charge. In contrast, poor recoveries were obtained with the LOABeads^{TM} AffiActive MBs. At this point and, taking into account that the binding capacity reported for

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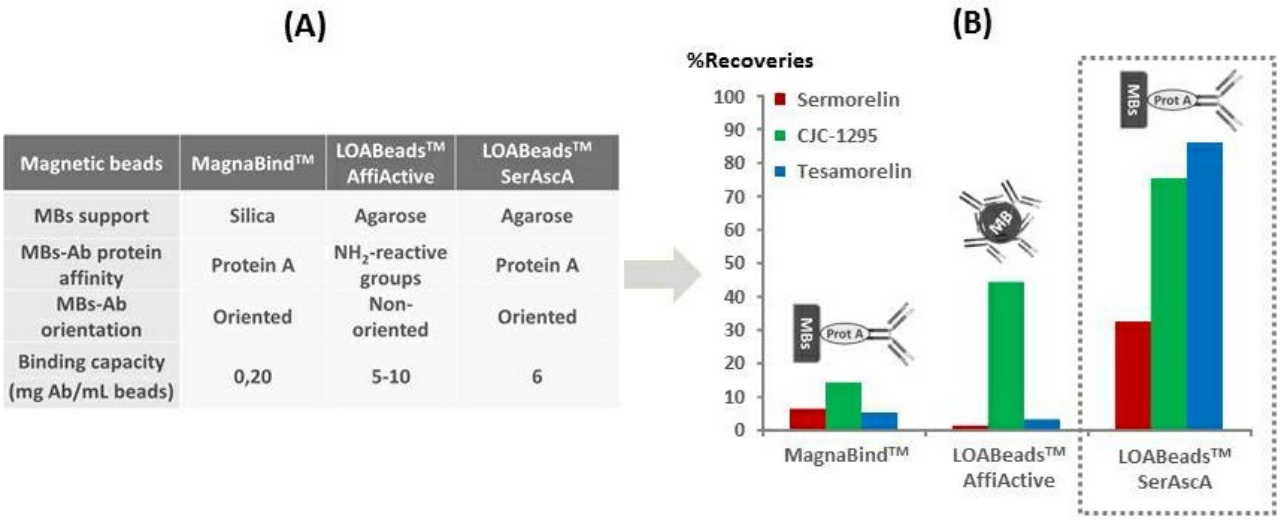
LOABeads™ SerAscA and LOABeads™ AffiActive is quite similar (see Figure 1A), we can conclude that, apart from the binding capacity, the orientation of the antibody is a critical point to obtain excellent recoveries during the IP step.

After optimization of the IP protocol with the LOABeads™ SerAscA MBs, the method was fully validated and found to be fit for purpose considering the parameters specificity, intra-day precision (lower than 15%), matrix effect, limit of detection (0.2 ng/mL) and limit of identification (0.5 ng/mL). Method validation was performed at the minimum required performance level (MRP) specified by WADA, demonstrating that the immunoaffinity-mass spectrometry-based method can be successfully applied to analyze GHRH peptides for doping control purposes.



Magnetic beads	MagnaBind™	LOABeads™ AffiActive	LOABeads™ SerAscA
MBs support	Silica	Agarose	Agarose
MBs-Ab protein affinity	Protein A	NH_2-reactive groups	Protein A
MBs-Ab orientation	Oriented	Non-oriented	Oriented
Binding capacity (mg Ab/mL beads)	0,20	5-10	6

Figure 1. (A) Properties of the MBs used for the optimization of the IP protocol and (B) recoveries obtained by LC-HRMS for all the target peptides (sermorelin, CJC-1295 and tesamorelin).

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Martin L[1], Maurice T[2], Audran M[1], Ericsson M[1], Marchand A[1]

Detection of a non-erythropoietic EPO, Neuro-EPO, in blood after intra-nasal administration in rat

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Abstract

Erythropoietin (EPO) is a hormone with several effects. Its main action consists of stimulating erythropoiesis. This has led to the production of recombinant EPO medicinal drugs to treat anemia. However it is also now well-described that EPO also possesses strong anti-oxidant properties as well as anti-apoptotic action and anti-inflammatory effects in a number of different tissues expressing EPOR-CD131 heterodimers. These protective non-erythropoietic effects of EPO are very promising to treat neurodegenerative disease (Alzheimer, Parkinson) and other brain injuries. To develop new treatments without secondary effects on erythropoiesis, it is necessary to dissociate the two effects of EPO. This was found possible by producing modified EPO with low or no sialic acid content (e.g.: asialo-EPO, carbamylated-EPO). They are nevertheless prohibited by WADA. A similar new drug candidate under clinical investigation to treat brain diseases is Neuro-EPO, produced by selecting EPO isoforms with low sialic acid content. Interestingly, intra-nasal administration was found to be an easy way to administer EPO into the brain and bypass the blood-brain barrier.

The aim of this project was to characterize Neuro-EPO with the anti-doping methods used to detect conventional rEPOs and to evaluate the window of detection of Neuro-EPO in blood after an intranasal administration in rats.

Neuro-EPO was analyzed by SDS-PAGE and IEF-PAGE. By IEF this modified EPO showed a very basic profile that needed the use of a 2-10 pH gradient instead of the conventional 2-6 pH gradient. Its characteristic profile consisted in 5 very basic bands that did not interfere with the endogenous EPO profile from human or rat. After separation by molecular weight using SDS-PAGE, a broad band was detected in the same area as endogenous EPO, making Neuro-EPO identification very difficult in the presence of endogenous EPO. Therefore, the IEF method was preferred to evaluate its presence in blood and tissue. We then evaluated the detection of Neuro-EPO in brain and blood up to 48h after intranasal delivery of a single therapeutic dose. Neuro-EPO was identified in blood 2h and 6h after the delivery. Fainter signals were then identified between 12h and 48h. However, some characteristic very basic isoforms remains detectable up to 48h. Surprisingly, brain extracts did not show the presence of Neuro-EPO even 2 hours after administration, showing a very fast elimination from the brain area to the bloodstream. Repeated administration might produce a cumulative effect and shall be investigated but this first experiment indicated the possibility to identify Neuro-EPO after intranasal delivery for a few days in blood and the utility to use a large pH gradient for IEF to identify very basic isoforms in case of particular forms of EPOs.

* The Manfred Donike Award 2020 for the best poster presentation went to Laurent Martin and co-

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workers from the Analysis Department of the French Anti-Doping Agency (AFLD) for their investigation of the detection possibilities of Neuro-EPO. This drug candidate is a low sialylated EPO that acts as an intranasally applicable neuroprotector. Their research results provide valuable information on method adaptation for future EPO analyzes.

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Kaliszewski P, Siek P, Zalewska Z, Michalak D, Grucza K, Wicka M, Kwiatkowska D

Method development for the detection of carbamylated erythropoietin (CEPO) for doping control purposes

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Abstract

Erythropoietin (EPO) has protective effects in several tissues and could be used for therapeutic purposes, but the doses of EPO that can be beneficial in case of hypoxic-ischemic conditions due to over induced erythropoiesis could be detrimental in treated patients. Carbamylation of erythropoietin maintains the tissue-protective effects of EPO but without erythropoietic effects. Carbamylated EPO (CEPO) is listed in the WADA Prohibited List in class S2 as “Innate repair receptor agonists” [1]. The CEPO was synthesized by using the method described previously [2]. Briefly, rhEPO (NeoRecormon, Epoietin beta) was mixed with sodium borate (pH 8.8) and potassium cyanate (KOCN) for a final concentration of 1 mol/L. The mixture was incubated in a water bath (37°C) for 24h and then concentrated by ultrafiltration and washed with Tris-HCl buffer pH 7.4. The digestion by endoproteinase Lys-C was used to distinguish rhEPO from CEPO. The digested samples containing recombinant EPO, urinary EPO (uEPO) or CEPO were analyzed by the SAR-PAGE method [3]. Endoproteinase Lys-C breaks the peptide chains of lysine. Lysine residues, converted to homocitrulline by carbamylation, cannot be cleaved by endoproteinase Lys-C. Therefore, the CEPO protein chain remaines unchanged in contrast to rhEPO and uEPO which allows for easy differentiation of them.

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Zhou X, He S, Zhang L, Shen L, He C

Assessment on rat EPO as Internal Standard in Doping Control Samples for EPO Detection with SAR-PAGE using Biotinylated Primary Antibody

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Abstract

According to the current TD for EPO, SAR-PAGE is the most commonly applied method for both screening and confirmation procedure. Although this method is effective and robust, there is no internal standard (IS) to monitor the efficiency of analysis with each sample covering every step of the whole procedure, including preparation, immunopurification, and western blot. This IS needs to be recognized by both of the two anti-EPO antibodies which are used for immunopurification and western blot, respectively. Besides that, the band of IS cannot be allowed to disturb the recognition of all types of targeted EPO and analogs. To meet these two principles, rat EPO was selected. In this study, for both urine and blood samples, rat EPO was spiked at the beginning of analysis. After preparation and immunopurification, single blotting with biotinylated AE7A5 as primary antibody was applied, followed by incubation of streptavidin coupled HRP. According to the comparison of different immunopurification methods, for urine samples, antibody AB-286-NA (R&D) coupled with M-280 magnetic beads (Invitrogen) is the better choice, while MAIIA column is suitable for blood samples. All these methods have been validated with selectivity, repeatability and sensitivity. It is shown that the modified method in this study can not only eliminate the cross reactivity between antibodies, but also monitor the whole procedure of EPO analysis with spiked rat EPO. Besides that, for urine samples, rat EPO also could be an indicator of monitoring the presence of protease.

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Zhou, X, He, S, Zhang, L, Shen, L, He, C. Research on spiking rat EPO as internal standard in doping control samples for detection of EPO using SAR-PAGE analysis with biotinylated primary antibody. *Drug Test Anal*. 2020; 12: 1054-1064. https://doi.org/10.1002/dta.2863

Reihlen P, Weiß P, Blobel M, Walpurgis K, Thevis M

Evaluation of PEGylated EPO-conjugates as internal standards for EPO analysis in doping controls

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Abstract

Immunopurification of doping control samples is a mandatory necessity in EPO analysis during a Confirmation Procedure; moreover, it has become common practice to also immunopurify samples for the Initial Testing Procedure. Typically used materials (e.g. Stemcell purification plate, MAIIA purification kit) rely on anti-EPO antibodies for purification. Also, the detection of EPO after electrophoretic separation and western blotting is based on a monoclonal anti-EPO antibody, clone AE7A5, directed against a 26 amino acid sequence of the N-terminal region of human EPO. While the electrophoretic separation and blot transfer efficiency can be monitored with reference standards and quality control samples, it is presently not possible to monitor the functionality of the entire sample preparation procedure. The reliance on antibodies for both purification and detection has complicated the implementation of an internal standard (ISTD). The Cologne laboratory has cooperated with Celares GmbH to synthesize customized EPO-polyethylene glycol (PEG)-conjugates and to identify those conjugates that can be co-immunopurifed from urine and blood samples using both common purification techniques (Stemcell, MAIIA). Moreover, avoidance of interferences of the ISTD with SAR-PAGE-based EPO analysis is vital. Preliminary results have shown that chosen EPO-PEG conjugates could be successfully co-immunopurified from blood and urine. A conjugate containing a 12 kDa PEG has been found to be most suitable for SAR-PAGE analysis. To date, only prototype conjugates were assessed to identify adequate ISTD candidates (especially concerning electrophoretic migration behaviors), and following the herein presented proof-of-principle analyses, larger-scale synthesis will be conducted, including thorough purification processes to ensure the absence of unconjugated rEPO residues in the final product.

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Novel Long-Term Markers for Methylnortestosterone Misuse in Human Urine

Doping Control Laboratory of Athens, Athens, Greece[1];
Sandoz GmbH, Tirol, Austria[2];
Cyprus Anti-Doping Authority, Nicosia, Cyprus[3];
Qatar Anti-Doping Lab, Doha, Qatar[4]

Abstract

Methylnortestosterone is a progestin and synthetic androgenic anabolic steroid that is prohibited under the S1 category of WADA's prohibited list. Methylnortestosterone misuse is commonly detected by the monitoring of the parent compound and its main metabolites 17α-methyl-5β-estrane-3α,17β-diol (M2) and 17α-methyl-5α-estrane-3α,17β-diol (M1) in the glucuronide fraction. In the current study a direct detection of methylnortestosterone sulfo-conjugated metabolites after extraction with ethyl acetate and analysis by liquid chromatography/quadrupole/time of flight mass spectrometry (LC/Q/TOFMS) in negative ionization mode was performed. Two main sulfate metabolites (S1, S2) were detected by this procedure.

For the characterization of metabolites, samples from the excretion study were additionally analysed by GC/MS after solvolysis and per TMS derivatization. RT and MS data collected, were compared with RT and MS data from metabolites of 17z-methyl-5β/α-estrane-3α/β,17z-diols structures with prefixed stereochemistry at 3 and 5 positions, synthesized through Grignard reaction from 19-noretiocholanolone, 19-norandrosterone and 19-norepiandrosterone. Based on these results the structure of the two sulfate metabolites was confirmed as S1, 17α-methyl-5α-estrane-3α,17β-diol-3α-sulfate and S2, 17α-methyl-5β-estrane-3α,17β-diol-3α-sulfate (main sulfate metabolite).

Furthermore applying targeted analysis based on RT and MS data of the synthesized metabolites in free and glucuronide fraction after hydrolysis with glucuronidase from E.coli two additional glucuronide metabolites M3, 17β-methyl-5β-estrane-3α,17α-diol and M4, 17β-methyl-5α-estrane-3α,17α-diol were detected after GC-MS/MS analysis. In the sulfoconjugated fraction after hydrolysis with H. Pomatia one more sulfate metabolite (S3) 17β-methyl-5β-estrane-3α,17α-diol-3α-sulfate was detected after GC-MS/MS analysis in lower abundance.

Interestingly methylnortestosterone's main sulfate metabolite (S2) could also be detected after the direct analysis of non-hydrolyzed steroid by GC-MS/MS as artifact, following normal ProcIV anabolic steroid procedure and using diethylether as extraction solvent. Glucuronide M2, M3, M4 and sulfate metabolites S2, S3, were detected up to until the end of the excretion time (192 h) while M1 and S1 were detected for 72h. PC was detectable on the glucuronide fraction for relatively narrow time window. These new reported metabolites can be used as additional markers of methylnortestosterone misuse.

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Martinez Brito D, Botrè F, de La Torre X

Mass spectrometric analysis of 7-oxygenated androst-5-ene steroids - an *in vitro* study

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Abstract

Rationale:
Several authors have described the generation of androsta-3,5-diene-7-one structures from androst-5-ene-3,7-dione or androst-5-ene-3β-ol-7-one under acidic conditions and/or at high temperatures. The goal of this study was to observe and to describe the results obtained after the chromatographic analysis of the trimethylsilyl derivatives of reference materials of 7-oxo-DHEA, 7α-hydroxy-DHEA, 7β-hydroxy-DHEA, and androsta-3,5-diene-7,17-dione known as arimistane.

Experimental:
The purity of the analyte reference materials was verified by liquid chromatography/quadrupole mass spectrometry. The trimethylsilyl derivatives obtained using several mixtures with MSTFA (N-methyl-N-trimethylsilyl trifluoroacetamide) in comparison with solely MSTFA were analyzed by gas chromatography coupled to a time-of-flight detector equipped with a multimode inlet or to a simple quadrupole detector with a split/splitless inlet.

Results:
The study showed that the formation of arimistane from 7-oxo-DHEA occurs using common derivatization reagents used for the analyses by gas chromatography (GC). In addition, the formation of the enolized TMS derivative of 7-oxo-DHEA was observed in considerable amounts when it was reacted with MSTFA. The analysis of 7α-hydroxy-DHEA resulted in the detection of close to 1% of arimistane. The formation of unexpected artifacts from derivatization is influenced by the reagent itself, the reaction temperature, the inlet used and its configuration.

Conclusions:
The derivatization reagent, instrumental conditions (inlet), as well as the chemical structures of the analytes present in the matrix, can influence the results. So, before describing a new feature as a potential “new” metabolite, special caution must be taken since we could actually be dealing with an artifact.

Published as:
Dayamin Martinez-Brito, Xavier de la Torre, Maria Kristina Parr, Francesco Botrè. Mass spectrometric analysis of 7-oxygenated androst-5-ene structures. Influence in trimethylsilyl derivative formation. *Rapid Commun Mass Spectrom*. 2020;34:e8834. https://doi.org/10.1002/rcm.8834.

Martinez Brito D, Iannone M, Tatangelo M, Molaioni F, Botrè F, de La Torre X

Old compounds, new alertness: *In vitro* and *in vivo* experiments using methyltestosterone

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

Abstract

Rationale:

Although the metabolism of methyltestosterone (MT) has been extensively studied since the 1950s using different techniques, the aim of this study was to investigate the hydroxylation in positions C2, C4 and C6 after *in vitro* experiments and *in vivo* excretion studies using gas chromatography time-of-flight (GC/TOF) and gas chromatography/tandem mass spectrometry (GC/MS/MS). The results could be influenced by the mass spectrometric analyser used.

Experimental:

Incubation were carried out with human liver microsomes (HLM) and six enzymes belonging to the cytochrome P450 family using Methyltestosterone (MT) as a substrate. The trimethylsilyl derivatives of the samples were analysed using GC/TOF and GC/MS/MS once the correct MS/MS transitions had been selected, mainly for 6-hydroxymethyltestosterone (6-OH-MT) to avoid artefact interferences. A urinary excretion study was then performed after the administration of a 10 mg single oral dose of MT to a volunteer.

Results:

The formation of hydroxylated metabolites of MT in the C6, C4 and C2 positions after both *in vitro* and *in vivo* experiments was observed (Figure 1). Sample evaluation using GC/TOF showed an interference for 6-OH-MT that could only be resolved in GC/MS/MS by monitoring specific transitions. The transitory detection of these hydroxylated metabolites in urine agrees with previous investigations that had described this metabolic route as being of little significance.

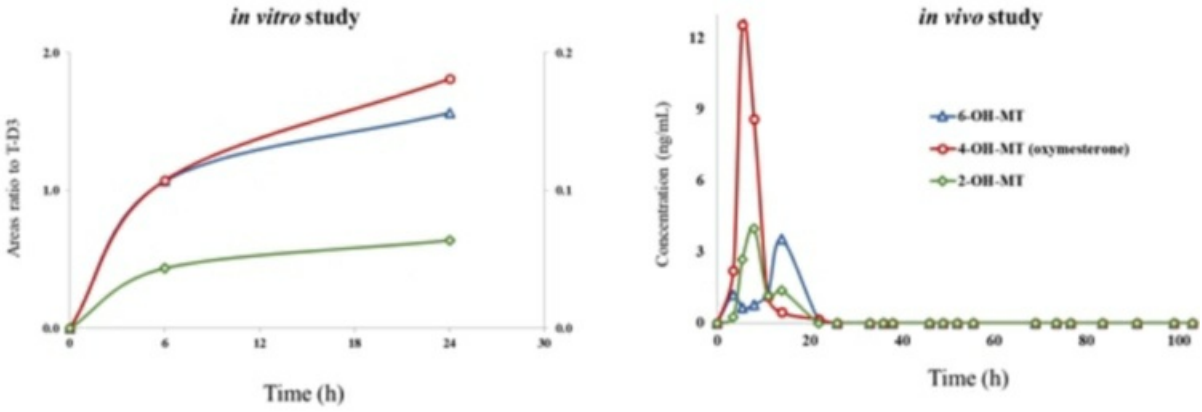

Figure 1. Formation of 6-hydroxy, 4-hydroxy and 2-hydroxy-methyltestosterone after incubation with HLM and after the administration of a single dose of methyltestosterone

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Conclusions:
In doping analysis, the formation of 4-hydroxymethyltestosterone (oxymesterone) from MT cannot be underestimated. Although it is only detected as a minor and short-term excretion metabolite, it cannot be overlooked as it was found in both in vitro and in vivo experiments. The use of a combination of different mass spectrometric instruments allowed reliable conclusions to be reached, and it was shown that special attention must be given to artefact formation.

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Deventer K, van Gansbeke W, van Eenoo P

Alternative approach for the detection of aminoglutethimide

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Abstract

In the field of doping, the use of aminoglutethimide is prohibited and it is classified under class S4 (hormone and metabolic modulators) because of its anti-estrogenic activity. The anti-estrogenic activity can be beneficial for athletes to circumvent side effects of intense anabolic steroid (AS) abuse by inhibiting the breakdown of the AS to estrogens. Additionally, by the inhibtion of their breakdown, the effect of the AS abuse can be prolonged. The MRPL of aminoglutethimide is set by WADA to 20 ng/mL. Investigation of detectability of aminogluthetimde in our laboratory showed poor detectability in intial testing procedures (ITP) on either GC-MS and LC-MS systems. An alternative strategy to detect aminoglutethimide can be obtained by investigating the metabolism. Acetyl aminoglutethimide has been described as a major urinary metabolite and hence, its detectability was investigated. Initially, the metabolite was synthesized by the acetylation of aminoglutethimide. In a next step, detection limits on either GC-MS and LC-MS systems were investigated. Finally, the metabolite was confirmed in authentic administration urine.

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Andersson A[1,2], Elings Knutsson J[3,4], Ekström L[1,2], Lindén Hirschberg A[3,5]

ABP profile in relation to transdermal testosterone administration in women

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Department of Women's and Children's Health, Karolinska Institute, Stockholm, Sweden[3];
Theme Children's and Women's Health, Stockholm, Sweden[4];
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Abstract

In times where the detection capability of steroid doping analysis is ever increasing, most studies are still performed on men. For all good intentions, this does leave us lacking in the understanding of female steroid doping, especially when involving testosterone (T). Recently published data (Handelsman et al. 2019) show none to low response of the classical steroid profiling parameters in urine, including for the important testosterone/epitestosterone ratio (T/E). This suggests that doping with T in women will not trigger an atypical passport finding.

In this study, 48 physically active women were recruited. The subjects were divided into two groups; half applied 10 mg testosterone (AndroFem 1) and the other half placebo crème. The women applied 1 mL crème daily for 10 weeks. Three samples were collected prior to treatment and 1 after its completion. Preliminary data of 5 subjects (and 3 placebo) resulted in 4 that did not arouse suspicion.

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Handelsman, DJ, Bermon, S. Detection of testosterone doping in female athletes. *Drug Test Anal*. 2019; 11: 1566-1571. https://doi.org/10.1002/dta.2689

Suominen T[1], Ericsson M[2], Ekström L[2], Schulze J[2]

ABP-profile in relation to the menstrual cycle

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Abstract

The intra-subject variations of all the urinary steroid metabolites and the ABP-ratios are larger in females than in males making the interpretation of female samples more challenging. It has been shown that T/E varies during the menstrual cycle as previously investigated in two small studies of six and nine participants, respectively. Here we aim to study if the concentrations of the ABP metabolites differ between the menstrual cycle phases as biological determined based on estrogen, progesterone and gonadotropins levels. Moreover, the present study will provide an understanding if the intra-individual fluctuation of the urinary steroid ABP-ratios is similar between two menstrual cycles. 17 healthy women with regular menses not using hormones were included. Urine and blood samples were collected 3 times/week and 1 time/week, respectively for two consecutive cycles. It was showed (in agreement with previous finding) that E concentrations increased approximately 150% in the ovulation (21 ng/ml ± 14.9) and luteal phase (17.7 ng/mL ± 12.3) as compared to follicular phase (8.5 ng/mL ± 3.8). For the other ABP metabolites 20% to 50% significant higher concentrations were observed during ovulation. There were large inter-individual variations in how the much the ABP ratios fluctuate during the cycles; CV% between 21-68 for T/E, and CV% between 9-66 for the other ABP ratios. Interestingly, when monitoring the ABP profile for two cycles we observed that the fluctuation pattern in respectively cycle mimic each other. This was true for all five ABP ratios. The women with consistently larger CV% show wider individual thresholds which may be associated with greater complexity to detect doping with EAAS.

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ABP urinary metabolites and how they correlate to serum hormone levels in female athletes

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Department of Gynaecology and Reproductive Medicine, Karolinska University Hospital, Stockholm, Sweden[4]

Abstract

Several studies have investigated the correlations between urinary steroids and serum concentrations of androgens in men showing that T and E both reflect the systemic levels of androgens, whereas studies in women are lacking. Since testosterone synthesis and metabolism in women is different it is of interest to study such correlations also in women. Here we have analyzed the urinary steroid profile with GC-MS/MS and correlated with serum levels of androgens (T, DHT, DHEA), Estrogens (E1, E2), gonadotropins and cortisol in 94 elite athletes.

Neither of the urinary ABP metabolites were correlated with the gonadotropins, except for E where a significant correlation with LH was noted (Rs=0.41, pË,0.001). The androgen index (FAI) showed weaker correlations to E than the other ABP metabolites, whereas the opposite was found for serum estradiol which show stronger correlations with E (Rs=0.62, pË,0.0001), than the other ABP metabolites (Rs 0.21-0.33, pË,0.05). So contradictory to men, E in women reflects the estrogens to large extent than the androgenic load. Serum DHEA showed correlations with all the urinary ABP metabolites (0.33-0.42, pË,0.01) except for E. This is in agreement with the hypothesis that E is formed from pregnenolone. We also studied if any correlations between cortisol and the ABP metabolites and interestingly we found borderline significant negative correlations with T (Rs=0.19, p=0.06), and significant negative correlations with A (Rs=-0.25, pË,0.05) and 5α (Rs=-031, pË,0.01). This is the first time correlations between cortisol and ABP have been studied, but there are some studies suggesting that stress influences the ABP profile.

In conclusion, all the urinary metabolites except E showed similar correlation profile to serum hormone levels. We will also study these correlations in sedentary women in order to study exercise impact.

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Brailsford A[1], Wojek N[2], Chih-Wei C[1], Walker C[1], Cowan D[1], Wolff K[1]

Determination of anabolic steroids in dried blood using micro-sampling and gas chromatography-mass spectrometry: application to a testosterone gel administration study

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Science and Medicine, UKAD, London, United Kingdom[2]

Abstract

Anabolic androgenic steroids (AAS) are the most commonly abused substances taken by both professional sportsmen and recreational bodybuilders. Although the detection of exogenous AAS is not particularly challenging, the misuse testosterone (T), particularly in low doses remains difficult as it is also produced endogenously, and thus detecting its administration requires more nuanced approaches such as GC-C-IRMS and steroid profiling.

The collection of dried blood (DB) obtained by finger pricking is a cost effective and potentially a complementary approach to traditional sample collection in anti-doping, as it offers advantages such as simplicity of sample collection and reduced invasiveness. Moreover, the introduction of the volumetric absorptive microsampling (VAMS) technology overcomes some of the limitations associated with spotting blood onto a filter paper such as non-homogenous sampling and quantification.

In the work presented, a fast and sensitive GC-MS/MS method was developed and validated for simultaneous quantification of AAS in DB collected by means of VAMS. T as well as eight of the most abused synthetic AAS (nandrolone, boldenone, mesterolone, drostanolone, metenolone, metandienone, oxandrolone, and dehydrochloromethyl T) were selected as the target analytes. The method exhibited acceptable precision, accuracy, recovery, stability, and sensitivity, with detection limits is as little as 0.10 ng mL^{-1} in 20 µL of DB). The developed method was then successfully applied for the analysis of samples collected from a T administration study, with participants given transdermal doses of 50 mg and 100 mg of T gel.

Our results demonstrated that using the developed method it was possible to detect the administration of the administered T in DB. However, significant inter-individual variation was noted in the analysed T concentrations, both in terms of the basal values and those observed post administration. Interestingly, the results for placebo control samples demonstrated that the within individual variation in T concentration was low, which raises the possibility of using athlete profiling in DB samples to provide a more sensitive approach for the detection of T administration.

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Cavalcanti G[1], Zhu Y[2], Wijeratne N[2], Martins C[2], Padilha M[1], Pereira H[1]

Application of VeriSpray ion source Triple Quadrupole Mass Spectrometry for stimulants and narcotics analysis in oral fluid

Chemistry, Chemistry Institute, Rio de Janeiro, Brazil[1];
ThermoFisher Scientific, San Jose, USA[2]

Abstract

Paper Spray ionization is an ambient ionization approach which allows the direct analysis of complex biological samples requesting no sample preparation step. On this ionization technique, the sample is spotted onto a piece of paper followed by the dispense of a small spray solvent volume. A high voltage is then applied to the paper positioned in front of MS promoting the ESI process at the paper tip. Recently, a plenty of promising applications of paper spray ionization Mass Spectrometry have been described in forensic field for the detection of several drugs in many biological matrices, such as: Dried Blood Spot (DBS), urine and oral fluid (OF). Nevertheless, there is a lack of the applicability of this ambient ionization in doping analysis employing OF as alternative matrix to track prohibited substances in competition only. The main antidoping testing approach for these class of drugs is to detect their recent use. For this purpose, OF is more suitable than the traditional urine matrix once these drugs and their markers filtered or transported to saliva have a short detection window indicating an early drug intake. Therefore, this alternative matrix can aid more accurately in the decision process management whether athlete was under influence of biological effects of specific prohibited drug during competition period. Here, we performed a preliminary assessment of paper spray ionization MS application for analysis of cathinone, amphetamine (AMPH), lisdexamfetamine methamphetamine, methylphenidate, ritalinic acid and tramadol in OF. The OF was collected, spiked with reference materials and labeled deuterated internal standards. The sample volume of 8 uL was spotted onto the paper strips. Data were acquired using a Thermo Scientific™ TSQ Quantis™ triple quadrupole mass spectrometer coupled to the VeriSpray™ PaperSpray ion source. The spray and rewet solvent employed was MeOH:CH_2Cl_2:NH_2OH (80:20:0.2) and it was programmed to dispense at a specific delay time. The total analysis time was 1 min (MRM acquisition duration). The blank samples were analyzed and compared with those samples spiked at lower levels. The signal to noise achieved for quantify MRM transition at this concentration level was higher than 3 when compared with the blanks. The ion ratio and RSD (%) was calculated as well and used to stablish the lower detection limit concentrations. All the RSD calculated were lower than 10% at lower concentration level showing a reasonable method precision. The detection limit concentrations was in agreement with their concentrations usually find in real OF samples. Also, a quantitative approach of these prohibited drugs in competition was evaluated by this method. Paper spray ionization MS provided a quite fast analysis time (no sample preparation and 1 min of analysis time) to determine some prohibited substances in doping control employing OF as alternative matrix so far. Further studies must be done to enlarge the drug panel and assess the method reliability over the gold standards LC MS/MS and HRMS methods. Furthermore, the method validation and real sample analysis shall be done to evaluate the fitness for purpose of the method.

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Kowalczyk K[1,2], Torres Elguera JC[1], Konopka A[1], Jarek A[2], Stanczyk D[2], Wicka M[2], Kwiatkowska D[2], Bulska E[1]

In vitro metabolic studies of emerging selective androgen receptor modulators (PF-0620414, TFM-4-AS-1, BMS 564929 and GSK 2881078) and implementation of the results into LC-MS/MS-based doping control analysis

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

Abstract

Selective androgen receptor modulators (SARMs) were developed as a treatment of choice for diseases that are currently cured with steroidal androgens. Owing to their high potency combined with a significant reduction of adverse side-effects, the compounds quickly grabbed both doctors' and athletes' attention. Despite the fact that SARMs have been included in the World Anti-doping Agency's (WADA's) Prohibited List since 2008, the number of doping cases, as well as product seizures, has been increasing.
The aim of the presented study was to explore the metabolic pathways of four compounds, namely PF-06260414, TFM-4-AS-1, BMS 564929, and GSK 2881078 in view of a potential toxicological or doping control analysis. Metabolites were synthesized using human liver subcellular fractions (microsomal and S9). Prior to a thorough analysis by means of LC-HRMS/MS, the samples were prepared according to procedures based on liquid-liquid extraction and dilute-and-shoot approach.
Detected metabolites, mainly resulting from single or multiple hydroxylations, methoxylation, demethylation, dehydrogenation, or the combination thereof, and the following II phase modifications were discussed as potential markers of the abuse of the substances of interest. The main metabolites, identified during the study are presented in Figure 1. Finally, a simplified method was proposed and validated according to the WADA technical documents.
For more details regarding the main metabolites and their use as markers of the intake, please refer to the full article which will be published shortly.

Acknowledgements

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The study was carried out at the Biological and Chemical Research Centre, University of Warsaw, established within the project co-financed by the European Union from the European Regional Development Fund under the Operational Programme Innovative Economy 2007–2013.

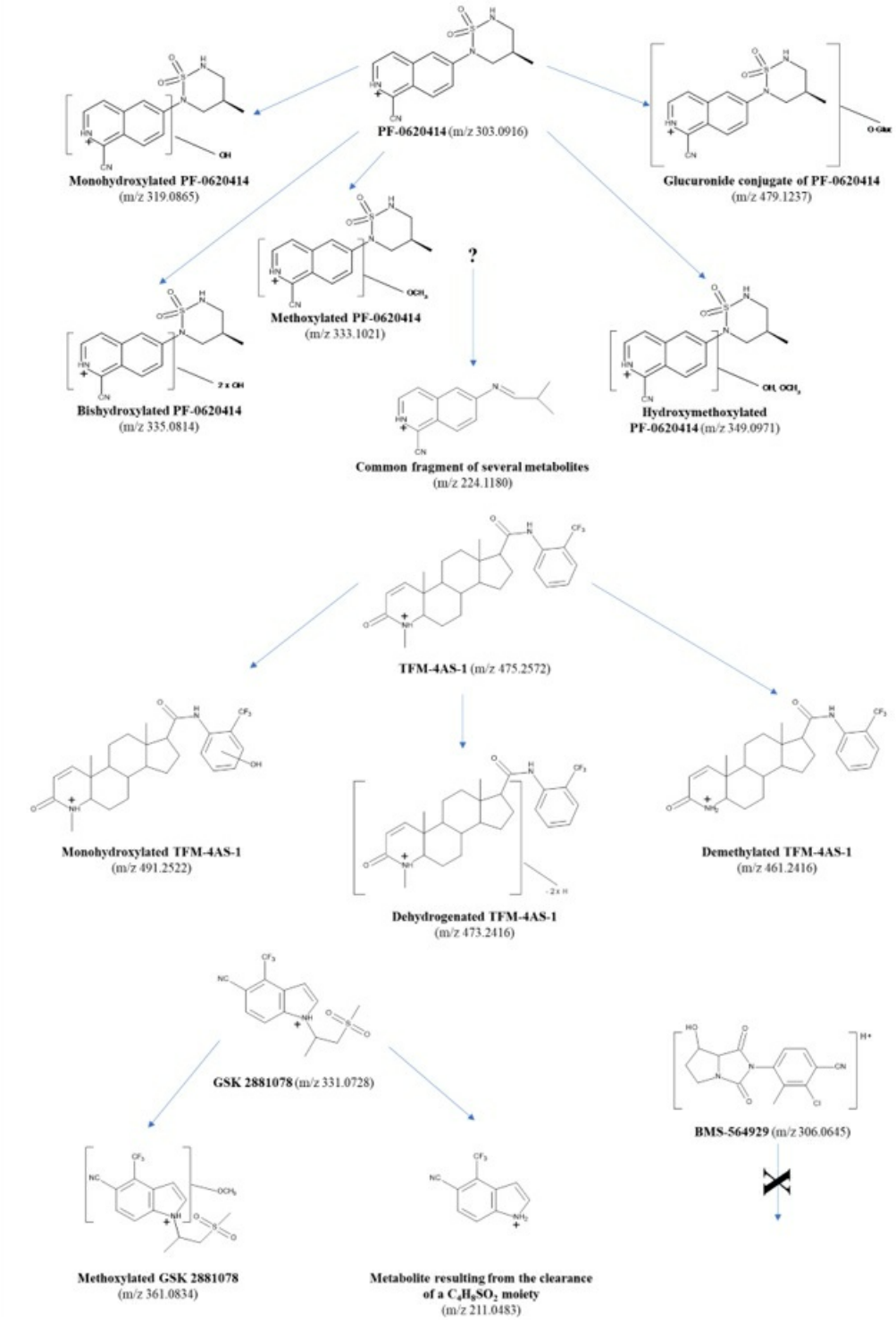

Figure 1. Metabolic pathways of PF-0620414, TFM-4AS-1, GSK 2881078, and BMS-564929

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Sato M, Kageyama S, Okano M

Detection of the selective estrogen receptor modulator Bazedoxifene in human urine by LC-MS/MS

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

Abstract

Bazedoxifene, a selective estrogen receptor modulator, has been explicitly included in the WADA Prohibited List since January 2020. A high resolution liquid chromatography–tandem mass spectrometric (LC–MS/MS) method was developed to identify bazedoxifene and the metabolites in human urine for sports drug testing. Bazedoxifene acetate (20 mg) was orally administered to seven volunteers and urine samples collected were analysed using the developed method. The quantitative method for urinary bazedoxifene was fully validated. Free bazedoxifene, bazedoxifene gluco-conjugate and bazedoxifene-*N*-oxide were identified in the urine sample. The profiles of urinary excretion showed small amounts of free bazedoxifene and bazedoxifene-*N*-oxide resulting in predominant bazedoxifene glucuronide. In all seven subjects, bazedoxifene (free plus glucuronide) could be detected up to 78 h after dosing.
The detailed results will be published elsewhere.

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Dubey S, Sah S, Sahu PL

Comprehensive screening of doping agents by ‘dilute and shoot’ urine analysis using high resolution/high accuracy mass spectrometry

National Dope Testing Laboratory, New Delhi, India

Abstract

The World Anti-Doping Agency (WADA) list of prohibited substances and methods includes molecules of diversified chemistries and pharmacological classes. Further, the International Standard for Laboratories (ISL) also necessitates the detection of numerous other substances either for informative or monitoring purposes. Therefore, a doping control laboratory work flow demands open and high throughput screening methods to accommodate new analytes and to have a faster turnaround time. The use of dilute and shoot urine analysis on tandem mass spectrometer has provided comprehensive and fast screening tools. However, multiple reaction monitoring (MRM) based data are selective and do not provide retrospective information on unknown molecules present in the sample, and the number of MRMs can be a limiting factor prohibiting inclusion of more analytes in a method. The use of hybrid high resolution mass spectrometry (HRMS) based analysis promises an endless possibility of analyte detection in a given mass range along with restrospectivity. Hence, the HRMS has been evolved as promising tool for initial testing in sports doping control analysis. Herein, we report a fast and simple ultra-high performance liquid chromatography-high resolution mass spectrometry based assay for screening of more than 230 analytes of different classes including phase two metabolites of several substances in human urine by diluted urine injection. The method was developed on a Thermo Scientific Vanquish ultra-high performance liquid chromatograph coupled to a Q Exactive-HF hybrid mass spectrometer. The target doping agents could be detected with-in/below the minimum required detection limits set by WADA. The method was successfully validated as per the requirements of WADA ISL for qualitative analysis. The applicability of the method has been demonstrated by simultaneous analysis of iQAS samples, few excretion urines and the samples previously reported as positive, using both the existing LC-QQQ and the newly developed LC-HRMS methods.

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Mongongu C[1], Marchand A[1], Buisson C[1], Coudoré F[2], Audran M[1], Ericsson M[1], Madi Moussa E[1]

A sensitive detection of synthetic IGF-I analogs using a mass spectrometric immunoassay and nano-ultra-high-performance liquid chromatography coupled to high resolution mass spectrometry

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Laboratoire de Neuropharmacologie, INSERM UMRS 1178, Université Paris-Saclay, Châtenay-Malabry, France[2]

Abstract

Insulin-like Growth Factor-I (IGF-I) is a growth factor synthesized by the liver under the biological effect of GH (growth hormone). The therapeutic use of recombinant IGF-I has been approved for the treatment of patients with a growth retardation due to a severe IGF-I deficiency. In the 1990s synthetic analogs such as Des (1-3)-IGF-I, LongR3-IGF-I and R3-IGF-I were developed to increase activity and half-life but these drugs were never approved for human use. Since 1999, Insulin-like Growth factor-I (IGF-I) and its analogs have been listed as prohibited substances by the WADA. Nonetheless, black market peptide companies sell these analogs as growth promoting agents and they could be used by athletes The present study describes a fast sample preparation and a sensitive method for analysis of IGF-I analogs, Long-R^3-IGF-I, R^3-IGF-I and Des(1-3)-IGF-I in serum.

In this work, a top-down analytical method was developed for the detection of IGF-I analogs. Briefly, the sample preparation consisted of an immunoaffinity purification of IGF-I analogs using a polyclonal rabbit anti-human IGF-I antibody. After isolation, the target substances were directly analyzed with a nano-LC-HRMS system. The combination of immunopurification and nano-LC-HRMS ensured sensitivity, specificity and selectivity. Analytical performances were studied using spiked human serum samples. The method was validated for the following parameters; linearity (0.25 to 200 ng/mL, R^2>0.99), specificity, detection limits (< 0.25 ng/mL), recovery (30-60%), precision (<25% at 0.5 ng/mL) and robustness. The validation confirmed the usefulness of this method for the detection IGF-I analogs.

In order to prove and validate the applicability of the developed approach, a single intramuscular administration of 100 µg/kg for each analog was carried out in rats and kinetic elimination was studied from 4h post injection to 36h post-injection. R^3-IGF-I and Des(1-3)-IGF-I were detected until 16h post administration (0.2-2 ng/mL) and Long-R^3-IGF-I was detected until 4h post administration (0.7-1.5 ng/mL). In addition to the intact molecule Long-R^3-IGF-I, three N-terminally truncated metabolites were observed: Des(1)-Long-R^3-IGF-I, Des(1-10)-Long-R^3-IGF-I and Des(1-11)-Long-R^3-IGF-I. Des(1-11)-Long-R^3-IGF-I was detected until 16h after administration while the other two were detected only until 4h after administration. No metabolites for Des(1-3)-IGF-I and R^3-IGF-I were identified. This administration study in rats demonstrated that the developed method can be used to detect IGF-I analogs after an *in vivo* administration. However the window of detection was small (less than 24h) due to rapid degradation/elimination of the analogs and the metabolites. An administration in human is needed to confirm if a similar detection window for the intact analogs and metabolites is found.

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

Metabolites of TB-500 peptide in various *in-vitro* enzyme systems by UHPLC-MS/MS

Doping Control Center, Korea Institute of Science and Technology, Seoul, Republic of Korea[1];
Division of Biomedical Science & Technology, KIST-School, Korea University of Sciecne and Technology, Seoul, Republic of Korea[2]

Abstract

TB-500 is a preparation of seven peptides prepared from the active site of thymosin β4. Thymosin β4 has effects on tissue regeneration, anti-inflammation, and fast repair capabilities with potential of abuse in athletes. TB-500 misuse was found in equine sports, and is known to be used illegally in athletes. In this study, we aimed to find out new metabolites of TB-500 peptide in various *in-vitro* enzyme systems such as human kidney microsome, rat liver microsome, rat liver cytosol, rat liver S9, human serum, and human skin S9. The metabolic reaction was conducted after prolonged incubation (22 hrs) with various enzymes and the metabolites were analyzed in a full-scan mode by liquid chromatography coupled with MS/MS (Q-Exactive). As the results, human kidney microsome and rat liver microsome systems showed higher concentration of metabolites than the rest of enzymes. We found *N*-acetylleucine (Acetyl-Leu-OH; m/z 174) as a new metabolite. The new metabolite, *N*-acetylleucine (Acetyl-Leu-OH; m/z 174), was also found in all enzyme systems used. The other metabolites such as acetyl-Leu-Lys-Lys-Thr-Glu (*m/z* 660), acetyl-Leu-Lys (*m/z* 302), and acetyl-Leu-Lys-Lys (*m/z* 430) were detected. We suggest, that human kidney microsome or rat liver microsome systems are better enzyme systems for TB-500 metabolism studies than cytosol, S9 and serum. We report for the first time *N*-acetylleucine as a metabolite of TB-500. Therefore, *N*-acetylleucine detection in serum and urine also can be a useful marker of detecting TB-500 abuse in doping.

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

In vitro phase I metabolism study of the diuretic clopamide by LC-MS/MS

Doping Control Center, Korea Institute of Science and Technology, Seoul, Republic of Korea[1];
Division of Biomedical Science & Technology, KIST-School, Korea University of Science and Technology, Seoul, Republic of Korea[2]

Abstract

Diuretics are often illegally used in different kinds of sports disciplines, and clopamide is included in the list of prohibited substances of the World Anti-Doping Agency. The objective of this study was to investigate clopamide metabolites from *in vitro* enzyme system using both negative ionization ($[M-H]^-$, *m/z* 344) and positive ionization ($[M+H]^+$, *m/z* 346), as its metabolism study was not reported yet.

In this study, the phase I metabolites were generated through an *in vitro* enzyme system with rat liver microsomes, and identified by using both negative and positive ionization modes of an ultra high-performance liquid chromatography/Orbitrap mass spectrometer (Q-Exactive). A full scan and *dd*-MS/MS mode were used to obtain structural information of the metabolites.

We have characterized 3 mono-hydroxylated ($[M-H+16]^-$, *m/z* 360) metabolites and 2 dehydrogenated metabolites ($[M-H-2]^-$, *m/z* 342) at collision energy of 40 eV in negative mode. In case of positive ionization, 3 mono-hydroxylated metabolites ($[M+H+16]^+$, m/z 362), and 2 dehydrogenated metabolites ($[M+H-2]^-$, *m/z* 344) were found at collision energy of 35 eV, based on their structures and mass spectra. These metabolites could be useful for further *in vivo* metabolites identification with the purpose of anti-doping analysis. The further *in vitro* phase II and *in vivo* studies are progressing to identify more metabolites as potential biomarkers for anti-doping analysis.

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Pettersson-Bohlin K, Oesterlind L, Börjesson A, Ekström L

Bis-(4-fluouphenyl)-methylpiperazine detection in urine from female and male Caucasians after oral lomerizine intake

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Abstract

The Japanese migraine medicine Migsis contains the non-prohibited substance lomerizine which metabolizes to the prohibited substance trimetazidine. In order to be able to distinguish between intake of lomerizine and trimetazidine in doping control samples, specific lomerizine metabolites are of importance. Okano et al studied the detection window of the lomerizine metabolite bis-(4-fluouphenyl)-methylpiperazine (M6) in Asians and found that M6 is detectable up to 276 h after administration. Due to the long detection window, M6 is the most useful lomerizine metabolite in order to differentiate lomerizine from trimetazidine intake. In order to study the metabolism of lomerizine in the Caucasian population five females and five males were given an oral dose of 5 mg lomerizine and followed for 144 h. The samples were analyzed by LC-HRMS on a Q Exactive in PRM mode after SPE clean-up using Chromabond columns (HR-XC).

Result: The lomerizine metabolite M6 was detectable for at least 144 h in all Caucasian samples which is in line with the previous study performed by the Japanese group.

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Chiral analysis of amphetamine and methamphetamine with Mosher derivatization by LC-MS^2

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Abstract

Stimulants are one of the most used classes in sport doping, second only to anabolic agents and diuretics. Among the stimulants used, amphetamine and methamphetamine are the most used substances in this class, being prohibited only in competition. Thus, the determination in biological specimens is a main issue in forensic toxicology and in antidoping analysis. It is important to distinguish between *d*- and *l*-isomers of amphetamine and methamphetamine due to their distinction in illicit or legal nonprescription use, as the enantiomeric analysis can allow to draw conclusion with respect to the origin of the stimulant. Besides that, the *l*-enantiomers of amphetamine and methamphetamine have five times more psychostimulant activity than the *d*-enantiomers. Because enantiomers have the same physical properties, they are not separated by chromatographic methods without derivatization. Generally, to distinguish between the *d* and *l* isomers of amphetamine and its derivatives, chiral reagent derivatization with subsequent GC-MS analysis is used. By selecting an optically pure reagent such as (R)-(-)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (R-MTPAC, Mosher), the isomers present can be chromatographically separated while maintaining the isomeric composition of the starting compounds, without racemization. In LBCD, most stimulant confirmation analyses in doping control are performed by LC-MS^2. The analyses of small stimulant molecules such as amphetamine are difficult by this technique without derivatization. Thus, the objective of this work was to develop a LC-MS^2 method for amphetamine and methamphetamine detection by Mosher derivatization. The sample preparation procedure consists of a liquid-liquid urine extraction using hexane and Mosher reagent in alkaline medium. The analytes are concentrated by evaporation of the solvent and dissolved in mobile phase water:methanol (7:3) with 0.1% formic acid and 5 mM ammonium formate. Initially, the LC method used had 15% of mobile phase B (methanol with 0.1% formic acid) in the initial mobile phase gradient, with a total of 20 minutes of chromatographic run. Using this mobile phase gradient, the separation of the R and S enantiomers was achieved, but the analytes left at the end of the chromatographic run, and methamphetamine was detected already in the return to the initial gradient (S/R-amphetamine RT 16. 7 and 16.9 and S/R-methamphetamine RT 17.9 and 18.2). Thus, the initial ratio of B was increased to 30% and the total running time extended to 30 minutes. Under these conditions, amphetamine and methamphetamine were detected at appropriate retention times around the middle of the chromatographic run (S/R-amphetamine RT 16.6 and 16.9 and S/R-methamphetamine RT 18.5 and 18.9). The analyses were performed in a triple quadrupole (QqQ) using 20V of collision energy for both compounds. The fragments for amphetamine and methamphetamine were *m/z* 91, 202 and 119 and 148, 216 and 119, respectively. The developed method was validated in relation to the parameters of selectivity, repeatability, capacity and limit of identification, robustness and carryover based on WADA ISL 2019. The developed method

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was used for amphetamine confirmation in a real athlete's urine sample, where amphetamine was detected below 50 ng/mL and the TD2015IDCR criteria were met. Thus, this procedure allowed the identification of amphetamine and methamphetamine enantiomers in human urine by liquid chromatographic separation using the Mosher chiral reagent.

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Quantification of intact human IGF-I in serum by LC-MS/MS

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Abstract

Background:

Insulin-like growth factor-I (IGF-I) is one of the biomarkers used to detect human growth hormone administration in GH Biomarkers method. Mass spectrometry-based approaches rely on trypsin digestion of IGF-I which may extend the operation time. In order to simplify operational processes, a simplified quantification method of intact human IGF-I in serum by LC-MS/MS was developed.

Methods:

IGF-I was extracted from human serum samples by acetonitrile precipitation. Separation was achieved via UPLC and MS analysis was conducted by triple-quadrupole mass spectrometry (ABI5500). Stable-isotope-labelled IGF-I was added as internal standard.

Results and conclusions:

A six-point calibration curve ranging from 50-1200 ng/mL of human IGF-I in rat serum was used to establish instrument response. The LOQ was 50 ng/mL with Sr lower than 10% and Sw lower than 15%. The parameters of specificity, capability, linearity, Sr, Sw, LOQ, robustness and bias were validated according to the Guidelines and other requirements. A quantitative method was developed for quantification of intact IGF-I in human serum samples. Further investigate should be carried out to evaluate the correlation of the method with other IGF-I testing methods.

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MS Behavior of Intact Phase-II Metabolites of Anabolic Androgenic Steroids after Derivatization for their Detection by LC/(MS)MS

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Abstract

Phase-II metabolism of anabolic androgenic steroids (AAS) is important for their excretion from the human body as the highly non-polar compounds are converted to easily excreted forms in urine mainly as glucuronated and sulfated metabolites [1]. While detection of intact Phase-II metabolites through LC/(MS)MS analysis is straight forward in negative ionization mode, their Collision Induced Dissociation (CID) patterns usually provide poor product ion mass spectra with ions that are related mainly or exclusively to the glucuronide or sulfate moiety. Derivatization can be considered as an alternative for the development of confirmation methods of intact AAS Phase-II metabolites producing intense molecular ions' signals with CID fragmentation that offers a number of ions with significant diagnostic value for the steroid backbone [2,3].

The objective of the present work was the study of three different derivatization reagents, i.e. Girard Reagent T (GRT), methoxamine and hydroxylamine, which were tested against androsterone glucuronide and androsterone sulphate as pilot compounds. The selection of the most suitable derivatization reagent was made taking into account the sensitivity enhancement on the detection of the pilot compounds, in positive ionization mode, compared (in arbitrary units) to its non-derivatized counterparts in negative mode and the evaluation of their product ion mass spectra in different collision energies (CE).

GRT was the preferred derivatization reagent and, subsequently, 17 synthesized sulfo-conjugated metabolites of AAS were studied by LC/MS after their GRT-derivatization. Their CID patterns were studied in order to reveal fragmentation patterns fulfilling the identification criteria of the World Anti-Doping Agency (WADA) relative technical document TD2015IDCR [4]. ESI signal enhancement using GRT derivatization reagent was at least 10-fold for androsterone glucuronide and androsterone sulphate derivatives in relation to their non-derivatized counterparts in negative mode (arbitrary units). The product ion mass spectra were also informative compared to their non-conjugated analogues giving abundant signals of protonated molecular ions [M^+] as well as ions with high CID diagnostic value related to the steroidal backbone. The CID fragmentation of the studied 17α-/17β-hydroxy isomers provided differences in main ions contributing to their effective identification as observed for testosterone sulfate and epitestosterone sulfate. The 3α-/3β- as well as the 5α-/5β-isomers of the studied AAS resulted mainly in identical mass spectra but differences in ion ratios were observed indicating the importance of their chromatographic resolution.

The present study revealed ions of AAS Phase-II GRT-derivatives that can be deemed useful for the structural elucidation of unknown steroid metabolites as different ions of 17α-/17β-isomers as well as

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different ion ratios of 3α-/3β- and 5α-/5β-isomers were observed in the studied compounds.

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Implementation of the HIF activator IOX-2 in routine doping controls – pilot study data

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Abstract

Hypoxia-inducible factor (HIF) activators are prohibited both in- and out-of-competition. A variety of drug candidates has been closely monitored in sports drug testing since 2012, and whilst clinical approval was completed so far only for one substance (roxadustat) in 2019, various adverse analytical findings were reported since 2015 for different HIF activators. One of the patented drug candidates identified in the context of preventive doping research in 2012 was detected early 2020 in a racehorse doping control sample, referred to as IOX-2, and the testing capability of human routine sports drug testing methods was revisited concerning this newly surfaced doping agent. By association (and elemental composition), the co-detection of IOX-2 and roxadustat as intact and unconjugated analyte using identical precursor/product ion pairs was utilized and since 2015, no adverse analytical findings were recorded. However, to date no metabolic reactions were assessed.

Roxadustat and IOX-2 exhibit identical sum formulae of $C_{19}H_{16}N_2O_5$, and the fact that the structurally related pharmacophores both comprise a glycineamide side chain suggested similar collision-induced dissociation behaviors as reported in 2017 and corroborated in this study. The monitoring of diagnostic product ions such as *m/z* 307, 296, and 278 enabled the detection of intact IOX-2 alongside intact roxadustat in LC-DIA-HRMS routine analyses, with both analytes separated in retention time by approximately 0.5 min. In addition to the unmodified intact substance of IOX-2, also its hydroxylated analog and the corresponding glucuronic acid conjugates were detected in post-administration urine samples, supporting both initial testing and confirmation procedures in routine doping controls.

IOX-2 can adequately be covered in routine doping controls by targeting the intact drug and its hydroxylated metabolite, and the capability of the combined detection of IOX-2 and roxadustat was demonstrated. Further studies are indicated in order to complement the pattern of urinary metabolites and their utility in terms of enhanced retrospectivity.

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