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# RECENT ADVANCES IN DOPING ANALYSIS (33)

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# Online 2D-LC with multiple heartcutting for urinary steroid purification prior to isotope ratio mass spectrometry in anti-doping

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## Abstract

To determine the isotope ratios or urinary steroids, the compounds need to be purified extensively prior to GC/C/IRMS analysis. The required purity is generally achieved by semi-preparative liquid chromatography. Currently, the Swiss laboratory for Doping Analysis (LAD) employs a method that uses a two-step purification. To increase automation and throughput, a semi-preparative method for online 2D-LC with multiple heartcutting was developed for the purification of urinary steroids. Positive and negative quality control samples that were purified with this method had purity and isotope ratios that were comparable with the routine method. Due to instrumental limitations, the robustness of the method was not yet sufficient for a routine application. Nonetheless, the approach seems promising and could be improved with future technical advances.

## Introduction

The detection of doping with endogenous steroids like testosterone (T) in urine currently requires the determination of carbon isotope ratios (CIR) by GC/C/IRMS. The CIR of T and its metabolites are compared to endogenous steroids that would not be influenced by the administration of anabolic steroids. Significant differences indicate an exogenous origin of the steroids in question.
However, the GC/C/IRMS analysis requires extensive sample preparation, which is generally achieved by semi-preparative liquid chromatography. The method currently employed at the LAD could be described as offline 2D-LC with multiple heartcutting, since three fractions are transferred to the second dimension, creating orthogonality through acetylation. Other anti-doping laboratories that use online 2D-LC for sample purification, use heartcutting 2D-LC of either acetylated [1] or underivatized [2] steroids, where some steroids are transferred to the second dimension and others are already sufficiently clean after the first dimension. Since not all fractions enter the second dimension, this could lead to unequal purification of some substances. Furthermore, some collected fractions contain more than one steroid, typically the 5α/5β-isomers. Mixed fractions could be an issue for samples with a wide and different ranges of concentrations of the substances of interest, as the linear range of the IRMS should be reached for all substances to guarantee accurate measurements.
Therefore, we wanted to address some of the mentioned shortcomings by developing a method for the purification of urine samples prior to GC/C/IRMS that uses online 2D-HPLC with multiple heartcutting.

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## Experimental

Positive and negative quality control samples were used for this study. Steroids were extracted from urine samples according to the routine protocol for GC/C/IRMS analysis as described in the literature [3]. Briefly, the urine sample (10 mL) underwent a solid phase extraction, enzymatic hydrolysis and liquid-liquid extraction before the whole extract (30 µL) was subjected to semi-preparative chromatography.

For the semi-preparative online 2D-LC with multiple heartcutting an Agilent 1260 Infinity II system was used. The setup included a 1260 injector, a 1260 binary pump (first dimension), a 1260 column oven, a 1290 2D-LC valve with two parking decks and 12 loops (120 µL volume), a 1290 high speed pump (second dimension), two 1260 diode array detectors, and a 1200 fraction collector.

The $^{1}$D-separation was performed on a Zorbax Eclipse XDB-C18 (300 x 1.0 mm, 3.5 µm) at 50°C using pure water (A) and ACN (B) as mobile phases. The initial conditions (33% B and 0.05 mL/min) were kept for 16 minutes, the proportion of B was then increased to 42% over 28.6 minutes, followed by an increase to 50% over 12.2 minutes and an increase to 98% over 8.2 minutes. The mobile phase composition was kept at 98% B and the flow rate was increased to 0.15 mL/min over 15 minutes before the mobile phase was set to the initial composition of 33%. Over the next 20 minutes the flow rate was reduced to the initial 0.05 mL/min and was kept there for equilibration at the initial conditions for additional 20 minutes, resulting in a total gradient time of 120 minutes.

The $^{2}$D-separation was performed on a Zorbax SB-CN column (50 x 4.6 mm, 3.5 µm) at 50°C using pure water (A) and ACN (B) as mobile phases at a flow rate of 2.5 mL/min. The $^{2}$D-separation consisted of an analytical and a flush gradient. For the analytical gradient, the initial proportion of 10% B was increased over 0.5 minutes to the isocratic conditions and held at those conditions for 4.5 minutes. Afterwards, the organic proportion was increased over 0.5 minutes to 98% B, held there for 0.5 minutes before 0.5 minutes of re-equilibration at the initial mobile phase composition of 10% B. The isocratic step for 11-O-Etio was set at 21% B, for T at 27% B, for 5βAdiol and 5αAdiol at 29%, for Etio and A at 30% B, for PD at 31% B and for 16EN at 41% B. The flush gradient was kept generic, starting at the initial mobile phase composition of 10% B increasing to 98% B over 1 minute, holding 98% B for 0.5 minutes followed by 0.5 minutes of re-equilibration at the initial conditions.

Fractions containing the aforementioned steroids are collected with a small margin before and afterwards the peak, determined through injection of standards, to ensure that no isotopic fractionation takes place. After evaporation of the solvent in the fractions, the extracts were acetylated and analysed by GC/C/IRMS as described in literature [3].

## Results and Discussion

### Results

During this proof-of-concept test, all collected steroids could be detected in the quality control samples, except 16EN. It was assumed, that this substance completely evaporated after acetylation due to its high volatility. Most isotope ratios of detected steroids were well comparable to routine measurements, falling within a range of ±1‰ of the validated values as shown in Table 1.

Larger differences below 1.5‰ were observed for 11-O-Etio and Etio; they could be attributed to differences in conditions of critical instrument parts (column and oxidation reactor) between the

validation and these measurements. Similarly, the cleanliness of the chromatograms was generally comparable with the routine purification. Except for the fraction containing T of the negative sample, which contained a large contaminating peak eluting directly before T. However, the isotope ratio of T appeared to be not influenced by this contamination.

| | | PD [‰] | A [‰] | Etio [‰] | 5αAdiol [‰] | 5βAdiol [‰] | T [‰] | 11-O-Etio [‰] |
|---|---|---|---|---|---|---|---|---|
| PQC | routine | -23.6 | -28.4 | -28.6 | -29.3 | -28.8 | -30.5 | -24.4 |
| | 2D-LC | -23.3 | -28.4 | -30.0 | -30.1 | -28.4 | -29.9 | -25.6 |
| NQC | routine | -24.0 | -24.0 | -24.8 | -25.1 | -25.0 | -24.3 | -24.6 |
| | 2D-LC | -23.9 | -23.2 | -23.9 | -25.7 | -25.3 | -23.5 | -23.7 |

**Table 1.** CIR comparison of positive and negative quality control samples purified by either the routine method or the online 2D-LC with multiple heartcutting method

**Discussion**

Online 2D-LC with multiple heartcutting requires a delicate balance between the gradient times of both dimensions. The cuts need to be short enough to fit in a reasonable number of loops and the $^{2}$D-gradient shall be fast enough to empty the occupied loops before the next cuts can be transferred onto the second dimension. The general approach to achieve this balance is the use of a slow gradient and long columns in the first dimension to achieve a good separation of the compounds of interest, while the second dimension employs fast gradients and short columns with an orthogonal separation to remove coeluting interferences.

As the stationary phase has the biggest influence on the separation, different types of columns were evaluated for both chromatographic dimensions. For the $^{1}$D-separation of steroids, only C18-columns were considered, as they are the most capable of separating all steroids of interest. The comparison between a classical C18-column (Zorbax Eclipse C18) and a C18-column with an embedded polar group (XBridge Shield RP18) demonstrated different retention behaviour of the steroids with the two columns using modelled gradients, as shown in Figure 1. On the XBridge Shield RP18, the separation between the 5α- and 5β-isomers of the steroids was excellent, unfortunately this led to other unwanted coelutions, notably 5αAdiol and Etio. The classical C18-column had an overall good separation between all steroids, including isomers and was therefore chosen for further investigations.

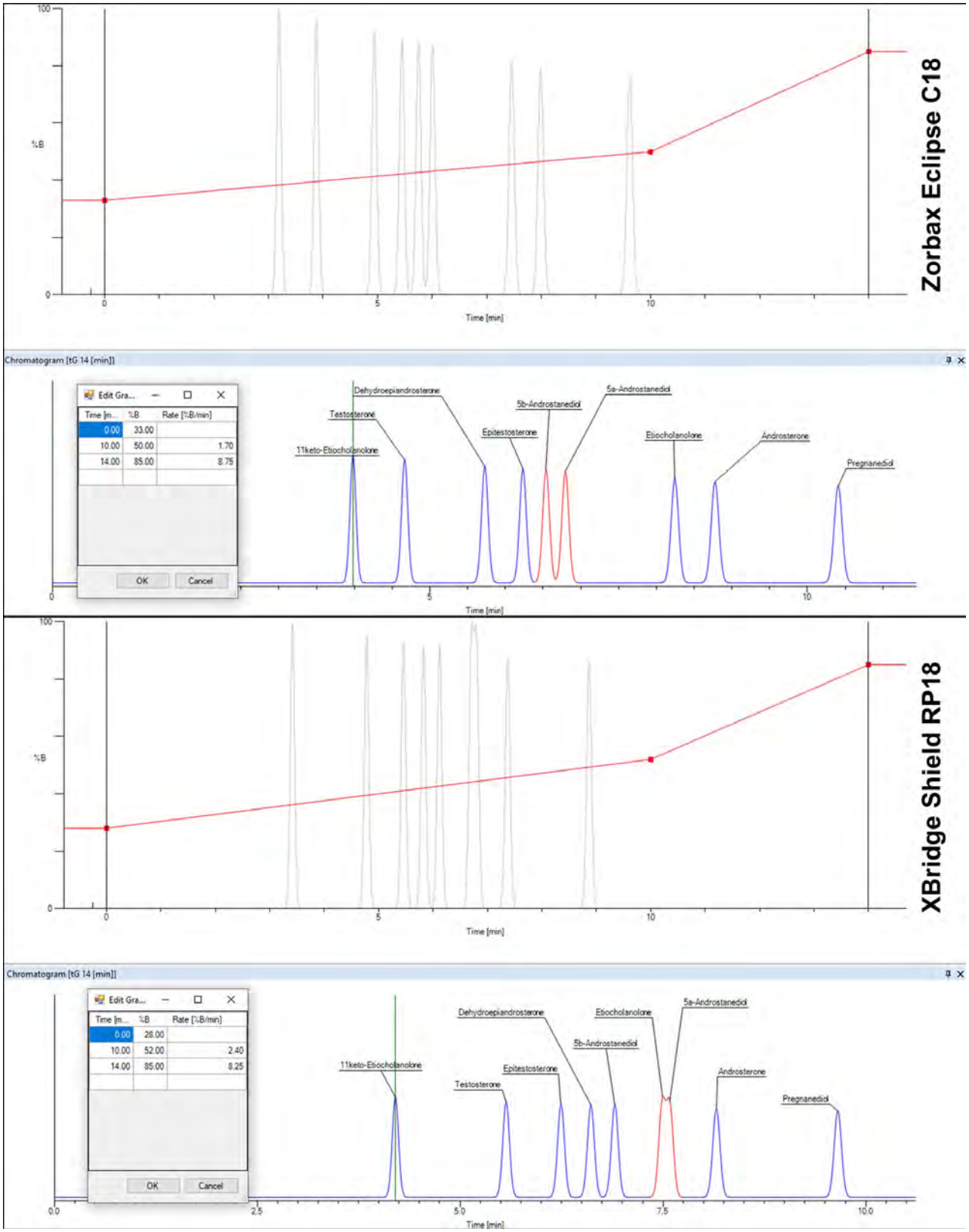


**Figure 1.** Simulated chromatographic gradients for the separation of steroids after computational optimisation on an Zorbax Eclipse C18 column (top) and a XBridge Shield RP18 (bottom)

With the standard conditions for sample purification prior to GC/C/IRMS analysis (columns with 4.6 mm inner diameter and mobile phase flow rates of 1 mL/min), all cuts would have had volumes above 1 mL. As the instrumental and software configuration allowed 12 loops with maximal 180 µL each, the available time for the $^{2}$D-separation would have been too short for adequate separation. To reduce the volume per peak, the gradient was geometrically scaled for a reduced column diameter and mobile phase flowrate, achieving a sufficient separation on a Zorbax Eclipse XDB-C18 (300 x 1.0 mm, 3.5 µm) over 120 minutes as shown in Figure 2.

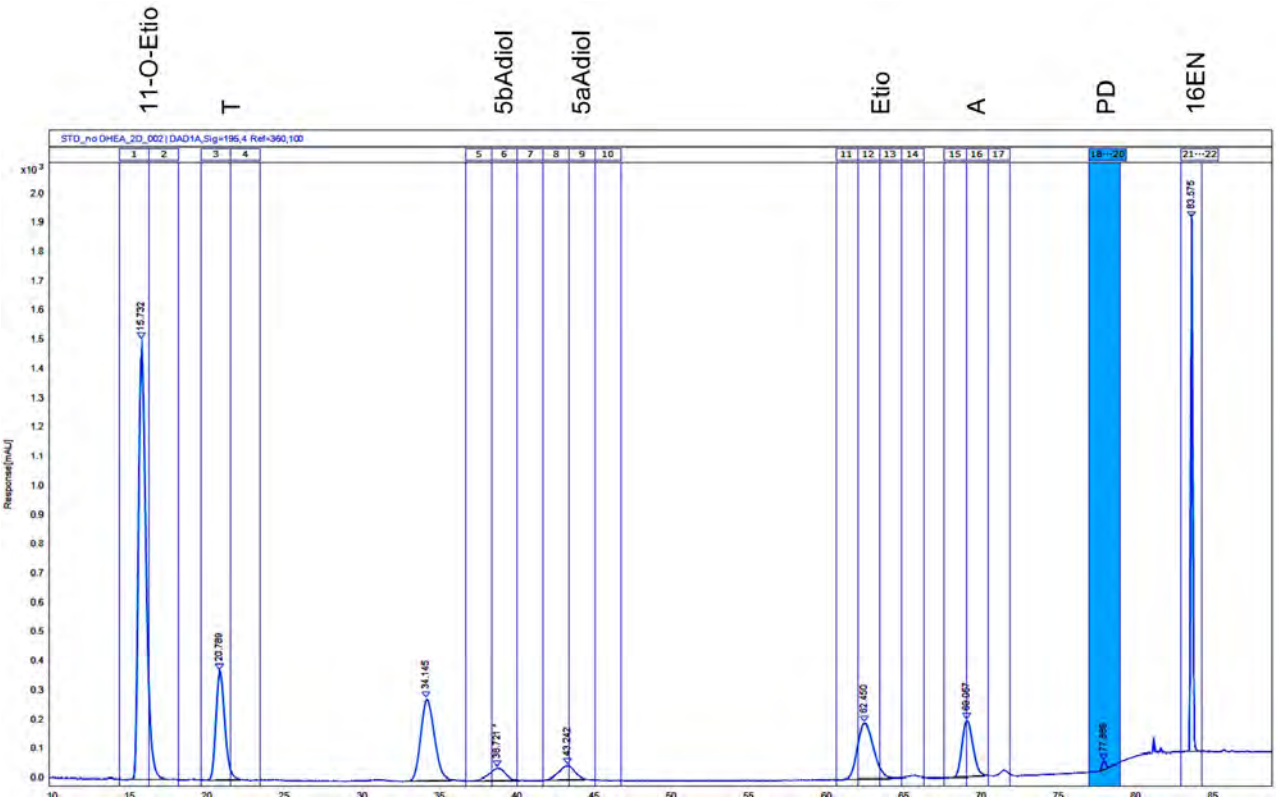


**Figure 2.** Chromatogram of a standard mixture in the first dimension. The transfer windows for the second dimension are indicated by rectangles (here PD fraction highlighted blue) and abbreviations of the corresponding substances are indicated above the chromatogram

For the second dimension, several stationary phases with the same column dimensions (50 x 4.6 mm, 3.5 µm) were evaluated considering orthogonality tests from literature [2]. HILIC (Zorbax HILIC Plus) showed no retention and C18 columns were excluded due to lack of orthogonality. Columns with Phenyl-groups (Zorbax XDB-Phenyl and Zorbax Eclipse Plus Phenyl-Hexyl) were tested but showed no improved separation in comparison to the $^{1}$D-separation alone. For these two stationary phases, the addition of a protic solvent like MeOH to the mobile phase could have led to increased π-π interactions and therefore increased orthogonality but would have also complicated the UV-detection at 195 nm. In the end, a cyano column (Zorbax SB-CN) was chosen, as the purity of the final extracts was better than after only $^{1}$D-purification.

Unfortunately, unstable $^{1}$D retention times, were observed during this project. Considering the column dimensions and the low flow rates, the chosen injection volume of 30 µL was too high for these conditions. But due to the sensitivity of the final GC/C/IRMS analysis, the whole extract has to be purified by semi-preparative LC, and it was not possible to solubilise the extract in less solvent.

A potential solution could be the use of larger loops, as the loop size defines the maximal cut-size. The installation of larger loops would allow the use of columns with larger inner diameter and therefore an increased mobile phase flowrate in the first dimension, which should consequently result in a more stable chromatography. However, the manufacturer only offers loops with a size of up to 180 µL and the software is not capable of adjusting to higher volumes, as the loop size is used to determine automatically the flush times.

## Conclusions

The method presented hereby for preparative online 2D-LC with multiple heartcutting was capable of purifying urinary samples for subsequent GC/C/IRMS analysis. In a proof-of-concept test, positive and negative quality control samples were analysed. The purity of the fractions was slightly worse in comparison with the routine method, especially for the fraction containing T in negative samples. Nonetheless, the determined CIRs were well comparable.

The method in its current state would not be yet robust enough for routine application, because the combination of low flowrates and relatively high injection volumes lead to unpredictable changes of $^{1}$D retention times. However, the use of larger sample storage loops might allow larger columns, higher flow rates and eventually a more robust chromatographic separation.

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# Stability of roxadustat and vadadustat in urine and dried blood spots: What should we be aware of?

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## Abstract

HIF-stabilizers are known to be unstable when exposed to intense UV light and this needs to be considered for sport drug testing. Indeed, urine and DBS samples are shipped at room temperature. Moreover, samples are usually not protected from the light during the sample collection and the sample processing once in the laboratory, including all the steps involved in the sample extraction procedure.
We have investigated the stability of two representative HIF-stabilizers (roxadustat and vadadustat) under the influence of temperature and exposure to light in dried blood spots (DBS) and urine. Temperature up to 37°C and exposure to intense UV light up to 30 min were considered as sufficiently harsh conditions to mimic an authentic scenario. Following exposure to these conditions, samples were analysed by LC-HRMS, acquiring the data in full scan mode to detect any additional species formed.
Vadadustat was stable under the conditions investigated in both urine and DBS. On the contrary roxadustat confirmed its susceptibility to light in urine samples, with the rapid formation of the photoisomer. This was not observed on DBS samples, where the compound remained stable even after a 30 min exposure to an intense light. The temperature had no influence on the stability of roxadustat in either DBS or urine. Finally, a roxadustat elimination urine was exposed to the light to verify whether the glucuronoconjugation might prevent the photoisomerization.

## Introduction

Erythropoiesis-stimulating agents are substances that increase the production of red blood cells by stimulating the bone marrow [1]. Despite being developed as medicaments to treat anaemia, they are known to be misused in sport to improve aerobic performance by increasing oxygen-carrying capacity [2]. Amongst these substances the so-called HIF-stabilizers act by reducing the breakdown of the hypoxia inducible factor (HIF) by the prolyl hydroxylase domain proteins (PHD), thereby enhancing their activity. This process ultimately stimulates red blood cell production mimicking the physiological adaptations observed during prolonged exposure to low oxygen levels (e.g. altitude training).
HIF-stabilizers are known to be unstable under certain conditions. For example, roxadustat degrades to a photoisomer when exposed to the light [3-5]. Potential degradation factors, such as exposure to light or temperature, might occur at various stages during the journey of the samples, from collection to shipping, but also during the different steps of sample preparation in the laboratory.
Moreover, the matrix itself might protect or facilitate the degradation compared to pure solvent. When transferring previously developed methods from urine to alternative matrices, such as the dried blood

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spots, this needs to be considered.

It was the goal of this project to investigate the stability of two HIF-stabilizers (roxadustat and vadadustat) in urine and dried blood spots when exposed to temperature and light conditions that could occur during the transportation of the sample to the lab or during the sample manipulation according to the sample preparation procedure.

## Experimental

The procedures described below were performed at room temperature (about 20 to 22°C) and avoiding the direct exposure to intense light, except for the temperature and light exposure experiments.

Urine sample preparation

Urine samples from 6 different volunteers were spiked with roxadustat and vadadustat at 10 and 1000 ng/mL. Urine samples (1 mL) were treated with 220 µL of a solution containing beta-glucuronidase in acetate buffer (0.5 M, pH 6.3), in the ratio 3/20. Samples were left in a Thermoshaker at 50°C for 60 minutes, then extracted using mixed-mode weak cation exchange sorbent (WCX) (Strata C-CW 30 mg, Phenomenex) in 96-well plate format. The procedure included a conditioning step with 1 mL of methanol followed by 1 mL of water, the sample loading followed by a wash step with 1 mL of 30% methanol in water. The elution was obtained using 1 mL of formic acid 6% in methanol/acetonitrile (75/25). After drying under a nitrogen stream, samples were reconstituted in 150 µL 0.1% formic acid in water/acetonitrile 70/30 and analysed.

DBS sample preparation

Venous blood from 6 different volunteers was collected in EDTA tubes and spiked with roxadustat and vadadustat at 10 and 1000 ng/mL. Samples were spotted (20 µL) onto Whatman protein saver cards and Tasso M20s supports, left to dry overnight at room temperature and then stored in the fridge in the presence of a desiccant until the analysis.

The sample extraction was performed by placing the dried blood spots into a 96-well plate and adding 500 µL of methanol. The plate was placed on a Thermoshaker for 25 minutes at 25°C. Afterwards, the supernatant was collected and transferred into a new 96-well plate, and 100 µL of 0.3M HCl in methanol was added to each sample. The plate was evaporated under nitrogen at 40°C for 30 minutes using a Biotage TurboVap®96 Dual system. Finally, samples were reconstituted by adding 100 µL of mobile phase (0.1% formic acid in water/acetonitrile 70/30; V/V) and analysed.

Sample exposure to temperature and light

For the temperature stability study, urine and DBS samples were placed at 37°C in a Sorvall® oven supplied by Kendro Laboratory Products for 8, 24, 48 and 72 hours. For the UV-VIS light exposure tests, these were performed using an OSRAM Ultra Vitalux 300W 230V E27 for 5, 10, 15 and 30 minutes. These steps were performed on the samples before the extraction procedure. In parallel, samples non exposed to light and temperature were analysed.

Sample analysis by LC-HRMS

LC-HRMS analyses were performed on a Vanquish UHPLC system from Thermo Fisher Scientific

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(Waltham, MA, USA) coupled to a Q-Exactive Plus high-resolution orbitrap mass spectrometer from the same manufacturer. The separation was performed on an Acquity UPLC Cortecs C18+ column (1.6 µm, 2.1 x 100 mm) from Waters (Milford, MA, USA).

Analyses were performed in full scan acquisition mode. Chromatograms were obtained by extracting the theoretical m/z value of each target analyte with an m/z range of ±10 ppm. The chromatographic and MS parameters are summarised in Table 1.

| | | | | | | |
|---|---|---|---|---|---|---|
| LC parameters | Time (min) | A% (0.1% formic acid in water) | B% (0.1% formic acid in ACN) | Flow rate (mL/min) | Column temperature | Injection volume |
| | 0 | 80 | 20 | 0.6 | 60C | 10 µL |
| | 6 | 2 | 98 | | | |
| | 7.42 | 2 | 98 | | | |
| | 7.49 | 80 | 20 | | | |
| | 9 | 80 | 20 | | | |
| | | | | | | |
| MS parameters | Spray voltage | Sheath gas flow | Capillary temperature | Auxiliary gas temperature | RS lens level | Resolution |
| | 3.5 kV | 40 | 320C | 250C | 55 | 35,000 |

**Table 1.** Chromatographic and MS conditions

## Results and Discussion

Temperature and light exposure can occur at any time during the sample journey to the laboratory or during the sample analysis. It is known that HIF stabilizers compounds, and particularly roxadustat, are sensitive and degrade when exposed to intense light [4].

Our preliminary tests confirmed that roxadustat in pure solvent degrades to the corresponding photoisomer when exposed to the light for a time as short as 5 minutes. Vadadustat under the same conditions was more stable, but the formation of a degradation product at *m/z* 263.0581 (compatible with a decarboxylation of the compound) was observed after a 30 min exposure to an intense light.

Less is known regarding the stability of these compounds when exposed to relatively high temperatures, that can occur especially when the samples come from hot countries and travel for several days before reaching the laboratory.

Finally, the different matrices (urine, DBS) might have the effect of facilitating the degradation or protecting the compounds compared to pure solvents, and this requires further investigation.

Urine and DBS samples were spiked with vadadustat and roxadustat at different concentrations. They were subsequently exposed to temperature (37°C) up to 72 hours or intense light up to 30 minutes. To estimate the degradation, identical reference samples were prepared in parallel and not exposed to temperature nor UV light. The peak areas obtained from the analysis of the reference samples were compared to those obtained from the analysis of the forced degradation samples.

Effect of the temperature

Urine and DBS samples were placed in an oven (37°C) for varying periods of time up to 72 hours. We believe that these conditions would represent a possible scenario during sample transportation, in those cases where samples remain in hot conditions a few days before reaching the laboratory.

At the end of the forced degradation procedure, all samples including the non-degraded reference samples were extracted and analysed according to the applicable procedure for urine or DBS.

The results of normalized peak areas for roxadustat and vadadustat in urine samples and Whatman cards DBS are summarised in Figure 1.

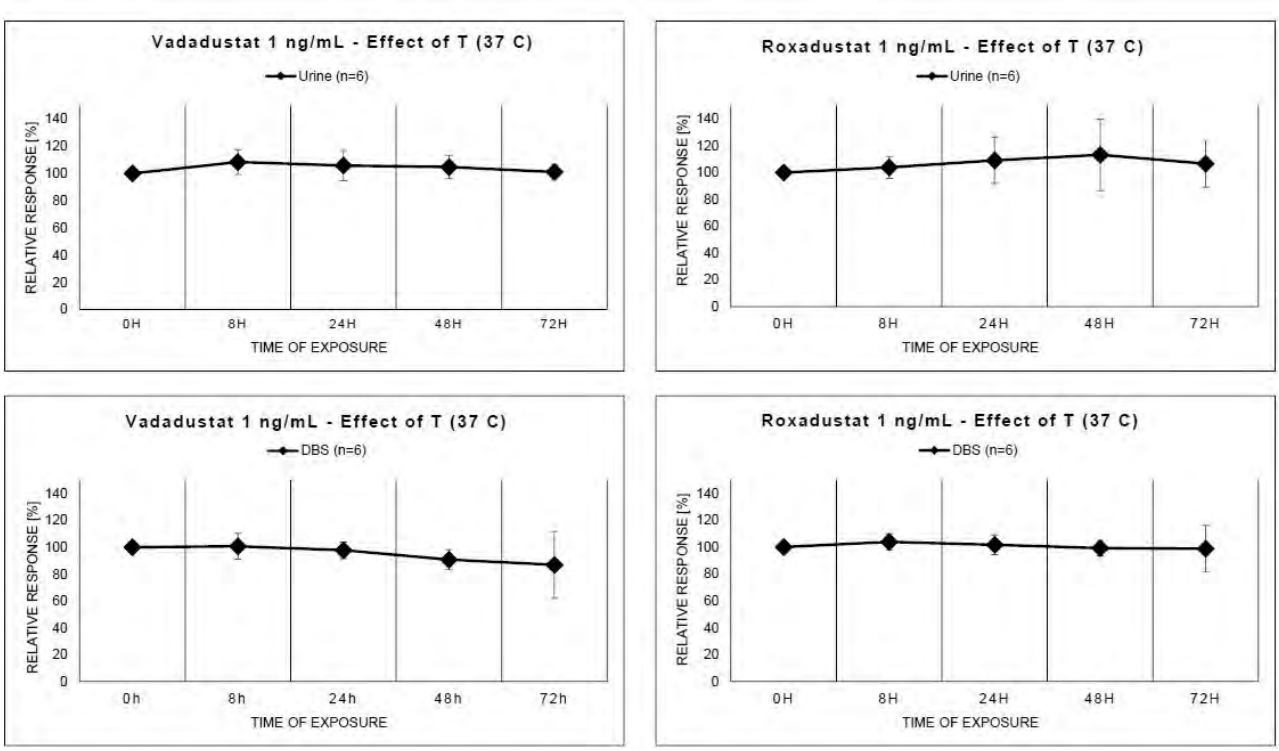


**Figure 1.** Effect of the prolonged exposure to temperature (37°C) on the stability of vadadustat and roxadustat in urine (top) and DBS (bottom). Data are reported as the average of 6 samples, expressed as the peak area relative to the reference sample (0h exposure)

The 0h exposure time corresponds to the reference sample. Roxadustat and Vadadustat were stable for up to 72 hours at 37°C in all the samples investigated. Longer exposure times or higher temperatures were not investigated as in our opinion, these would have been an implausible scenario for a doping control sample.

Effect of the exposure to light

To investigate the effect of the light on the stability of the target compounds, samples (urine and DBS) were placed under the light produced by an OSRAM Ultra Vitalux lamp. According to the manufacturer description, this lamp should produce a relatively strong radiation not only in the visible region, but also in the UVB (3.0 W) and UVA (13.6 W) region, thus mimicking an intense direct sunlight. Samples were placed under the lamp for 5, 10, 15 and 30 minutes. In parallel, reference samples were prepared and maintained protected from the light until the sample extraction. All samples were extracted and analysed as described above.

The results obtained are summarised in Figure 2, that reports the normalised peak area values for the reference samples (0’ exposure) and the forced degradation samples.

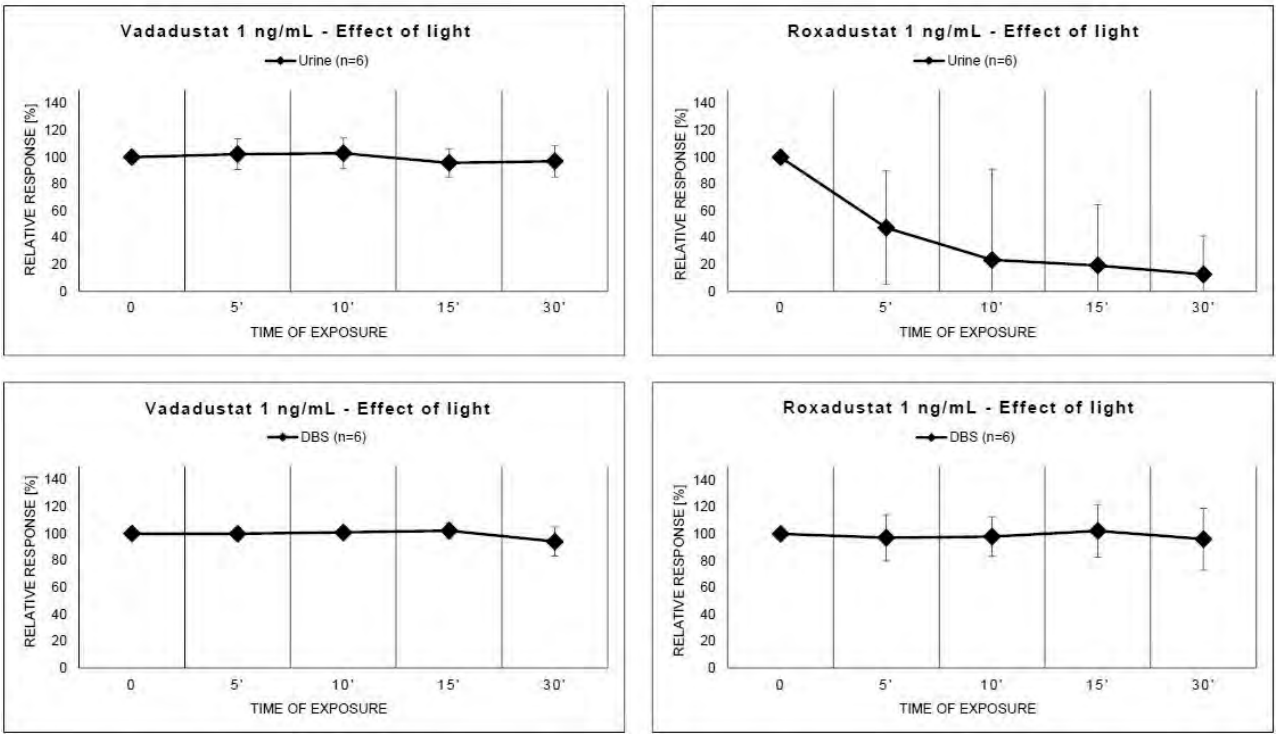


**Figure 2.** Effect of the exposure to intense UV-Vis light on the stability of vadadustat and roxadustat in urine (top) and DBS (bottom). Data are reported as the average of 6 samples, expressed as the peak area relative to the reference sample (0 min exposure)

In urine, vadadustat appears stable while roxadustat rapidly degrades. The formation of the photoisomer observed in urine samples was inversely proportional to the disappearance of the parent compound (Figure 3), supporting the hypothesis that the two compounds are directly related.

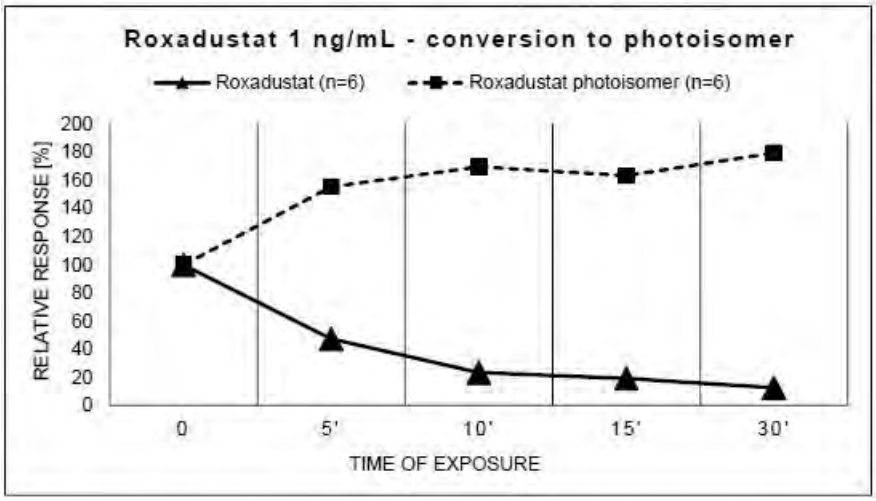


**Figure 3.** Degradation of roxadustat and formation of the photoisomer under exposure to UV-Vis light. Data are reported as the average of 6 samples, expressed as the peak area relative to the reference sample (0 min exposure)

In DBS samples, on the contrary, both compounds appear stable, suggesting that the matrix might shield somehow the compounds from the action of the light. To verify whether the effect was actually due to the matrix and not to the fact the DBS are dried or that the support might prevent the light from hitting the compound, roxadustat samples were prepared in pure solvents and spotted onto DBS (both cards and Tasso), to mimic DBS samples without the biological matrix. It was observed that in the absence of the matrix and even under these DBS-like conditions roxadustat was unstable when exposed to the light (Figure 4), demonstrating that the protective effect is, indeed, due to the blood matrix.

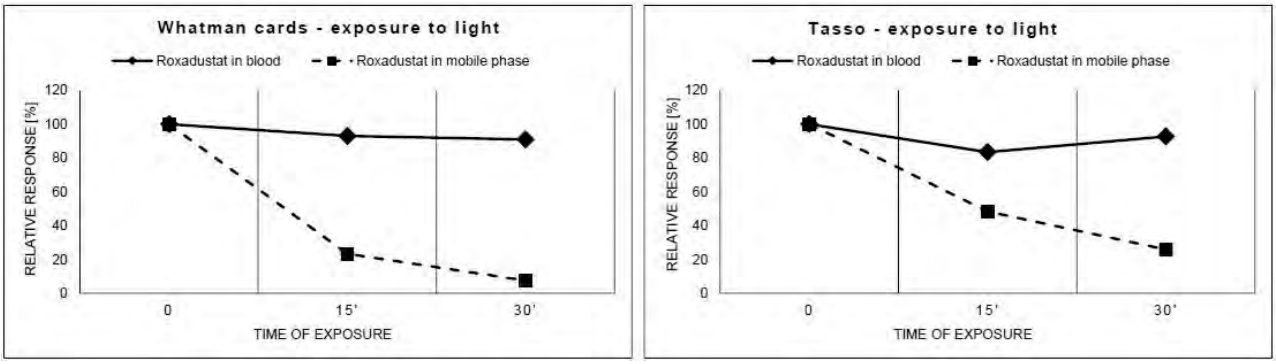


**Figure 4.** Effect of the UV-Vis light on roxadustat spotted on DBS supports and in the presence or absence of the blood matrix

Analysis of an authentic sample

In human metabolism, roxadustat is eliminated at least to some extent as glucuronoconjugated. This might stabilise the compound preventing the photodegradation. To investigate this possibility, an authentic excretion urine sample after oral roxadustat administration was analysed with a modified procedure involving either direct injection of a diluted sample, or the diluted total fraction resulting from enzymatic hydrolysis using beta-glucuronidase. Furthermore, additional aliquots of the same sample were exposed to the UV light at different stages of the procedure and precisely before or after the deconjugation. Results are summarised in Figure 5. The presence of the glucuronide moiety seems to protect the compound from photodegradation, but once the conjugation is removed roxadustat is quickly degraded.

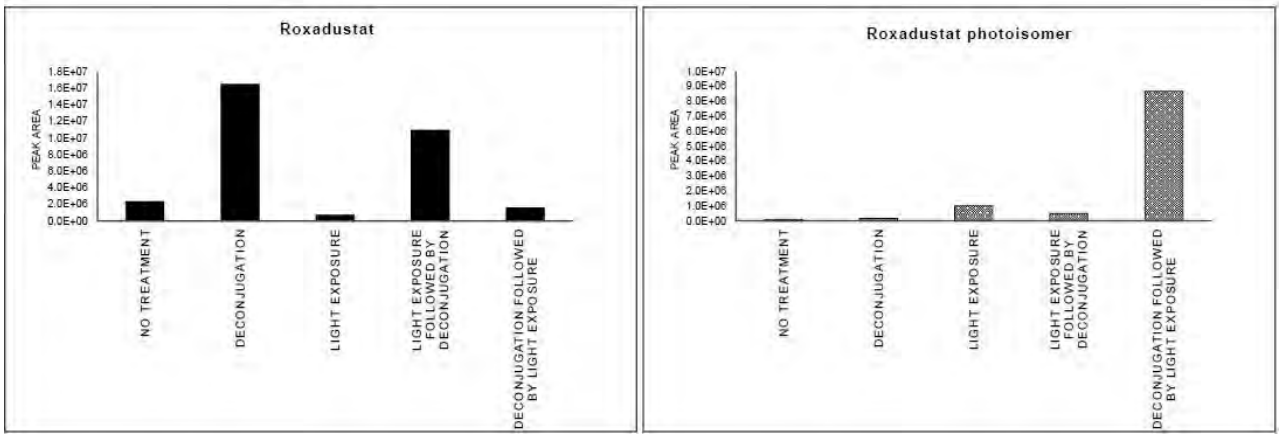


**Figure 5.** Roxadustat photodegradation in a real sample: protective effect of the glucuronoconjugation

## Conclusions

We have investigated the stability of roxadustat and vadadustat in urine and DBS samples when exposed to relatively high temperature and strong light.

While vadadustat appears stable, roxadustat rapidly degrades in urines when exposed to the light. The presence of a blood matrix (DBS) has a protective effect on the degradation even after a 30 min exposure to the light.

Our study demonstrates the importance to operate carefully by protecting the samples from intense light in the laboratory. This is particularly relevant for urine samples, but also for DBSs for example once the matrix is removed during the sample extraction and the compound is no longer protected from degradation. We also advise monitoring the roxadustat degradation product for doping control.

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# The Laboratory on the Screen Approach: Real-time integration in an anti-doping laboratory - The Brazilian experience

Bazilian Doping Control Laboratory - LBCD, Rio de Janeiro, Brazil

## Abstract

Technological advancements have significantly improved professional practices, especially within antidoping laboratories. This paper presents the "Laboratory on the Screen" (LoS) developed by the Brazilian Doping Control Laboratory (LBCD). LoS integrates analytical and administrative sectors using strategically placed visual screens and web technologies, enabling continuous monitoring and real-time visualization. This solution has substantially enhanced operational efficiency, reducing daily analyst workload and supporting compliance with strict antidoping guidelines.

## Introduction

Technological advances significantly transform professional practices, particularly in antidoping laboratories, where stringent timelines and large sample volumes demand efficient data management solutions. Laboratory Information Management Systems (LIMS) have emerged as indispensable tools, improving workflow integration and analytical efficiency. Herein, we discuss the "Laboratory on the Screen" (LoS) initiative, developed by the Brazilian Doping Control Laboratory (LBCD), aimed at real-time integration and streamlined operational management [1,2].

## Experimental

The LoS integrates analytical and administrative departments through strategically positioned visual displays connected directly to the LIMS. This integration is achieved using SQL databases, web technologies (ASP.NET, C#), and front-end frameworks (HTML, JavaScript, Bootstrap). Spanning 2,000 m² across two floors and encompassing 10 different rooms or laboratories, the system provides department-specific, real-time information on critical aspects of laboratory workflow, including sample reception, aliquoting, analytical status, result management, and overall laboratory management. The method is illustrated in the Figure 1.

Each of the 10 screens installed in critical operational areas offers tailored data. In the reception and aliquot preparation area, metrics such as sample receipt anomalies, aliquot status, and document tracking are clearly visible. Analytical screens provide real-time equipment statuses, calibration reminders, and reporting deadlines, promoting immediate intervention capabilities. Presumptive findings, particularly from GC-MS analyses, are disseminated instantly across relevant departments, aiding in comprehensive decisionmaking.

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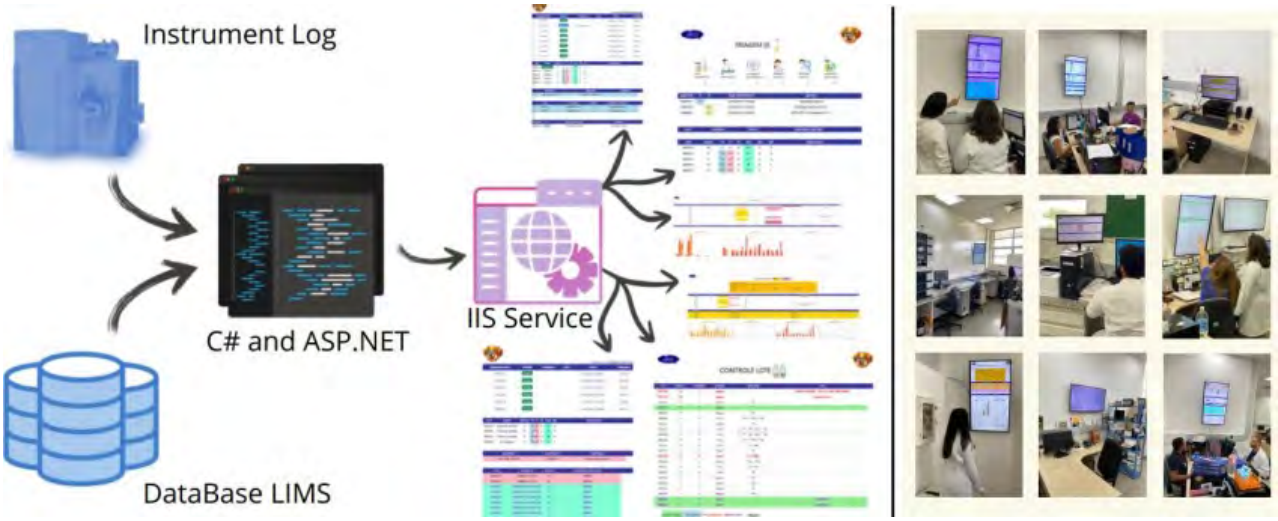


**Figure 1.** Laboratory implementation method

## Results and Discussion

The LoS initiative substantially enhanced operational productivity by eliminating redundant workflow steps and consolidating fragmented communications into a unified visual platform. In the results-interpretation department alone, the system generated daily time savings of up to one hour per analyst, contributing to measurable improvements in task throughput. Over three years, LoS supported the management of approximately 18,000 samples, demonstrating robustness and stability in a high-demand analytical environment.

Beyond time savings, preliminary quantitative indicators suggest broader performance gains. Internal monitoring revealed reductions in manual transcription events and sample-routing discrepancies, reflecting improved process reliability. Although a full audit-level assessment is ongoing, initial compliance checks indicate more consistent adherence to turnaround-time targets during periods of high workload, particularly surrounding major sporting events.

Human-factor aspects also played a critical role in the system's successful adoption. Analysts and administrative staff reported rapid adaptation due to the intuitive interface and task-oriented dashboards. Short structured training sessions (typically under two hours) were sufficient for full operational use, and informal feedback collected across departments indicated increased confidence in task monitoring, reduced cognitive load, and higher perceived coordination between teams. These elements collectively reinforce the practical feasibility of implementing LoS in other laboratory contexts.

## Conclusions

LoS exemplifies effective technological integration within antidoping laboratories, optimizing resources, strengthening operational control, and supporting compliance with stringent turnaround requirements. Its real-time, cross-departmental visibility has generated measurable productivity gains while also improving workflow reliability and user experience. The combination of technical performance, positive human-factor outcomes, and minimal training needs demonstrates the system's maturity and scalability.

As such, LoS represents a compelling model for laboratories seeking to modernize operations through digital transformation and structured process integration.

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# The DopinGPT: Machine learning with an intelligent chatbot integrated with WhatsApp for knowledge storage in doping control

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## Abstract

Technological innovations are based on information management, work practices, and design in the use of technologies [1]. Although anti-doping professionals yearn for technological advances in the analytical area, the use of practical solutions such as those available on cell phones can also facilitate work in laboratories. One increasing challenge in routine lab work is the volume of documents (ISL, TDs, TLs, TNs, Guidelines) made available by WADA. Their quantity and diversity make continuous consultation a common practice, impacting decision-making speed. To streamline this, LBCD developed DopinGPT, an intelligent system using Large Language Models (LLMs) and a Retrieval-Augmented Generation (RAG) mechanism. DopinGPT was designed to provide intelligent access to WADA documents via WhatsApp. The system operates with two access levels: Community (open WADA documents) and Enterprise (internal/confidential documents). The tool ensures traceability and efficiency in information retrieval, enabling analysts to resolve doubts quickly without manually searching documents. DopinGPT has shown promising performance in preliminary testing and is positioned for future multilingual and secure deployments.

## Introduction

Technological innovations have significantly advanced various scientific fields worldwide [1], including anti-doping, where the implementation of a Brazilian solution provides the anti-doping community with a tool designed to optimize time and streamline access to information from documents governing WADA-accredited laboratories. To address this challenge, LBCD has developed DopinGPT, an intelligent system leveraging large language models (LLMs) [2] and a Retrieval-Augmented Generation (RAG) mechanism [3]. DopinGPT enhances accessibility and efficiency in laboratory workflows by storing, updating, and intelligently providing access to WADA documents. This machine learning-based approach ensures accurate and efficient information retrieval, tailored to the needs of laboratory professionals.

## Experimental

DopinGPT integrates a LLM with WhatsApp via API to support anti-doping professionals. Two knowledge streams power the system:

- Community Layer: Public documents such as the ISL, TDs, TLs, and the 2025 Prohibited List are ingested into the LLM for general guidance.
- Enterprise Layer: Confidential internal documents (e.g., technical notes, EQAS results) are securely

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processed and used by a protected LLM instance. In this version, secure access is enforced by restricting usage to pre-authorized phone numbers, ensuring that only verified users can interact with the chatbot via WhatsApp. Access events can be logged for traceability, and communication is protected by WhatsApp's end-to-end encryption.

This dual-layer approach enables fast, secure, and context-aware responses to doping-related queries through WhatsApp.

In the initial phase, 38 public WADA documents were incorporated into the system as part of the Community Layer of DopinGPT. From these, 11 documents were selected, and 38 evaluation questions were developed by a senior doping control analyst, with at least three questions formulated per sampled document. Each response from DopinGPT was evaluated independently by two analysts using a binary scoring system (0 = incorrect/incomplete, 1 = correct/acceptable), following best practices in LLM and chatbot assessment [4-6]. Five parameters were considered: Response Accuracy, reflecting overall correctness; Recall, measuring the inclusion of all relevant content; Citation Accuracy, assessing whether the cited source truly supported the answer; Hallucination Rate, indicating the frequency of unsupported or fabricated information; and Response Time, tracking how quickly the system replied.

## Results and Discussion

DopinGPT's performance was evaluated across five key metrics widely recommended in the assessment of LLMs and conversational agents:

Response Accuracy (86.8%):
The majority of answers were judged as correct and relevant to the question context, demonstrating that the system effectively understood the intent and retrieved appropriate content from WADA documents. This level of accuracy is consistent with initial benchmarks observed in domain-specific chatbots [4].

Citation Accuracy (89.5%):
Most responses included appropriate document references that aligned with the answer content, reinforcing traceability. High citation fidelity is critical in compliance environments, where the presence of supporting evidence is a key success factor [7].

Recall (76.3%):
Recall analysis indicated that some relevant elements were occasionally omitted. This suggests room to optimize retrieval strategies and document chunking, as incomplete answers—while not necessarily incorrect - may lead to operational gaps. Similar trends were reported, where recall variance was notable even in high-performing LLMs [6].

Hallucination Rate (5.3%):
Only a few responses included unverifiable or fabricated content. This rate is within acceptable thresholds for an early-stage domain application [5,6]. Nevertheless, further model fine-tuning and stricter retrieval constraints could help reduce residual hallucinations, especially in contexts requiring high factual precision [4].

Average Response Time (10.12 seconds):

The system generated complete answers quickly, balancing retrieval depth and latency. For end-users, this response time represents a significant efficiency gain compared to traditional manual document searches, which are not only slower but depend heavily on the user's familiarity with WADA documentation structure.

In contrast to manual lookup - which often requires navigating dozens of technical documents and cross-referencing multiple sources - DopinGPT delivere immediate, context-aware responses regardless of the user's experience level. This reinforces the tool's potential to democratize access to regulatory knowledge and reduce cognitive and operational load on analysts [5].

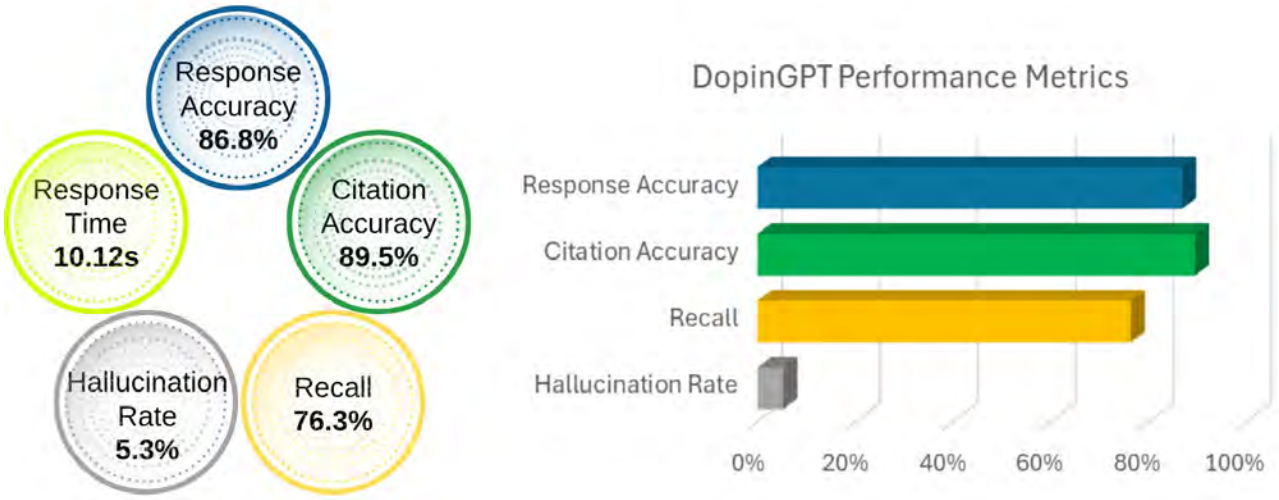


**Figure 1.** Performance Metrics of the DopinGPT Chatbot: This graphic presents key performance indicators used to evaluate the effectiveness of DopinGPT in supporting doping control operations

## Conclusions

DopinGPT integrates LLMs with a RAG mechanism to provide intelligent, contextualized responses based on WADA regulatory documents. By combining machine learning with structured retrieval, it ensures traceability, accuracy, and accessibility, with 38 WADA documents incorporated so far. Validated as an effective consultation tool, DopinGPT streamlines information retrieval and compliance processes. The Enterprise version offers an additional advantage: access to historical laboratory data, enabling more informed decision-making and enhanced operational efficiency. Future enhancements include multilingual capabilities and advanced ETL testing to further optimize RAG-based retrieval, improving recall, response time, and overall system accuracy. Continuous validation through performance monitoring and user feedback will strengthen DopinGPT as a trusted AI-powered solution for anti-doping laboratories.

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# Automation of urinary specific gravity measurement by refractive index detector

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## Abstract

The possibility of using a liquid chromatograpy-refractive index detector (LC-RID) system for the automatization of the specific gravity (SG) measurement of urine samples in doping control analysis was investigated. An LC-RID method was developed and its performance and fitness for purpose was studied. Correlation of SG results of different urine samples measured by the developed method and by a conventional manual refractometer was very good and within-day repeatability of measurements met the necessary requirements. The developed method provides a simple automated way for SG determinations.

## Introduction

According to WADA's instructions, SG must be measured in all urine samples taken for antidoping purposes to detect possible adulteration or manipulation of the sample and for normalizing the concentrations of the steroid profile compounds [1]. Furthermore, for compounds with a threshold, the SG is used for adjusting the decision limit (DL), in case the SG is higher than 1.018 [2]. In the ITP either a digital refractometer or a densitometer can be used but in the CP the use of a digital refractometer is mandatory [1]. In laboratories urine SG is usually measured manually with a refractometer designed for the purpose, although automated systems also exist.

In combination with LC, RI detectors have commonly been used as universal detectors to detect compounds such as carbohydrates, that are not possible to be measured by UV detection [3]. In this project we investigated whether it would be possible to automate urinary SG measurement by using a LC-RID system. The principle of measurement of the refractometer and the RI detector (Figure 1) is basically the same [4,5]. Automating the measurement would reduce the workload and possibility of human errors, especially when analyzing large amounts of samples.

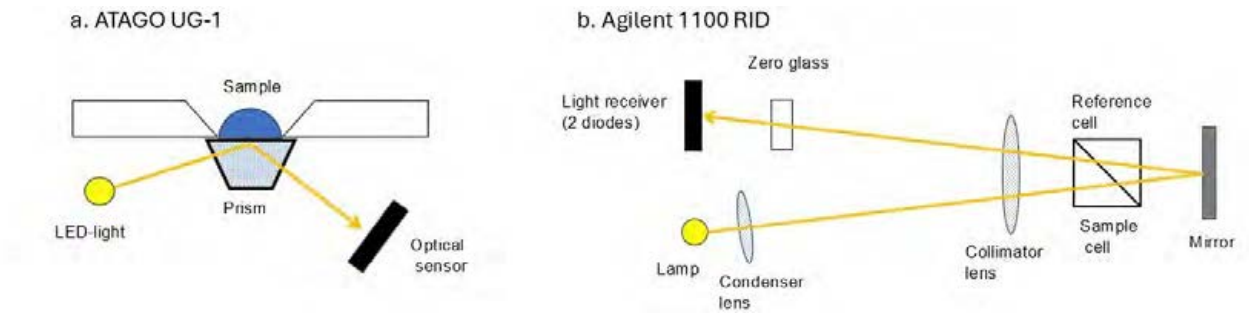


**Figure 1.** Detection principle of a) Atago UG-1 digital refractometer and b) Agilent 1100 RI-detector

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## Experimental

An Agilent Series 1100 HPLC equipped with an autosampler, binary pump and refractive index detector was used for the study. The detection principle of the detector is presented in Figure 1. Milli-Q water (ultra-purified grade water, Millipore, Bedford, USA) was used as an eluent. Because of the high sensitivity of the detector, only 5 µL of urine was injected, and instead of an analytical column, an Agilent static solvent mixer was used to dilute the sample (Figure 2). The flowrate was 1 mL/min, with a run time of 3 minutes (2 minutes + 1 minute post-time). The RI detector was set to 35°C. For manual determination of SG, a digital refractometer (Atago UG-1, Atago, Tokyo, Japan) was used.

The positive QC sample used for the preliminary validation of the LC-RID method was the same QC used in the laboratory for determining SG in urine samples by the manual digital refractometer. Shortly, the positive QC is prepared by dissolving 7.9 g of sodium chloride (Merck, Darmstadt, Germany) and 0.1 g sodium azide (Merck, Darmstadt, Germany) into 100 mL of Milli-Q grade water.

Linearity of the RI detector response was investigated using 5 different sucrose solutions corresponding to urinary SGs of 1.008, 1.013, 1.020, 1.027 and 1.040. The solutions were prepared as described by F. Wardenaar *et al.* [6] by spiking different amounts of sucrose (Sucrose BioXtra, Sigma-Aldrich, Massachusetts, USA) in Milli-Q grade water. The within-day variability of the developed method was studied by analyzing replicate samples of a QC sample with a SG of 1.035 normally used for the manual determination of SG.

To test the performance of the method, different urine samples (n=10) were measured both by LC-RID and with the manual refractometer and the test results were compared. The RI detector was calibrated daily with a one-point calibrator (sample with a SG of 1.035).

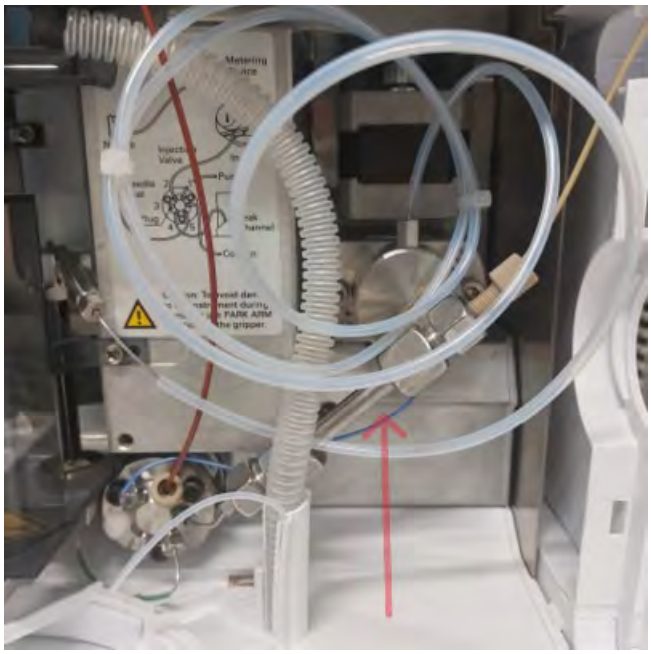

**Figure 2**. HPLC analytical column was replaced with a static solvent mixer (indicated by red arrow) used for mixing the urine sample with the mobile phase before the RI detector

## Results and Discussion

The detector response of LC-RI was linear between a SG-range from 1.008 to 1.040 (Figure 3). The within-day repeatability was ± 0.001, which is compliant with the maximun allowed uncertainty for SG ($U_{MAX\_SG}$) defined by WADA [2]. The correlation between urinary SGs measured by the ATAGO UG-1 digital refractometer and by the developed LC-RID method was good ($R^2$ = 0.992) (Figure 4). Based on the preliminary validation results, the developed method is well suited for the automatization of SG measurements, even though a more thorough validation is needed before taking the method into use for routine samples.

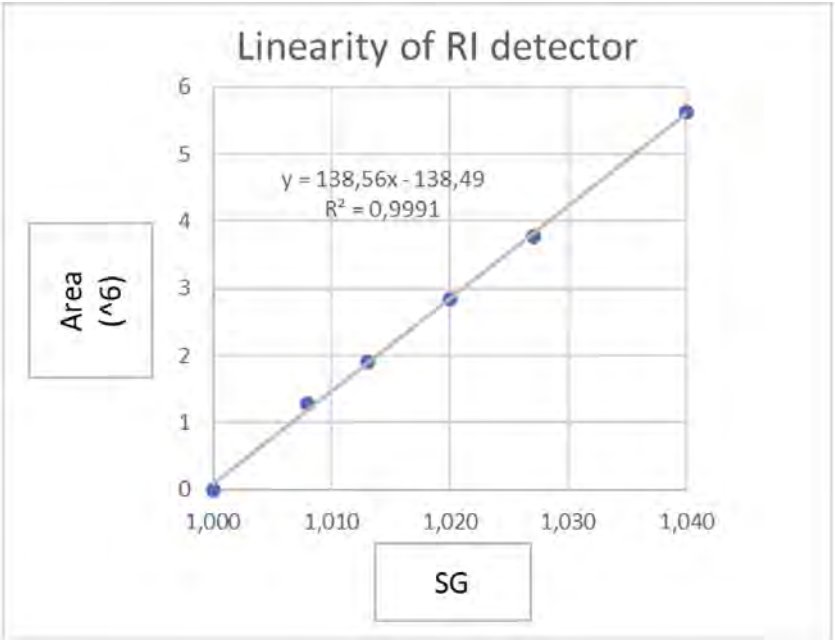


**Figure 3.** Linearity results of the RI detector

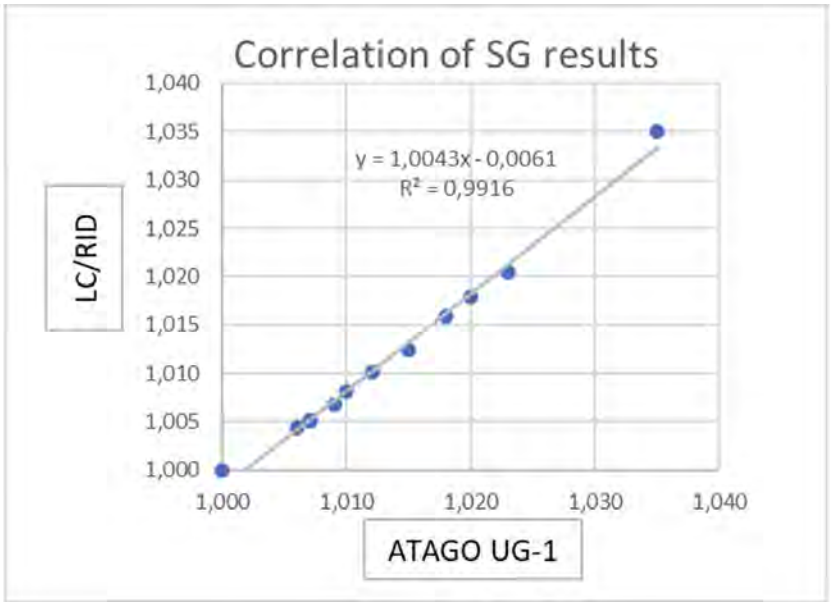


**Figure 4.** Correlation of SG results measured by Atago UG-1 and LC-RID

## Conclusions

Based on this preliminary study, the developed LC-RID method works well for determining the SG of urine samples. The setup is simple and provides a simple automated way for SG determinations.

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# Molecular isotopic structure as a novel tool for doping controls to detect 4-androstenedione misuse

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European Monitoring Center for Emerging Doping Agents (EuMoCEDA), Cologne/Bonn, Germany[3]

## Abstract

In order to differentiate between naturally elevated urinary levels of testosterone and testosterone doping, isotope ratio mass spectrometry is the method of choice. The carbon isotope ratios (CIR) of target analytes like testosterone or its direct metabolites are compared to those of endogenous reference compounds like pregnanediol. In rare cases, the CIR of the administered testosterone might exhibit CIR similar to those of the endogenous steroids, which may hamper the unequivocal detection. And due to the permanent isotopic dilution of the exogenous signal by endogenously produced steroids, the detection window employing classical target analytes may be relatively short.

The purpose of this research project was to develop a novel stable isotope-based test that examines the intramolecular distributions of rare isotopes, i.e., proportions in a sample of molecules containing specific atomic positions of rare isotope substitution. Success in this endeavor could allow for measurements that discriminate more reliably between synthetic and endogenous sources, thus increasing the challenge of mimicking endogenous patterns of isotopic substitution through engineered syntheses.

The only technology that is capable of constraining proportions of numerous isotopologues of complex organic molecules in small samples is a recently-developed, highly specialized form of Fourier-Fourier-transform mass spectrometry using Orbitrap mass analyzers; this method was introduced just 8 years ago but has already achieved technical bench marks that could enable significant improvements in the specificity of steroidal forensics [1].

This study provides preliminary insights into the isotopic characterization of androsterone, a testosterone metabolite, in urine samples after administration of synthetic androstenedione or testosterone, highlighting the forensic value of site-specific isotopic analysis in distinguishing synthetic from endogenous sources. The research outcomes underscore the capability of isotopic fingerprinting to not only detect the presence of synthetic steroids but also to trace their metabolic pathways and temporal degradation within the human body in the near future.

## Introduction

Current doping control methods are based on molecular-average $^{13}C/^{12}C$ isotope ratios in steroidal compounds. While this method is used to identify synthetic doping agents, such as testosterone or boldenone, it may yields inconclusive results due to overlapping isotope ratios between synthetic steroids and natural human hormone variations. Furthermore, athletes can try to circumvent these tests by sourcing or synthesizing steroids with isotope profiles resembling natural human signatures. This

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possibility, particularly accessible to well-funded programs, highlights a vulnerability in one of the most essential doping control tests. To address these limitations, the field requires more sophisticated and conclusive methods capable of overcoming these overlaps and resist attempts to mask synthetic origins.

## Experimental

The primary tool used in this study is the Orbitrap Fusion Lumos Tribrid mass spectrometer, a highly advanced device known for its high sensitivity, capability to achieve high-resolution (up to 500,000), high scan speed that can reach up to 15 Hz, and accurate mass measurements (Figure 1). This instrument was chosen for its unique ability to differentiate between isotopologues of complex molecules, such as steroids, based on its exceptional mass resolving power.

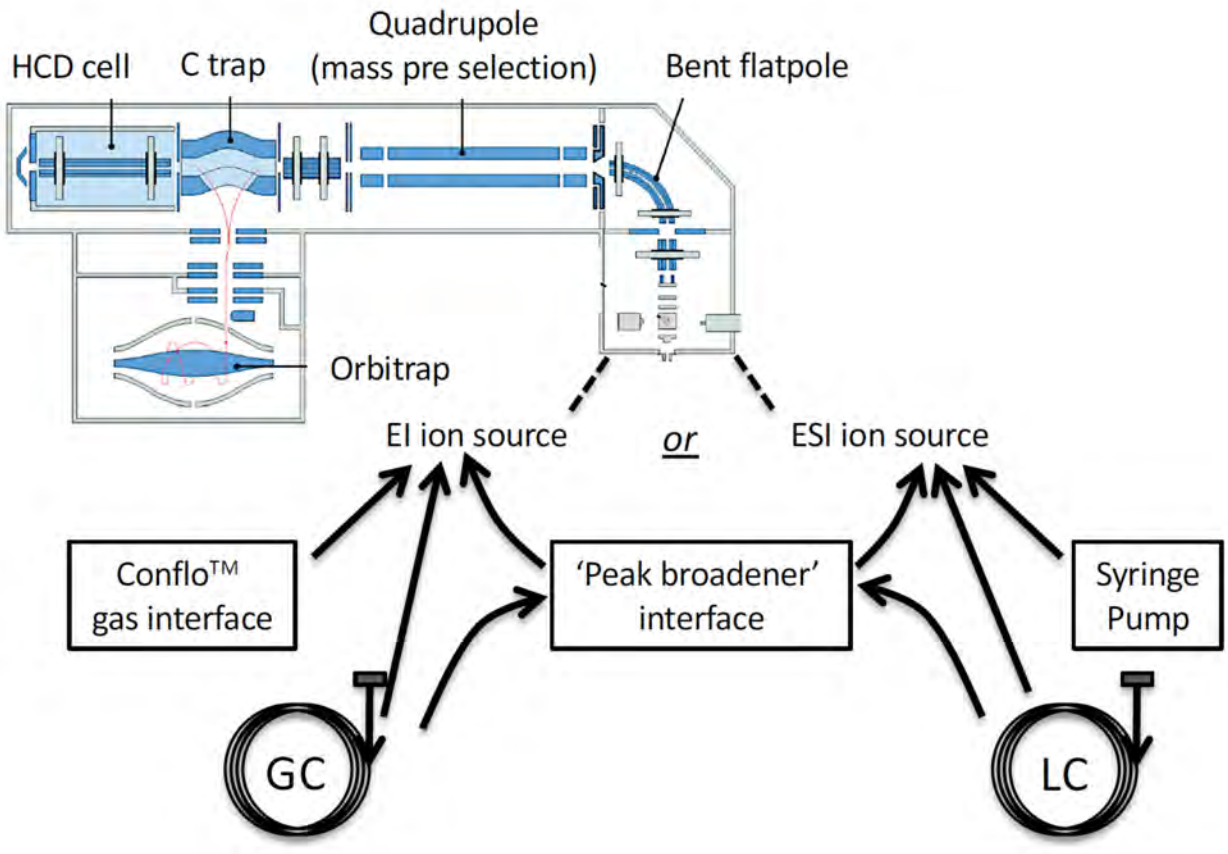


**Figure 1.** Schematic illustration of the major components used for sample introduction, ion production, ion transfer, mass pre-selection, collision-cell experiments, and Orbitrap mass spectrometry on the Q Exactive series platforms [1]

Androsterone, one of the main urinary metabolites of 4-androstenedione, was introduced into the Orbitrap's ion source through a dual inlet syringe pump. Once ionized, these steroid molecules were subjected to either carbon skeleton (represents the entire-molecule) analysis or controlled fragmentation in the CID cell. The molecular fragments employed include 255 Da, representing the entire carbon skeleton of the steroidal hormone (covering rings A, B, C, and D); 124 Da, which represents ring D and two carbons of ring C, including the carbonyl carbon in ring D; 199 Da, representing rings B, C, and D; and 173 Da, representing rings A, B, and two carbons of ring C (Figure 2).

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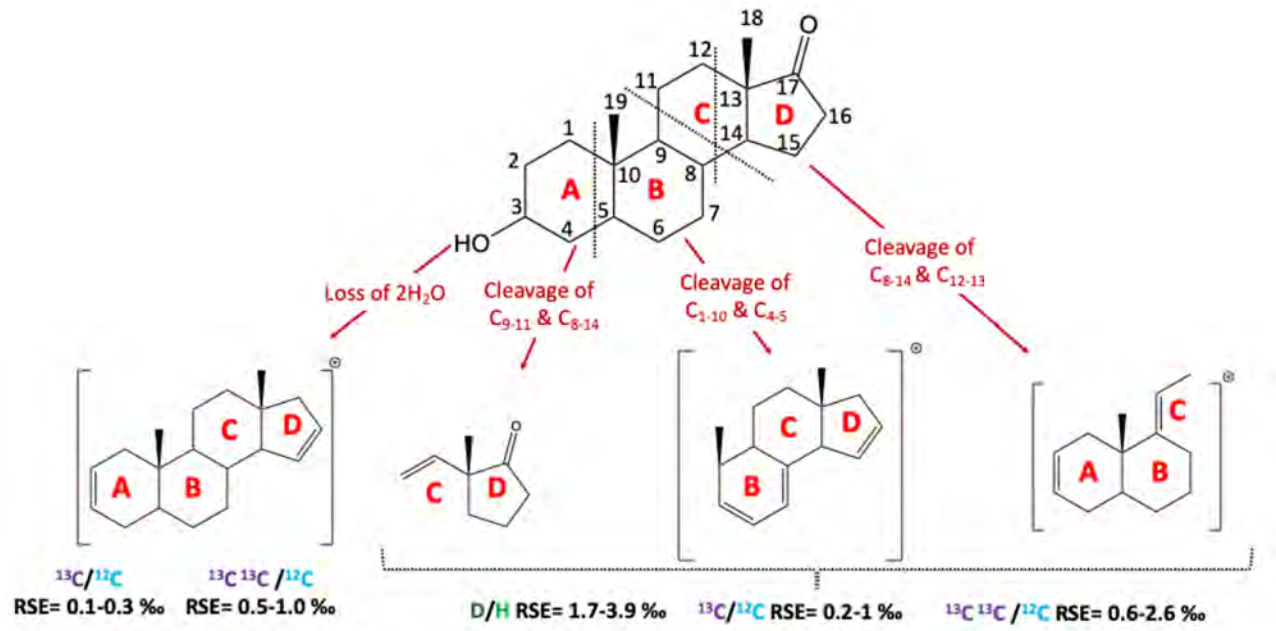


**Figure 2.** Illustration of targeted molecular fragments in androsterone analysis highlighting the specific fragments and their corresponding relative standard errors (RSE)

## Results and Discussion

High sensitivity and resolution was achieved and allowed precise site-specific isotope measurements, including $\delta^{13}C$, $\delta D$, and doubly substituted 13C isotopologues across various sites within androsterone molecules. Measurement designs with low relative standard errors (RSE) as given in Figure 2 were achieved.

Existing samples, which were taken 6, 10, 22, 28, and 38 hours after a previous oral administration of 80 mg of 4-androstendione with a $\delta^{13}C$ value of -31.8 ‰, were examined and the fragment specific results are shown in Figure 3. At least three measurements were conducted per experiment.

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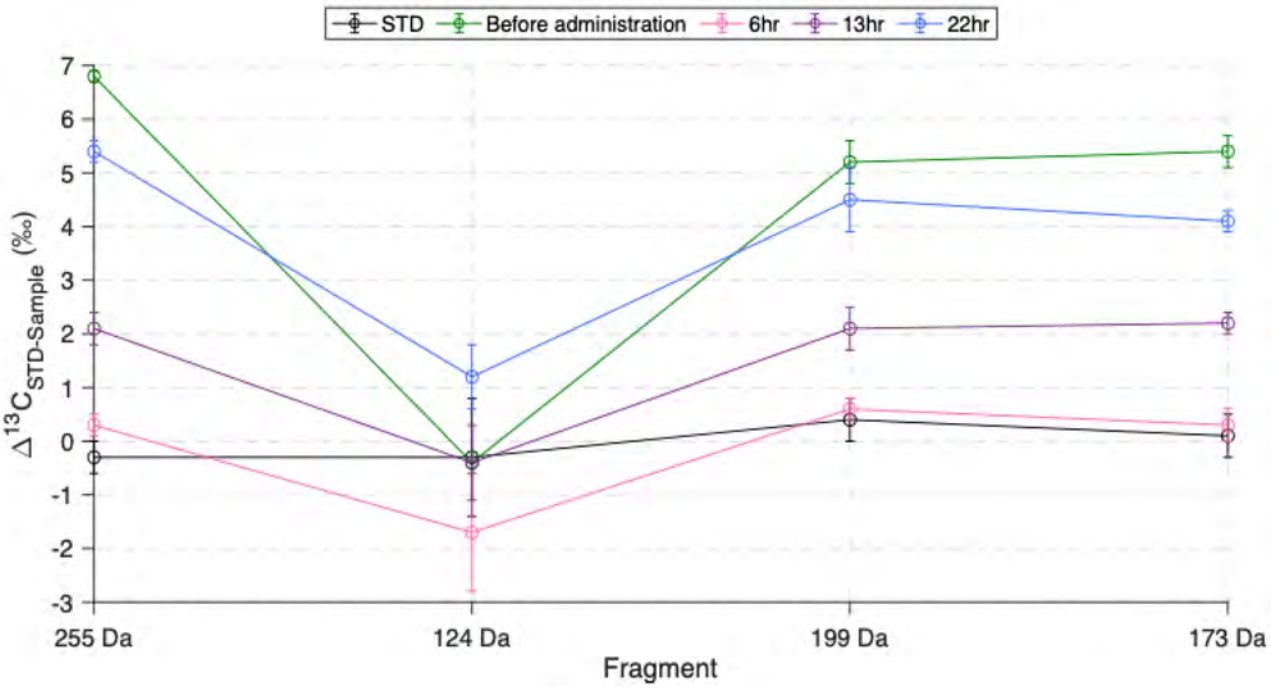


**Figure 3.** The δ¹³C isotopic shifts in various molecular fragments (255 Da, 124 Da, 199 Da, 173 Da) after the administration of synthetic 4-androstenedione. Measured at several time points post-administration (6 h, 13 h, 22 h), the shifts are compared against standard (STD) levels and baseline values before administration

Regarding δD, only the 124 Da fragment was measurable showing an inverse trend compared to δ¹³C as shown in Figure 4. After 22 h the influence of the administered 4-androstenedione is still clearly visible in contrast to the values measured for carbon isotope ratios.

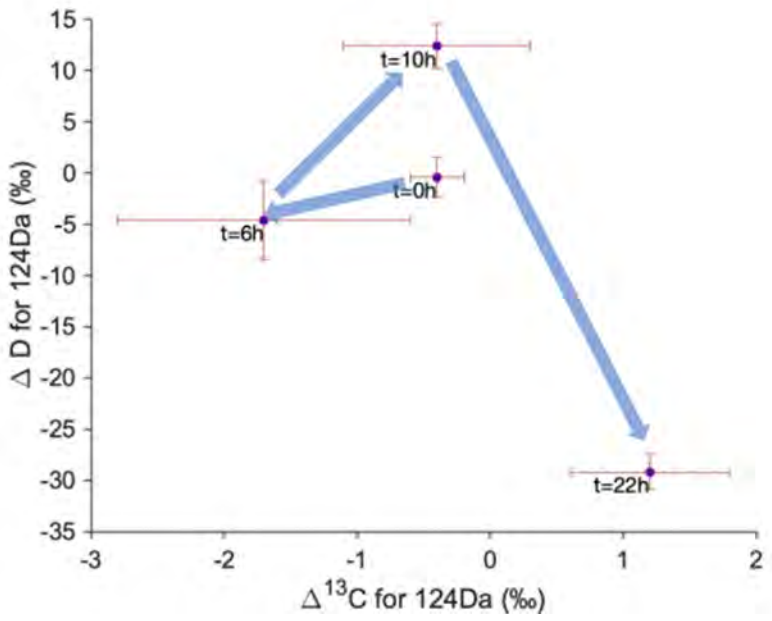


**Figure 4.** Dynamic changes in both δD and δ¹³C values for the 124 Da fragment at different time points following administration with 4-androstenedione

## Conclusions

This study provides crucial insights into the isotopic characterization of androsterone in urine samples after administration of 4-androstenedione. Especially the 124 Da fragment provides an intriguing counterpoint. This fragment exhibits a more moderate shift in $\delta^{13}C$ after administration (Figure 3). Additionally, the δD values of this fragment show an inverse relationship with its $\delta^{13}C$ values, diverging further from the biogenic values observed prior to administration (Figure 4). Neither the δD nor the δ13C values of this fragment approached the biogenic levels observed in the 124 Da fragment before administration. This unique isotopic behavior may be attributed to the presence of a carbonyl carbon in the 124 Da fragment, which is integral in the biochemical conversion of synthetic 4-androstenedione into androsterone via testosterone. The chemical change in the hybridization at this active site affects the immediate isotopic environment, thereby inversely impacting the isotopic signatures of $\delta^{13}C$ and deuterium [2]. Another potential explanation would be that the underlying human metabolism of 4-androstenedione is not straightforward but different routes of conversion from 4-androstenedione to androsterone may be exist accompanied by different isotopic fractionations. Changes in conversion rates over time would also result in fluctuating isotope ratios. More data will be necessary to further investigate this finding and to figure out if this might be useful in the context of sports drug testing.

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# Unintentional dermal dorzolamide application as possible source of Adverse Analytical Finding(s) – A case study

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## Abstract

Dorzolamide and Brinzolamide are listed in the WADA Prohibited List with the note that their topical ophthalmic administration is not prohibited. However, following the ophthalmic administration, these drugs reach systemic circulation and are accumulated in erythrocytes. Because of the long elimination half-life, the presence in urine can be detected for months/years after the drug administration is discontinued.

In the last three years, Seibersdorf Laboratory reported 23 Adverse Analytical Findings (AAFs) for Dorzolamide and 12 for Brinzolamide. Out of these 35 samples, the estimated concentrations were higher than 200 ng/mL (Minimal Required Performance Level) only in three samples, while they were below 2 ng/mL in 16 samples and between 2 - 20 ng/mL in an additional 11 samples. In one of the Dorzolamide positive cases (estimated concentration 0.32 ng/mL), the athlete indicated that the potential source of the Dorzolamide was an unintentional dermal application of eye drops while administering medication to their dog.

To simulate this scenario, an excretion study was conducted by applying six drops of Dorzo-Vision ® eye drops (20 mg/mL Dorzolamide) on the hands of one volunteer. The detectability of Dorzolamide and its potential confounding effect on the steroid profile were investigated. Dorzolamide was detected in all collected samples (c = 0.01-0.11 ng/mL; $T_{max}$ = 6h). No effect on the steroid profiles was observed. The current study demonstrates that an Adverse Analytical Finding in athletes' urine samples from repeated unintentional dermal exposure to Dorzolamide is plausible.

## Introduction

Dorzolamide and Brinzolamide are commonly used as eye drops for the treatment of ocular hypertension or glaucoma. After systemic use, these carbonic anhydrase inhibitors can have diuretic effects. In the WADA Prohibited List, they are listed under section S5 Diuretics and Masking agents with the note that topical ophthalmic administration is not prohibited [1]. However, following the ophthalmic administration, these drugs reach systemic circulation and are accumulated in red blood cells [2]. Because of the long elimination half-life (≥ 4 months) [3,4], the presence in athletes' urine can be detected for months/years after the ophthalmic administration of the drug is discontinued [5]. In this work, an excretion study was conducted to evaluate possible transdermal absorption of Dorzolamide.

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## Experimental

### Historic data evaluation

The AAFs reported by Seibersdorf Laboratory between years 2022 and 2024 were re-evaluated and the Dorzolamide and Brinzolamide AAFs were identified.

### Excretion study

One healthy Caucasian male volunteer applied six drops of Dorzo-Vision® eye drops containing 20 mg/mL Dorzolamide, i.e. 6 mg of the substance, to his hands. Urine samples were collected 30 min pre-application and up to 32 hours post-application (1+10 samples in total). pH and specific gravity were 6.25 ± 0.62 and 1.019 ± 0.003 (mean ± SD), respectively.

### Sample preparation

Urine aliquots (5 mL) were mixed with 100 µL of ISTD (Salbutamol d9) and 1 mL of sodium acetate buffer (pH 4.8). The analytes were extracted with 6 mL of ethyl acetate. The organic phase was evaporated to dryness, and the samples were reconstituted by the addition of 200 µL of water:methanol:formic acid (90:10:0.1, v/v) mixture. The extracts were placed on a heating block (60°C) for 10 mins, transferred to a vial and injected into the LC-HRMS system.

### LC-HRMS measurement conditions

LC-HRMS measurements were performed on a Vanquish Horizon UHPLC+ System coupled to Q-Exactive Orbitrap mass spectrometer (Thermo Fisher, Austin, Texas, USA), which was equipped with a heated electrospray ionization source operated in positive ionization mode (ESI+). The spray voltage was set at 3.8 kV. The capillary temperature was adjusted to 350°C, while the auxiliary gas heater temperature was set to 325°C. The sheath and auxiliary gas (nitrogen) flow rates were 25 and 8 arbitrary units, respectively. The system was operated in parallel reaction monitoring (PRM) mode using the transitions indicated in Table 1 (mass tolerance ± 10 ppm).

The obtained urine samples were analyzed by a fit-for-purpose LC-HRMS confirmation method for Dorzolamide. The chromatographic conditions and selected mass spectrometry parameters are presented in Table 1.

| Instrument | Q-Exactive Orbitrap HRMS |
|---|---|
| Column | Zorbax Eclipse XDB-C8, 4.6x150 mm, particle size 3µm |
| Column temp. | 25 °C |
| Mobile phase A<br>Mobile phase B | water + 0.2% formic acid<br>methanol + 0.1% formic acid |
| Gradient | 0% B (0.0-0.2 min), 0% → 40% B (0.2-4.0 min), 40% → 100% B (4.0-6.0 min), 100% B (6.0-9.0 min), 100% → 0% B (9.0-9.1 min), 0% B (9.1-12 min) |
| Flow rate | 0.5 mL.min$^{-1}$ |
| Injection volume | 10 µL |
| Precursor ion$^{+}$ (m/z) | 325.03450 |
| Product ions+ (m/z)<br>CE = 30 eV | 151.0211, 198.9881,<br>216.0147, 235.9502 |

**Table 1:** Chromatographic conditions and selected mass spectrometry parameters of the confirmation LC-HRMS method

To estimate the potential confounding effect of Dorzolamide on the steroid profile, all samples were measured with the validated accredited GC-MS/MS method which is routinely applied to determine steroid profile markers in all doping control urine sample analysed by the Laboratory.

## Results and Discussion

According to 2023 WADA's Anti-Doping Testing Figures, 74 Adverse Analytical Findings (AAFs) for Dorzolamide were reported, accounting for 14% of occurrences in the S5 Drug Class [6]. In the last three years, Seibersdorf Laboratory reported 23 AAFs for Dorzolamide and 12 for Brinzolamide (Figure 1). In 16 out of 35 cases (46%; 9 for Dorzolamide, 39%; 7 for Brinzolamide, 58%), the estimated concentrations were below 2 ng/mL. Additionally, 11 cases (31%; 9 Dorzolamide, 39%; 2 Brinzolamide, 17%) showed concentrations within the range of 2-20 ng/mL. Concentrations higher than the current Minimal Required Performance Level (MRPL) of 200 ng/mL were found only for three Dorzolamide cases (8.6% of total cases, 13% of Dorzolamide cases). In one of the Dorzolamide positive cases, where the concentration was below 2 ng/mL ($c_{est}$ = 0.32 ng/mL), the athlete indicated as a potential source of Dorzolamide an unintentional dermal application of eye drops while administering medication to their dog, which was being treated for glaucoma. An excretion study was conducted in collaboration with the responsible Testing Authority to simulate the athlete's declaration.

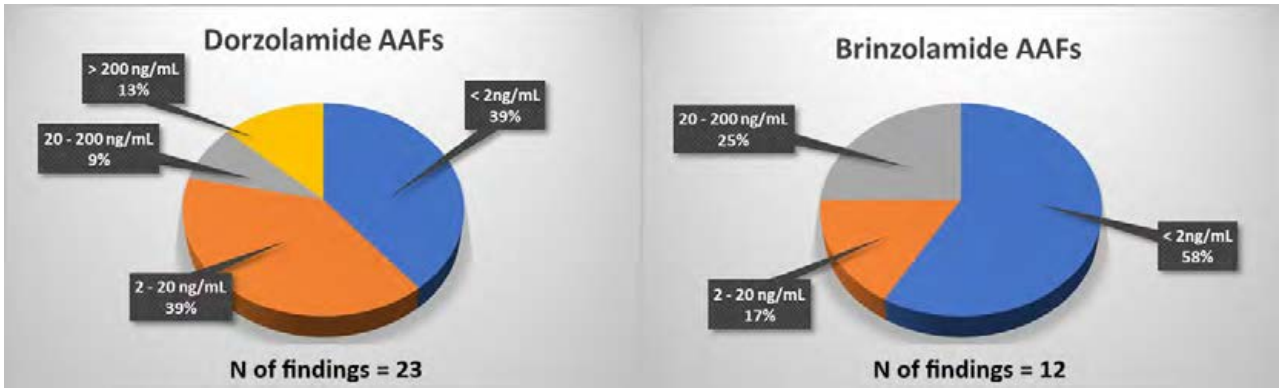


**Figure 1:** Overview of Dorzolamide (left panel) and Brinzolamide (right panel) estimated concentrations in AAFs detected by Seibersdorf Laboratory in the last three years

All urine samples collected during the excretion study were prepared and analyzed according to the procedures described in the Experimental section. No signal for Dorzolamide was detected in the sample collected before the administration of the medication. For the samples collected post-administration, Dorzolamide was detected in all samples with concentrations ranging from 0.01 to 0.11 ng/mL, with the maximum concentration detected 6 hours post-application (Figure 2).

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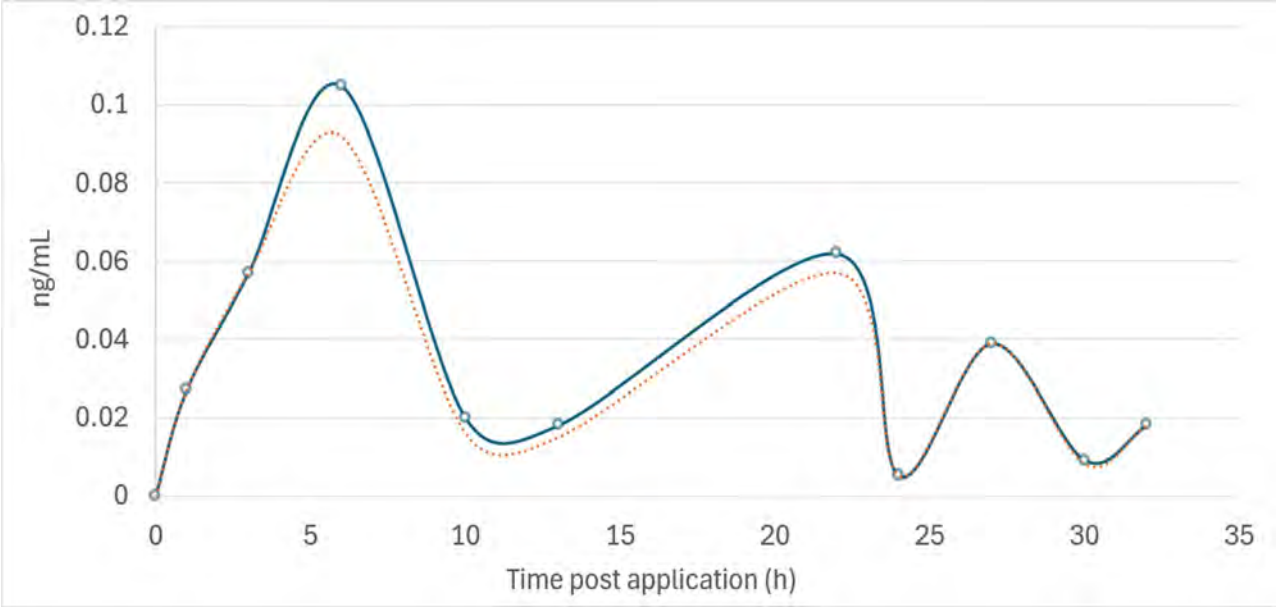


**Figure 2.** Excretion profile of Dorzolamide after dermal application of 6 mg Dorzolamide. The full line represents estimated concentration in urine over time after administration without adjustment for specific gravity. The dotted line represents the concentrations after correction for specific gravity

No effect was observed at any of the respective steroid profile markers or the relevant respective ratios after single-time dermal application of Dorzolamide (Figure 3). All the steroid profile markers followed the normal urinary circadian rhythm.

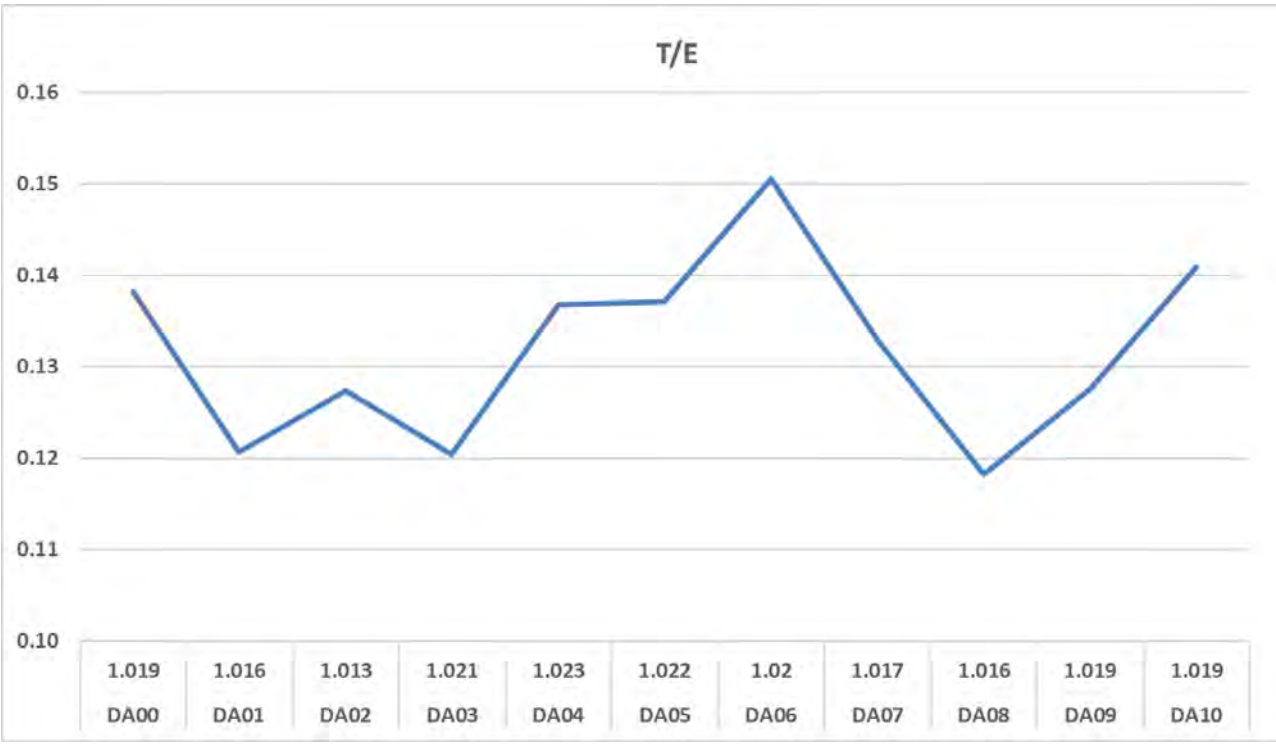


**Figure 3.** Urinary excretion profile (concentration-time curve) of testosterone/epitestosterone (T/E)

The results after single transdermal dose of 6 drops of Dorzolamide medication showed ca. 3-times lower maximal urinary concentrations compared to the estimated concentration in the athlete's sample. This clearly shows that Dorzolamide can be detected in the urine after skin application. The athlete applied the medication to their dog at least 5-times per day over the course of 6 months. Thus, it cannot be excluded (considering intraindividual differences) that the AAF in athlete's sample was caused by unintentional single application of more than 6 drops of dorzolamide medication to the skin or most probably by repeated exposure to lower amounts of the medication over the course of several administrations.

## Conclusions

The current study demonstrates that transdermal absorption of Dorzolamide is possible, and due to its cumulative effect in erythrocytes and long half-life, an AAF in athletes' urine samples as a result of repeated unintentional dermal exposure is plausible. No effect on the steroid profile was observed after a single Dorzolamide dermal application.

Due to the similarities in the chemical structure, long half-life, and incorporation into erythrocytes, a similar excretion behaviour can be expected also for Brinzolamide.

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# Colonoscopy - potential positive result of anti-doping test

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## Abstract

Colonoscopy, an endoscopic examination of the large intestine, can be performed under short intravenous sedation (analgosedation). A combination of a sedative (e.g. benzodiazepine) with painkiller (e.g. opioid) is widely used in analgosedation during colonoscopy. Fentanyl as synthetic primary μ-opioid receptor agonist with a potent narcotic and analgesic properties is frequently used as analgesic drug during colonoscopy. In the WADA Prohibited List fentanyl is listed in the group S7 – narcotics, and hence its use by athletes during competitions is banned. Administration of fentanyl during endoscopic procedure can result in a positive doping test.
The aim of the present work was to study the metabolic profile of fentanyl in urine following intravenous administration during colonoscopy. Detection was conducted by means of ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS).

## Introduction

Fentanyl is a highly synthetic primary μ-opioid receptor agonist which was developed for its efficacious analgesic profile [1]. Pharmaceutical fentanyl is routinely used for anesthesia and analgesia, e.g. during colonoscopy procedure [2]. It is highly lipophilic relative to other opioid agonists (like heroin), and when combined with is low molecular weight, it facilitates swift penetration into biological membranes, including the central nervous system [1]. Fentanyl is eliminated from the body mainly in the form of its metabolites in urine. Fentanyl elimination is facilitated by metabolism, which is heavily dependent on hepatic phase 1 cytochrome-P450 (CYP)-mediated reactions. The primary biotransformation is N-dealkylation at the piperidine ring to norfentanyl, which is mediated by CYP3A-enzymes. Norfentanyl is not the only metabolite of fentanyl, minor metabolic pathways include hydroxylation and hydrolysis, and are presented in Figure 1 [3]. In the WADA Prohibited List fentanyl and its metabolites are listed in the group S7 – narcotics, and hence their use by athletes during competitions is banned [4]. In accordance with WADA Technical Document TD2022 MRPL, the Minimum Reporting Level (MRL) for fentanyl and its metabolites is 1 ng/mL [5]. The aim of the present work was to study the metabolic profile of fentanyl and fentanyl analogues in urine, classified in the S7 groups following intravenous administration during colonoscopy in accordance with Table 1A.

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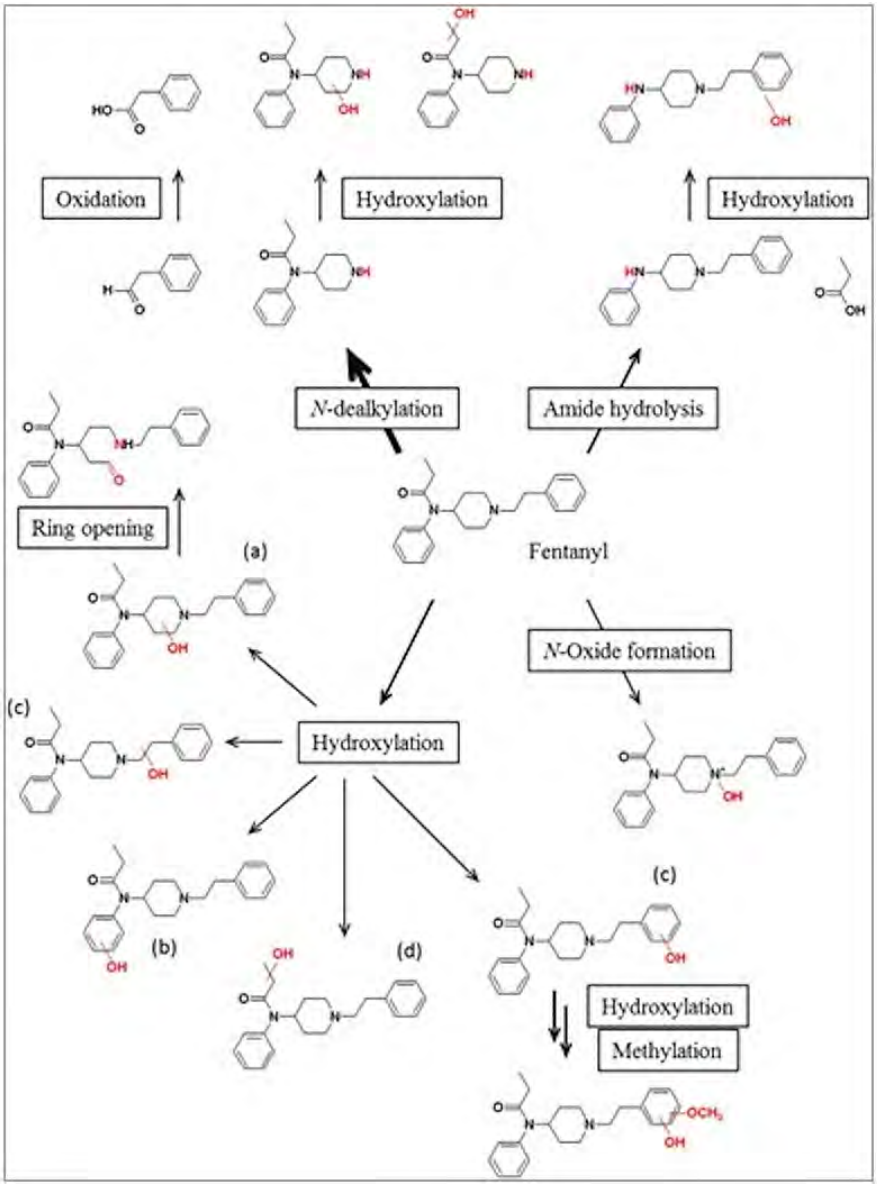


**Figure 1.** Metabolic profile of fentanyl in humans. Main metabolic pathways are marked by bold arrows *(source: based on [3])*

## Experimental

An excretion study was conducted in an opioid-naive female volunteer (age 54 years, 162 cm, 55 kg) after an intravenous administration of 100 µg of pharmaceutical fentanyl (1 ampule, 2 mL, 50 µg/mL) during an anesthetized colonoscopy procedure.

Urine samples (S1- S5) were collected according to Figure 2 and were treated as routine athlete samples. First of all, the Internal Testing Procedure (ITP) were performed to detect and pre-estimate the concentrations of fentanyl and/or its metabolites and/or analogs. Then, based on the ITP results listed in Table 1B, the Confirmation Procedure was conducted using selected MRM transitions shown in Table 1C.

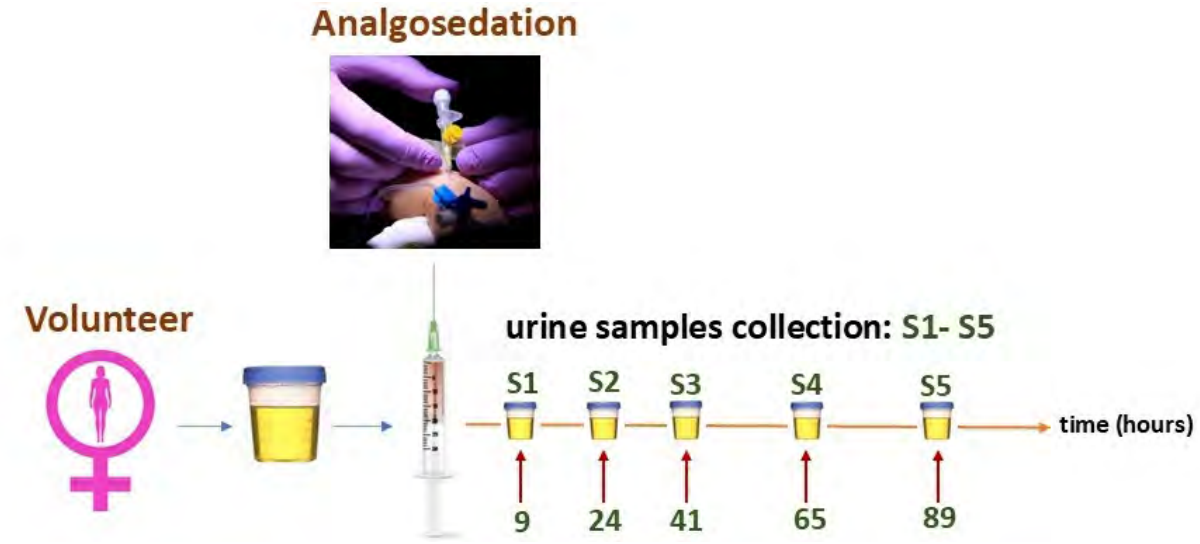


**Figure 2.** Scheme of samples collection

**Sample pre-treatment**

Samples were prepared in compliance with a dilute-and-shoot approach (used in a routine anti-doping initial testing analysis (ITP)) and a method involving double liquid-liquid (LL) extraction with ethyl acetate at acidic and basic pH (for Confirmation Procedure (CP)). In the case of the DAS method, the supernatant was transferred to a 96-well plate. In the second method (LL), the residue was reconstituted in 150 µL of mobile phase (acetonitrile/water, 1/9, v/v), transferred in a vial and was injected into the LC-MS/MS system.

**Instrumental analysis**

Chromatographic separation was conducted by means of a Waters Acquity I-Class UPLC System liquid chromatography with HSS T3 column (1.8 µm, 100 mm x 2.1 mm, Waters). The mobile phase consisted of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B), and the LC gradient was employed at the constant flow rate of 300 µL/min at 45°C. MRMs of the studied substances were traced with a Xevo TQ-S mass spectrometer equipped with UniSpray$^{TM}$ source (for ITP) and a Xevo TQ-XS mass spectrometer equipped with an Electrospray source (for CP), respectively. All analytes were investigated in the positive ionization mode. Desolvation gas flow was set at 800 L/h at 500 °C with ion source temperature at 150°C for both instruments.

**Method validation**

The validation process was performed in accordance with the WADA technical document TD2023IDCR [6]. Limit of identification (LOI) for fentanyl and norfentanyl determined at the concentration level of 0.25 ng/mL respectively. Selectivity was assessed by analyzing 6 blank samples from different individuals (3 males and 3 females).

## Results and Discussion

The elimination of fentanyl and its metabolite (norfentanyl) was studied using double liquid–liquid extraction, and the data are presented in Figures 3A and 3B. The highest concentrations of fentanyl and norfentanyl were observed 9 hours after administration.

Based on the available data, norfentanyl was detectable in urine above the WADA Minimum Reporting Limit (MRL) for at least 24 hours after administration. However, due to the limited sampling between 24 and 42 hours, it is not possible to precisely determine how long norfentanyl concentrations remain above the MRL beyond the 24-hour time point. In contrast, fentanyl concentrations were below the WADA MRL at all measured time points.

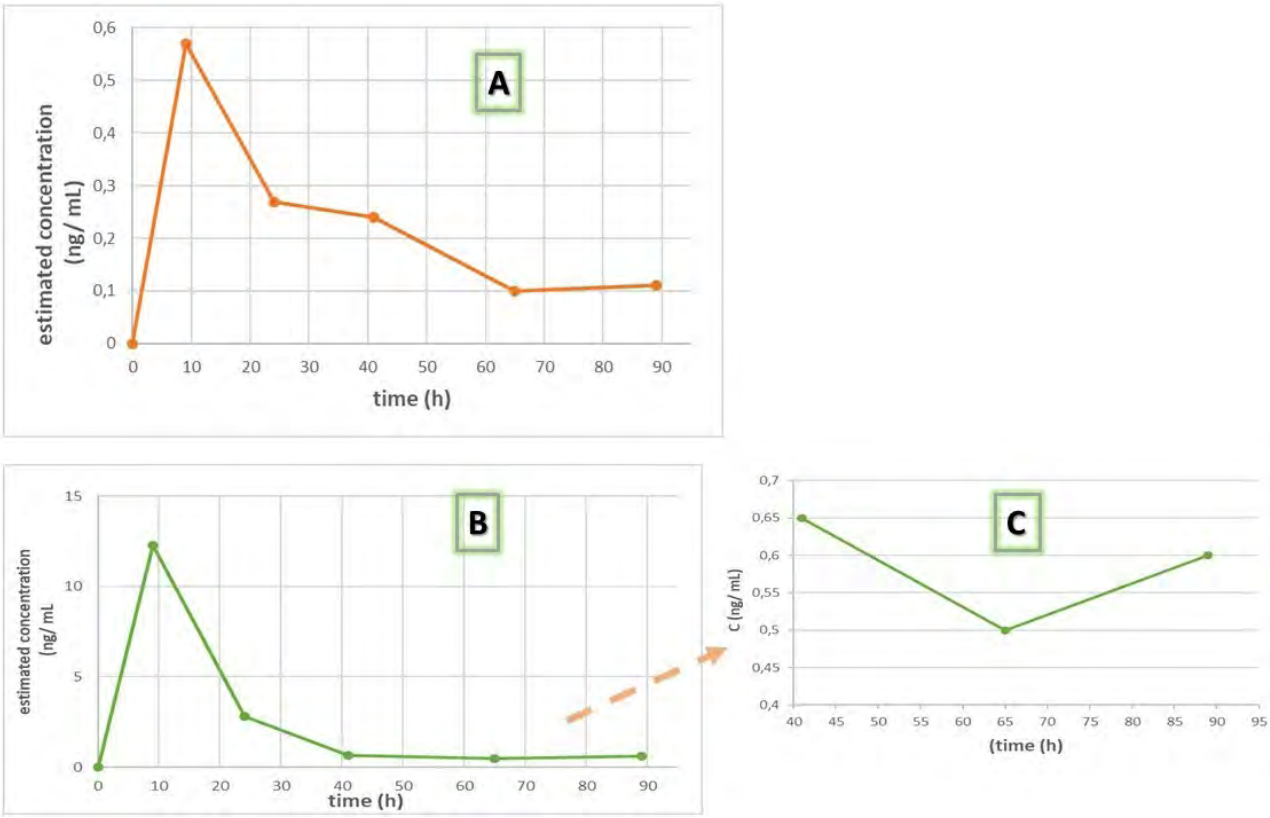


**Figure 3. A:** Excretion profile of fentanyl; **B:** Excretion profile of norfentanyl; **C:** Zoom of B++

**A**

| Compound | Parent ion (m/z) | Product ion (m/z) | Collision energy (eV) | Application | LOD (ng/mL) |
|---|---|---|---|---|---|
| Fentanyl | 337.23 | 188.1 | 24 | Screen | 0.5 |
| Norfentanyl | 233.17 | 83.99 | 16 | Screen | 0.5 |
| 3-methylfentanyl | 351.24 | 104.88 | 40 | Screen | 0.5 |
| Furanyl Fentanyl | 375.21 | 104.88 | 45 | Screen | 0.5 |
| Norcarfentanyl | 291.17 | 259.16 | 10 | Screen | 0.5 |
| Normethylfentanyl | 247.35 | 98.14 | 20 | Screen | 0.5 |
| p-FBF | 369.23 | 188.21 | 25 | Screen | 0.5 |
| Alfentanyl | 417.26 | 268.15 | 18 | Screen | 0.5 |
| Sufentanyl | 387.21 | 238.18 | 20 | Screen | 0.5 |
| Norsufentanyl | 277.19 | 96.01 | 24 | Screen | 0.5 |

**B**

| Initial Testing Procedure | | |
|---|---|---|
| Compound | Identified substance | Not detected |
| Fentanyl | √ | |
| Norfentanyl | √ | |
| 3-methylfentanyl | | √ |
| Furanyl Fentanyl | | √ |
| Norcarfentanyl | | √ |
| Normethylfentanyl | | √ |
| p-FBF | | √ |
| Alfentanyl | | √ |
| Sufentanyl | | √ |
| Norsufentanyl | | √ |

**C**

| Compound | Parent ion (m/z) | Product ion (m/z) | Collision energy (eV) | Application |
|---|---|---|---|---|
| Fentanyl | 337.23 | 188.1<br>104.96 | 25<br>40 | Confirmation |
| Norfentanyl | 233.17 | 84<br>177.23<br>150.1 | 20<br>15<br>20 | Confirmation |
| *Mefruside (ISTD)* | 382.97 | 129.05 | 20 | - |

**Table 1. A:** Traced MRM transitions and MS set-up for Initial Testing Procedure (ITP); **B:** Results of ITP; **C:** Traced MRM transitions and MS set-up for Confirmation Procedure (CP)

## Conclusions

- The intravenous administration of pharmaceutical fentanyl during a colonoscopy procedure may result in a detectability of the fentanyl metabolite (norfentanyl) above the WADA Minimum Reporting Limit for at least 24 hours, while detectability beyond this period remains uncertain.
- Athlete participation immediately after colonoscopy is unlikely due to the risk of bleeding. However, this possibility cannot be entirely excluded in certain cases, which is why the pharmacological risk related to the detectability of fentanyl and norfentanyl should still be considered, particularly if these substances were administered for procedural sedation.
- Fentanyl has a long pathway of excretion. Therefore, there is a high possibility that its level can significantly rise and then abruptly drop („fentanyl rebound”).

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Hülsemann F, Walpurgis K, Thevis M

# Detection of synthetic urea and bovine albumin in a specimen provided as human urine sample

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## Abstract

Manipulation of urine samples for drug testing or doping control analysis is a well-known problem, including dilution of urine, chemical adulteration or substitution by other liquids. Different analytical strategies have been developed to identify diluted or adulterated urine samples, as well as the identification of urine samples substituted with other liquids such as apple juice, alcohol-free beer, water or synthetic urine. Synthetic urine may contain different substances, including creatinine, uric acid and urea and show a specific gravity and pH similar to natural human urine samples. Several methods have been evolved within the last years to identify synthetic urine samples with routine analytical methods. These methods are based either on the identification of substance solely present in synthetic, but not natural urine, or on the identification of the absence of typically present urinary biomolecules.
In doping control analysis, the total absence of naturally occurring endogenous steroids (markers of the steroid profile) in a urine sample is strong evidence for a manipulated e.g. highly diluted or substituted sample. However, additional information supporting the assumption of a substitution of the original sample by synthetic urine can be particularly interesting.
A suspicious doping control urine sample without detectable amounts of endogenous steroids, a specific gravity of 1.014 and a pH of 8.3 revealed significant amounts of urea and protein. However, the nitrogen and carbon isotopic composition of urea and total urine indicated a synthetic origin, similar to commercially available urea standards and synthetic urine. The protein composition of the sample was investigated by SDS-PAGE and the pattern observed following Coomassie-staining was found to be rather unusual as only one intense band at an apparent MW of ~ 70 kDa and very few faint other signals were visible. In-gel tryptic digestion revealed the presence of bovine albumin instead of its human analog, clearly demonstrating a case of urine substitution.

## Introduction

Manipulation of urine samples for drug testing or doping control analysis is a well-known problem, including dilution of urine, chemical adulteration or substitution by other liquids. Different analytical strategies have been developed to identify diluted or adulterated urine samples, as well as the identification of urine samples substituted with other liquids such as apple juice, alcohol-free beer, water or synthetic urine. Synthetic urine may contain different substances, including creatinine, uric acid and urea and show a specific gravity and pH similar to natural human urine samples. Several methods have evolved within the last years to identify synthetic urine samples with routine analytical methods. These methods are based either on the identification of substances solely present in synthetic but not natural urine, or on the absence of biomolecules typically present in human urine [1].

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In doping control analysis, the total absence of naturally occurring endogenous steroids (markers of the steroid profile) in a urine sample is strong evidence for a manipulated e.g. highly diluted or substituted sample. However, additional information supporting the assumption of a substitution of the original sample by synthetic urine can be particularly interesting.

## Experimental

A suspicious doping control urine sample without detectable amounts of endogenous steroids, a specific gravity of 1.014 and a pH of 8.3 was checked for the presence of urea and protein. The test for urea was carried out using the xanthydrol-method, which also allows to analyze the nitrogen isotope ratio of urea present in a sample [2].
Additionally, the isotope ratio of the total carbon content of the dried sample was determined. Urea nitrogen and total carbon isotope ratio measurements were carried out on a EA-IRMS system. For comparison, synthetic urea samples (Sigma-Aldrich, Steinheim, Germany; IVA Analysentechnik, Meerbusch, Germany) and two synthetic urine samples (Sigmatrix from Sigma Aldrich, Steinheim, Germany; Synthetic Urine e.K, Eberdingen, Germany) were analyzed.
The protein composition of the sample was investigated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE)[3]. For that purpose, 4 mL each of the suspicious urine sample as well as a blank specimen were concentrated using Amicon® centrifugal filter units with a cut-off of 10 kDa. Subsequently, 20 µL of the resulting retentate were subjected to protein reduction and separation on NuPAGE Bis-Tris Mini Gels.

## Results and Discussion

The nitrogen and carbon isotopic composition of the suspicious sample provided as human urine sample was $\delta^{15}N$ = -2.0 ‰ for urea and $\delta^{13}C$ = -36.5 ‰ for total carbon, indicating a synthetic origin, comparable to the commercially available urea standards and synthetic urine (Figure 1). Nitrogen isotope ratios of human urea as well as the total carbon isotope ratios of human urine are significantly different than the isotopic composition found in the suspicious sample. Analysis of 1971 different samples of urea from human urine samples revealed $\delta^{15}N$ values between +8.2 and +1.4 ‰, whereas $\delta^{13}C$ values of the total carbon content of 746 different human urines samples were between -10.8 and -27.7 ‰.

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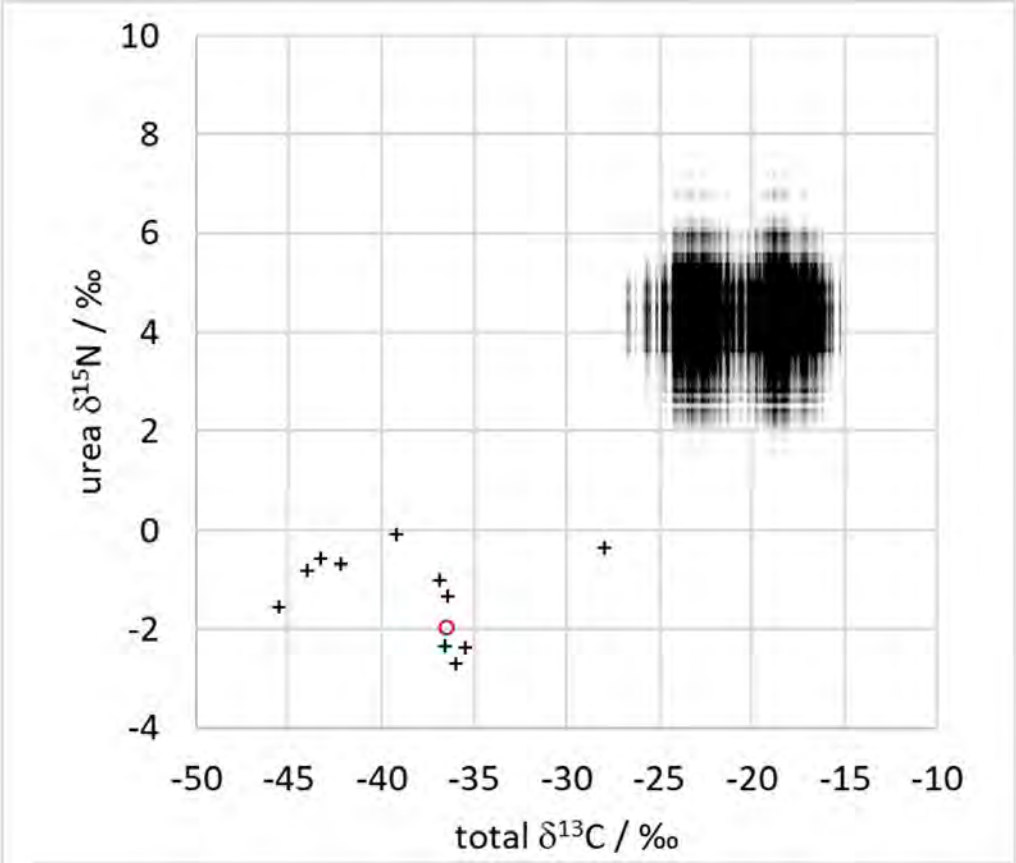


**Figure 1.** Isotope ratios for total carbon and urea nitrogen of suspicious sample (circle), synthetic urea and synthetic urine (crosses) and human urine samples (filled squares). Isotope ratios for human urine samples are represented as probability plot based on measurements of total carbon (n = 746) and urea nitrogen (n= 1971) of human urine samples

The protein composition observed following Coomassie-staining was found to be rather unusual as only one intense band at an apparent MW of ~ 70 kDa and very few faint other signals were visible. In-gel tryptic digestion, LC-HRMS/MS analysis and a database search using Proteome Discoverer Software revealed the presence of bovine albumin (Uniprot accession # P02769) instead of its human analog, clearly demonstrating a case of urine substitution.

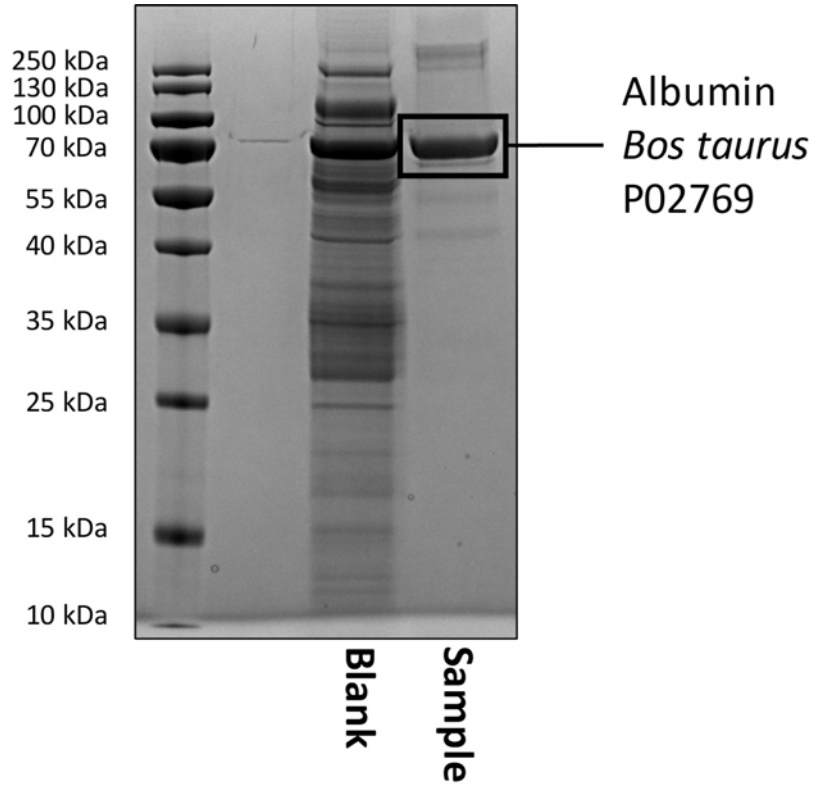


**Figure 2.** Coomassie-stained gel of the urinary retentates obtained from a blank urine specimen and the doping control routine sample suspicious for manipulation. The marked band was subjected to in-gel tryptic digestion and LC-HRMS/MS analysis

## Conclusions

The analysis of the nitrogen and carbon isotopic composition of urea and total urine as well as the protein composition of a specimen provided as a human urine sample can provide additional information about the potentially non-human origin of the constitutents in a suspicious sample.

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## Acknowledgements

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Hernández D, Coll S, Benítez L, Ventura R

# Presence of arimistane in samples with sings of bacterial degradation

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## Abstract

The formation of arimistane (androst-3,5-diene-7,17-dione) from 7-keto-DHEA (androst-5-en-3β-ol-7,17-dione) has been reported during various sample preparation procedures. However, to the best of our knowledge, a bacterial formation pathway has not been described. In our laboratory, we observed the presence of arimistane in samples exhibiting signs of bacterial degradation. The aim of this study is to determine whether arimistane is formed via bacterial pathways in degraded samples.
Data of a total of 95 samples were statistically evaluated. All samples were tested using the routine screening method for the quantification of anabolic steroids according to TD2021EAAS. Correlation tests were performed to determine relationships between arimistane levels and indicators of bacterial degradation (5αAND/A; 5βAND/Etio; DHEA; 3α5-cyclo-DHEA; androstenedione; and pH), as well as with potential arimistane precursors (7-keto-DHEA). Statistical analysis revealed significant correlations between arimistane, 7-keto-DHEA, 7αOH-DHEA concentrations, and pH. A weak correlation was also observed with degradation markers 5αAND/A and 5βAND/Etio. The observed correlations between arimistane and both 7-keto-DHEA and 5βAND/Etio suggest that arimistane may be formed from 7-keto-DHEA through microbial degradation. Therefore, arimistane alone is not a reliable biomarker for detecting its exogenous intake.

## Introduction

Arimistane (androst-3,5-diene-7,17-dione) is included in the World Anti-Doping Agency Prohibited List due to its aromatase-inhibiting activity [1]. Its metabolism has been described. Several hydroxylated metabolites were identified and the main metabolite described is 7β-hydroxy-arimistane. Notably, arimistane itself was not detected following administration [2,3]. Formation of arimistane from 7-keto-DHEA (androst-5-en-3β-ol-7,17-dione) via C3 dehydroxylation has been reported under chemical conditions such as dissolution in protic solvents, derivatization and acid hydrolysis [4,5].
In our laboratory, we detected arimistane in samples with bacterial degradation, in the absence of its main metabolite, suggesting possible microbial formation. These findings may complicate interpretation of results in anti-doping testing. The present study aimed to investigate whether arimistane can be produced via bacterial pathways in degraded samples.

## Experimental

Samples
In this study results of 95 urine samples received in the Catalonian Antidoping Laboratory between 2022 and 2024 were evaluated.

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Sample Preparation

All samples were prepared according to the standard operating procedure for the detection and quantification of anabolic steroids following TD2021EAAS. In summary, conjugated and unconjugated steroids were extracted at pH 9.6 using TBME after enzymatic hydrolysis (β-glucuronidase from *E. coli*) at pH 7. After centrifugation, the organic layer was transferred to a clean glass tube and evaporated to dryness. The residue was derivatized using 50 µL of MSTFA/NHâ„‚l/ethanethiol (1000:2:6, v/w/v) at 60°C for 20 minutes.

Instrumental analysis

The extracts (2 µL) were analysed by GC-MS/MS using an Agilent 7890A Gas System / 7000 MS triple Quad (Agilent Technologies). The column was a HP-Ultra1 (17 m x 0.2 mm, 0.11 µm) from J & W Scientific. The GC temperature was ramped as follows: initial 180°C, increase at 3°C/min to 220°C, increase at 10°C/min to 240°C, increase at 70°C/min to 310°C (2 min). The injector and transfer lines were kept at 315°C. A split ratio 1:10 was used. Helium was used as a carrier gas working under constant pressure. The source was set at 250°C. The MS was operated in electron ionization mode (70eV, 150°C). Dynamic Multi-Reaction Monitoring (dMRM) mode was employed.

Specific gravity and pH measurements

Specific gravity and pH were measured using a J457 refractometer (Rudolph Research Analytical) coupled with a Metrohm 780 pH meter and an Autoflex R837 autosampler. Measurements were made after calibration, using centrifuged aliquots.

Statistical Analysis

Pearson correlation tests were used to evaluate the relationship between arimistane concentration (corrected for specific gravity) and indicators of bacterial degradation (5α-androstandione/androsterone (5αAND/A); 5β-androstandione/etiocholanolone (5βAND/Etio); dehydroepiandrosterone (DHEA); 3α5-cyclo-DHEA; androstenedione; and pH), as well as potential precursors (7-keto-DHEA and 7α-hydroxy-DHEA (7α-OH-DHEA)). Statistical analyses were performed using XLSTAT 2024 version 4.1.

## Results and Discussion

Arimistane and 7β-hydroxy-arimistane were identified by comparing retention times and transitions with reference-spiked and excretion urine samples (Figure 1). Arimistane, in the absence of its main metabolite (7β-hydroxy-arimistane), was found in the 95 samples evaluated. All 95 samples showed signs of degradation (elevated pH and/or 5αAND/A or 5βAND/Etio > 0.1).

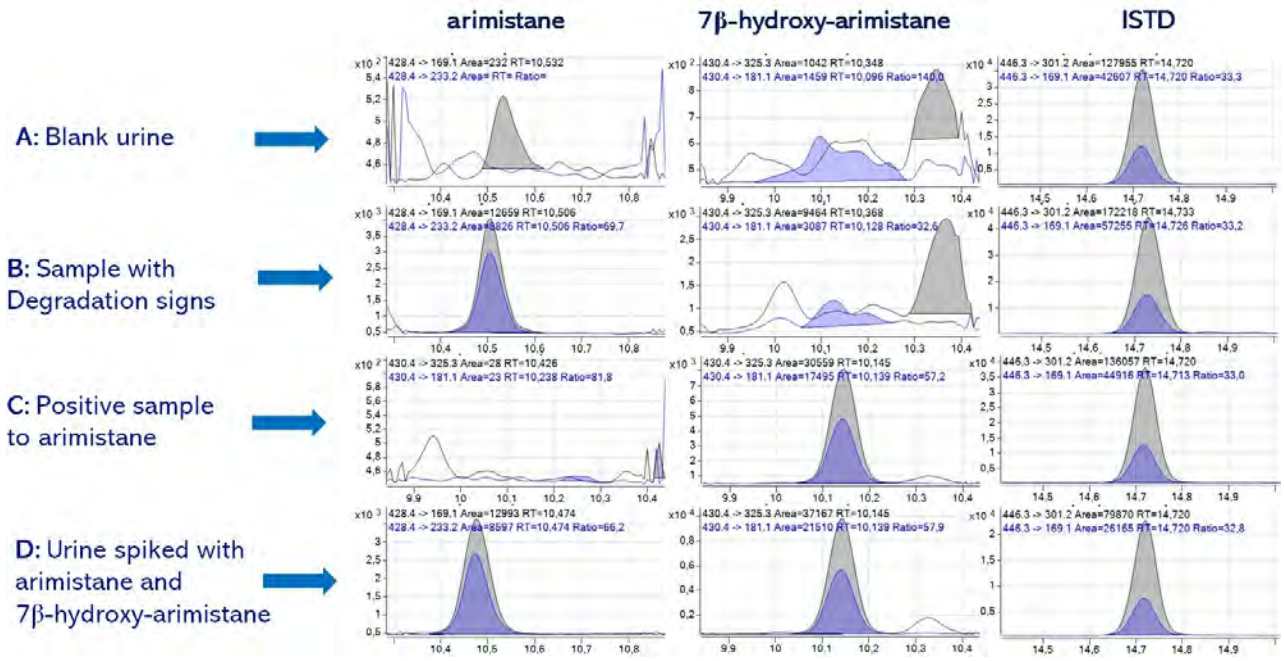


**Figure 1.** Chromatograms of: (A) Blank urine sample; (B) Urine sample showing signs of bacterial degradation (presence of arimistane without 7β-hydroxy-arimistane); (C) positive sample to arimistane and (D) Blank urine spiked with arimistane and 7β-hydroxy-arimistane (reference standard)

Descriptive statistics for arimistane levels, degradation markers, and potential precursors are shown in Table 1. Arimistane levels ranged from 2 ng/mL to 21.5 ng/mL. About 25% of the samples (3rd quartile) showed elevated 5βAND/Etio ratios, indicating moderate to high bacterial degradation [6]. Most samples (1st quartile) had pH > 8, further supporting bacterial degradation. Nearly half of the samples showed 7-keto-DHEA levels higher than those reported in a Caucasian athlete population [7].

| | Minimum | 1st Quartile | Median | 3rd Quartile | Maximum |
|---|---|---|---|---|---|
| arimistane | 2,0 | 2,9 | 4,1 | 6,1 | 21,5 |
| pH | 5,1 | 8,5 | 9,1 | 9,2 | 9,6 |
| 7-keto-DHEA | 0,3 | 1,4 | 2,4 | 4,4 | 22,3 |
| 7αOH-DHEA | 1,6 | 7,7 | 12,5 | 20,6 | 35,2 |
| DHEA | 5,1 | 27,1 | 39,3 | 60,8 | 266,5 |
| 3α5cydo-DHEA | 2,1 | 14,6 | 77,5 | 177,7 | 7706,8 |
| AED | 0,7 | 2,9 | 5,9 | 18,1 | 191,4 |
| 5βAND | 0,3 | 2,8 | 16,2 | 101,6 | 954,6 |
| 5αAND | 0,2 | 0,7 | 3,5 | 45,5 | 1263,9 |
| 5αAND/A | 0,00 | 0,00 | 0,00 | 0,01 | 1,23 |
| 5βAND/Etio | 0,00 | 0,00 | 0,01 | 0,06 | 1,30 |

**Table 1.** Descriptive statistics of the 95 samples evaluated. Concentrations of arimistane, 7-keto-DHEA, 7α-OH-DHEA, DHEA, 3α5-cyclo-DHEA, androstenedione (AED), 5βAND, and 5αAND are expressed in ng/mL

Significant correlations (95% confidence) were found between arimistane concentrations and 7-keto-DHEA (p=0.001), 7α-OH-DHEA (p=0.005) and pH (p=0.005) (Figure 2). These findings support 7-keto-DHEA as the precursor of arimistane, especially under conditions of of high pH and potential microbial

activity. A positive correlation was observed, suggesting increased formation of arimistane at higher pH levels, potentially due to bacterial degradation (Figure 3).

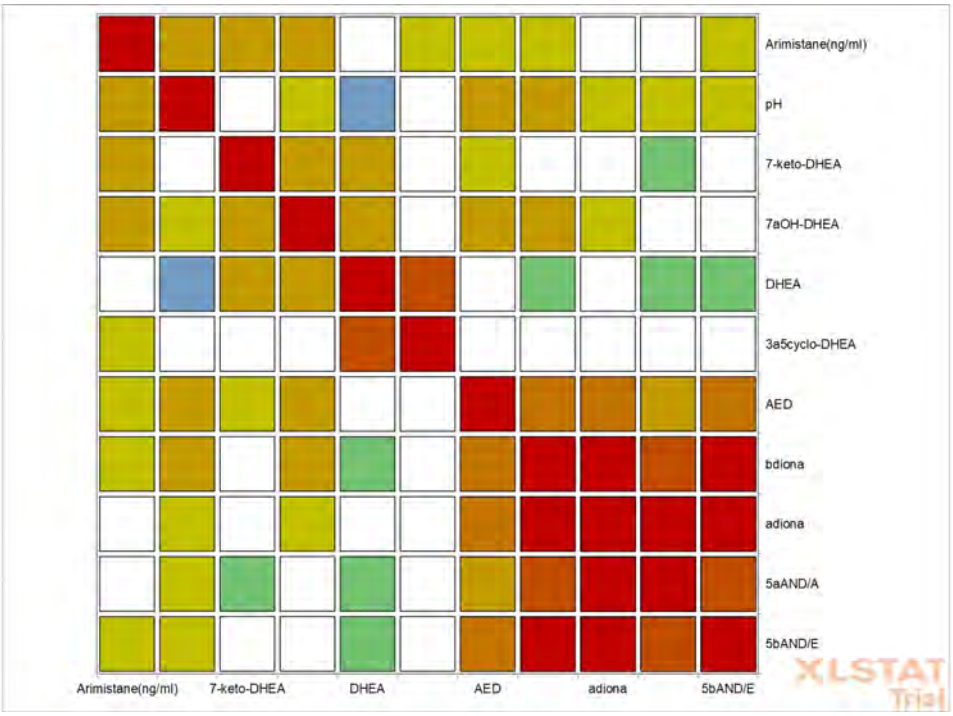


**Figure 2**. Correlation matrix between arimistane and related variables. Color intensity represents the strength of the correlation: **red** indicates a strong positive correlation, while **white** indicates no correlation. Correlation coefficients are based on Pearson's r values (n = 95)

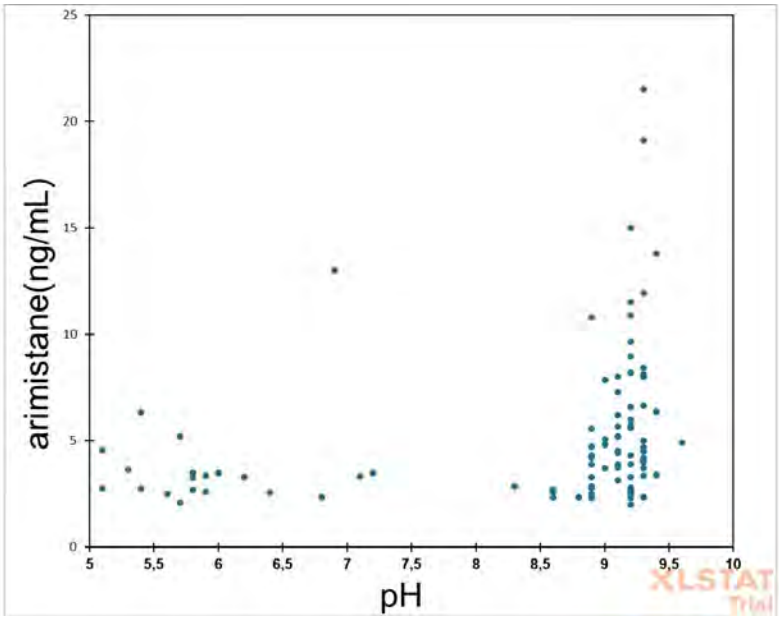


**Figure 3.** Relationship between arimistane concentration and urinary pH

Arimistane levels showed a slight correlation (no statistically significant) with 3α5cyclo-DHEA, AED, 5βAND and 5βAND/Etio levels, while DHEA, 5αAND and 5αAND/A levels showed no correlation with arimistane (Figure 2).

Our results suggest the following evaluation criteria after arimistane detection:

- Case 1: Presence of 7-hydroxy-arimistane, absence of arimistane, normal 7-keto-DHEA levels, no degradation → likely arimistane intake
- Case 2: Presence of arimistane, absence of 7-hydroxy-Arimistane, high 7-keto-DHEA levels, degradation signs → arimistane microbial formation
- Case 3: Presence of both arimistane and 7-hydroxy-Arimistane, high 7-keto-DHEA, degradation signs → likely arimistane intake plus bacterial degradation

In addition, the recommendations of TL20 have to be followed [8].

## Conclusions

Arimistane was detected in urine samples with signs of bacterial degradation at concentrations up to 21.5 ng/mL. The main metabolite detected after arimistane consumption, 7β-hydroxy-arimistane, was not detected in these urines. Although results of this study showed no linear correlation between arimistane and pH, most of the samples containing arimistane had a pH higher than 8. A slight correlation was observed between arimistane concentrations and both 7-keto-DHEA and the 5βAND/Etio ratio, suggesting that arimistane may be formed from 7-keto-DHEA as a result of microbial degradation. Our results indicate that arimistane is not a reliable target analyte for detecting arimistane consumpttion.

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# Analysis of microgreens for the presence of doping-relevant substances of natural origin

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## Abstract

Microgreens represent a modern option for the dietary strategies of competitive athletes and can be considered as products ranging between conventional food and nutritional supplements. Hence, to clarify whether microgreens contain plant-synthesized doping-relevant substances of natural origin, three microgreen species (radish, mustard and arugula) as well as their seeds and seed mats were analyzed. None of the 24 target substances were detected in the microgreens at any time. The risk of an unintentional antidoping rule violation through the consumption of microgreens is considered to be negligible. However for a final decision a more comprehensive investigation enclosing more microgreens would be necessary.

## Introduction

Nutritional supplements have been identified as the source of unintentional anti-doping rule violations at various occasions in the past [1]. As a plant-based natural alternative to supplements, so-called „microgreens“ of various species are advertised. Microgreens are young plants, which are rich in nutrients and can be cultivated at home. The WADA Prohibited List [2] includes a variety of naturally occurring and plant-derived substances, mainly covering the categories stimulants, narcotics and cannabinoids, but also ß2 agonists. For nearly all target analytes of the study, a minimum reporting level (MRL) is established [3], with the exception of cannabinoids (apart from THC, with a fixed urinary decision limit of 180 ng/mL [4]). However, an unintentional anti-doping rule violation can only be excluded when the target substances are not detectable in measurable quantities.
Three variants of microgreens as well as their seeds and seed mats were analyzed for the presence of 24 plant-synthesized doping-relevant substances in order to estimate the risk of an unintentional antidoping rule violation.

## Experimental

Microgreens of the species radish, arugula and mustard were grown according to the manufacturer’s instructions (Figure 1) and harvested on day 5, 8 and 13 (Figure 2). The microgreens, their seeds and seed mats were analyzed for the presence of selected doping-relevant substances according to established methods by means of GC-MS and LC-MS [5-7].

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**Figure 1. Microgreens day 1 (left to right: radish, arugula, mustard)**

**Figure 2. Microgreens day 13 (left to right: radish, arugula, mustard)**

Target substances

GC-MS/MS (detection level: 10 ng/g)
Androst-4-ene-3,17-dione, Cannabichromene, Cannabicyclol, Cannabidiol, Cannabidiolic acid, Cannabidivarin, Cannabidivarinic acid, Cannabigerol, Cannabigerolic acid, Cannabinol, Cannabinolic acid, Tetrahydrocannabidivarin, Tetrahydrocannabidivarinic acid, Δ8-Tetrahydrocannabinol, Δ9-Tetrahydro-cannabinol (THC), Δ9-Tetrahydrocannabinolic acid

LC-MS/MS (detection level: 100 ng/g)
Cathine, Ephedrine, Pseudoephedrine, Higenamine, Cocaine, Morphine, Strychnine

LC-MS/MS (detection level: 200 ng/g)
Methylhexaneamine, Octopamine

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## Results and Discussion

None of the target substances was detected in the microgreens, their seeds and seed mats at any time.

## Conclusions

The risk of an unintentional antidoping rule violation through the consumption of microgreens is considered negligible. However, the conclusion is based on the investigation of only three species of microgreens. For a final decision, a more comprehensive investigation enclosing a variety of microgeens would be necessary.

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# Analysis of hemp cosmetics for the presence of cannabinoids

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## Abstract

Substantial risks for an antidoping rule violation for athletes consuming cannabidiol (CDB) or hemp products are known.
To clarify whether hemp cosmetics contain $\Delta^9$- tetrahydrocannabinol (THC) or other cannabinoids relevant for doping controls, a total of 33 different commercially available hemp cosmetics was tested for the presence or absence of the selected cannabinoids.
In 25 products, the detection of prohibited cannabinoids was possible. Most abundant were cannabingerol (CBG), cannabidivarin (CBDV) and cannabidiolic acid (CBDA). In 11 products THC was detected. All products that declared the constituent “CBD” contained cannabinoids. Products declared to be composed of “synthetic CBD” contained also other cannabinoids as well as THC.
In light of these data, a risk for an antidoping rule violation for athletes utilizing hemp cosmetics may be given and should be further investigated by application studies of the products.

## Introduction

The interest in the consumption of hemp products is continuously growing, with an expanding scope of applications. Hemp products are prepared from cannabis plants and, therefore, might contain a variety of different natural cannabinoids. Synthetic CBD is also used as ingredient in hemp cosmetics. According to the regulations of the World Anti-Doping Agency, all natural and synthetic cannabinoids are prohibited in-competition, with the explicit exemption of CBD. Substantial risks for an antidoping rule violation for athletes consuming CBD [1] or hemp products [2] are known. To clarify whether hemp cosmetics contain THC or other cannabinoids relevant for doping controls, a total of 33 different commercially available hemp cosmetics (shampoos, shower gels, hand creams, body lotions, massage and body oils, hair tonics, mouth and muscle sprays, lip sticks - Figure 1) was tested for the presence or absence of the selected cannabinoids.

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**Figure 1.** Investigated hemp cosmetics

## Experimental

A total of 33 products was analyzed for the presence of doping-relevant cannabinoids (Table 1) according to an established method by means of GC-MS [2].

| Compound | Abbreviation | Compound | Abbreviation |
|---|---|---|---|
| Cannabidivarin | CBDV | Cannabinol | CBN |
| Cannabidiol | CBD | Cannabigerol | CBG |
| Cannabicyclol | CBL | $\Delta^9$-Tetrahydrocannabivarinic acid | THCVA |
| Cannabichromene | CBC | Cannabidiolic acid | CBDA |
| $\Delta^8$-Tetrahydrocannabinol | $\Delta^8$-THC | $\Delta^9$-Tetrahydrocannabinol acid | THCA |
| $\Delta^9$-Tetrahydrocannabinol | THC | Cannabinolic acid | CBNA |
| Cannabidivarinic acid | CBDVA | Cannabigerolic acid | CBGA |

**Table 1.** Cannabinoid target analytes

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## Results and Discussion

The detection of prohibited cannabinoids was possible in 25 of 33 products (76%). The most abundant cannabinoids besides CBD were CBG, reaching concentrations up to 1.5 mg/g, followed by CBDV (maximum concentration ~ 247 µg/g), and CBDA (maximum concentration ~ 277 µg/g).
All parts of the hemp plant can contain cannabinoids. According to literature, hemp seeds do not contain cannabinoids. If cannabinoids are found in oil and seeds, these residues are attributed to the external contact of the seed hulls with cannabinoids-containing resins in bracts and leaves during maturation, harvesting and processing. Due to the fact that hemp products are commonly prepared from cannabis plants, the occurrence of natural cannabinoids including THC in the respective products cannot be excluded. In 11 of 33 products, THC was identified (Table 2), for five of the products CBD was declared in the list of ingredients, and for two further products “synthetic CBD" was mentioned.
The detection of prohibited cannabinoids was possible in all products declared to contain CBD, including those labeled with “synthetic CBD”. Here it should be noted that the chemical transformation of CBD into cannabinoids under acidic reaction conditions is possible [3].

| | | | CBDV | CBD | CBL | CBC | Δ8-THC | Δ9-THC | CBDVA | CBN | CBG | THCVA | CBDA | THCA | CBNA | CBGA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Product | [µg/g] | [µg/g] | [µg/g] | [µg/g] | [µg/g] | [µg/g] | [µg/g] | [µg/g] | [µg/g] | [µg/g] | [µg/g] | [µg/g] | [µg/g] | [µg/g] |
| 1 | | Shower gel | | 0.014 | | | | 0.023 | | 0.013 | | | | | | |
| 2 | # | Shower gel | 0.025 | 252.279 | | 0.105 | 0.242 | 0.066 | 0.126 | 0.503 | 0.433 | | 0.571 | | | |
| 3 | # | Hand cream | 4.194 | 7,015.890 | | | | 0.135 | | 0.601 | | | | | | |
| 4 | | Hand cream | 0.089 | 5.031 | | 0.164 | | 0.323 | | 0.210 | | | 3.345 | | | 0.067 |
| 5 | | Ointment | 0.147 | 69.144 | 0.119 | 17.195 | 0.947 | 7.055 | 1.030 | 0.448 | 1.403 | 0.072 | 277.369 | 9.389 | | 2.331 |
| 6 | * | Muscle spray | 106.208 | 17,576.006 | | 5.043 | | 58.519 | | | | | 1.881 | | | |
| 7 | # | Sports gel | 1.853 | 3,202.375 | | 56.937 | | 4.619 | | | | | 3.986 | | | |
| 8 | * | Massage oil | 1.292 | 5,075.626 | | | | 0.529 | | | | | | | | |
| 9 | | Massage oil | 0.240 | 20.730 | | 1.376 | 1.733 | 3.933 | | 1.284 | 0.928 | 1.131 | 60.643 | 4.721 | 10.100 | 2.660 |
| 10 | # | Mouth spray | 6.069 | 137,249.492 | | | | 25.723 | | 2.463 | | | | | | |
| 11 | # | Lip stick | 247.183 | 29,369.244 | 10.363 | 46.990 | | 13.472 | | 8.565 | 1,547.470 | | 30.999 | | | |

* declaration list of ingredients: „synthetic CBD“
# declaration list of ingredients: CBD

**Table 2.** Cannabinoids in hemp cosmetics

## Conclusions

- Hemp cosmetics contain a variety of cannabinoids at occasionally substantial concentrations.
- Elimination studies with the investigated hemp cosmetics should be performed to evaluate the risk of an unintentional antidoping rule violation, caused by the absorption of cannabinoids contained in hemp cosmetics

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# Risk of unintentional antidoping rule violations by application of hemp cosmetics

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## Abstract

To clarify whether the application of cannabinoid containing hemp cosmetics can lead to findings of prohibited cannabinoids in urine, controlled single and multiple dose administration studies were conducted with 25 different hemp-based cosmetics, for which the presence of cannabinoids was previously confirmed.
For 5 of 25 hemp cosmetics (20%), cannabinoids were observed in the excretion study urine samples collected 3 hours after use of the cosmetic product. In particular the repeated application of a lip stick yielded enriched urinary concentrations for cannabinoids in the monitored time-course.
The application of hemp-based cosmetics can lead to findings of prohibited cannabinoids in urine and, consequently, contributes to the risk of an unintentional violation of anti-doping regulations.

## Introduction

Recently performed administration studies with hemp-based and cannabidiol (CBD)-containing products showed a substantial risk of an antidoping rule violation for athletes [1,2]. To clarify whether also cosmetics declaring hemp-derived constituents contain $\Delta^9$-tetrahydrocannabinol (THC) or other cannabinoids relevant for doping controls, a total of 33 different commercially available hemp-based cosmetics was analyzed. The investigated products were found to contain a variety of cannabinoids at, occasionally, substantial concentrations. In 25 products, the detection of prohibited cannabinoids was possible [3]. In light of these data, a risk for an antidoping rule violation for athletes utilizing hemp-based cosmetics may be given and was further investigated and assessed by application of the products.

## Experimental

For 25 different hemp-based cosmetics (Figure 1) with previously confirmed presence of prohibited cannabinoids [3], elimination studies were performed. Healthy volunteers applied the products following the manufacturer´s dosage recommendations and provided urine samples, collected before and 3, 6, 9 and 12 hours after product application. For one product (#5), multiple applications were carried out on three consecutive days. The excretion studies were performed with approval (number 138/2022) of the ethics committee of the German Sport University Cologne (Germany) and written informed consent was obtained from all participants. Urine samples were analyzed for the presence of doping-relevant cannabinoids according to an established method by means of GC-MS [1]. The cannabinoid concentrations were adjusted to a urine specific gravity (SG) of 1.020 [4].

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**Figure 1.** Hemp cosmetics for application studies

## Results and Discussion

Following single-dose administrations, urine samples were collected 3, 6, 9 and 12 hours after product application. In case of five (Table 1) of 25 hemp-based cosmetics application experiments (20%), the detection of cannabinoids was possible in specimens collected 3 h after use (Table 2). CBD as the main cannabinoid of the hemp plant was detected in urine after application of 4 of these 5 cosmetics in variable concentrations. Further, the prohibited cannabinoids cannabidivarin (CBDV), cannabinol (CBN) and cannabigerol (CBG) were detected in post-application urine samples at concentrations ranging from 0.07 – 1.36 ng/mL.

| Product | Formulation | Hemp ingredients (ingredient list) | Application |
|---|---|---|---|
| 1 | Hand creme | Hemp seed oil, hemp seed extract | single |
| 2 | Ointment | Extracts from cannabis leaf and tribe | single |
| 3 | Mouth spray | Hemp extract, hemp seed oil, CBD | single |
| 4 | Lip stick | Hemp seed oil | single |
| 5 | Lip stick | Hemp seed oil, CBD | multiple |

**Table 1.** Hemp cosmetics with detected cannabinoids in excretion study urine samples

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| | Cannabinoid concentration (ng/mL) | | | |
|---|---|---|---|---|
| Product | CBD (Cannabidiol) | CBDV (Cannabidivarin) | CBN (Cannabinol) | CBG (Cannabigerol) |
| 1 | 0.29 | | 0.07 | |
| 2 | | | | 0.83 |
| 3 | 113 | 1.08 | | |
| 4 | 0.06 | | | |
| 5 | 0.30 | 0.26 | | 1.36 |

**Table 2.** Estimated urinary concentrations of cannabinoids (ng/mL) in excretion study urine samples, collected 3 hours after application of hemp cosmetics according to the manfacturer-recommended dosage

The urine specimens obtained after repeated application of product number 5 (lipstick) were shown to return findings for cannabinoids at 3 and 12 h after each use (Table 3), yielding enriched urinary concentrations for cannabinoids in the monitored time-course.

Cannabinoids present in formulations such as “mouth spray” and “lip stick” appear to have a higher risk of entering the body, presumably resulting from a reduced barrier function of the skin in the mouth and lip area and the unintentional oral absorption of products when applying the cosmetics.

| | Cannabinoid concentration (ng/mL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | CBD (Cannabidiol) | | CBDV (Cannabidivarin) | | CBN (Cannabinol) | | CBG (Cannabigerol) | |
| Collection time | 3 h | 12 h | 3 h | 12 h | 3 h | 12 h | 3 h | 12 h |
| Day 1 | 0.30 | 1.17 | 0.26 | 0.29 | | | 1.36 | 6.28 |
| Day 2 | 2.17 | 3.35 | 0.61 | 0.68 | 0.01 | 0.04 | 1.72 | 2.26 |
| Day 3 | 8.25 | 2.69 | 2.00 | 0.47 | 0.05 | | 4.38 | 1.09 |

**Table 3**. Estimated urinary concentrations of cannabinoids (ng/mL) in excretion study urine samples (multiple application of product 5), collected 3 and 12 hours after application of 3 subsequent days

## Conclusions

- The application of hemp-based cosmetics can lead to findings of prohibited cannabinoids in urine.
- Comprehensive information and thorough education of athletes concerning the risk associated with the consumption of hemp-based products is necessary and important.
- Revisiting reporting levels for cannabinoids might be warranted.

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# Findings of undeclared doping substances in nutritional supplements in follow-up investigations of adverse analytical findings (2021 – 2024)

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## Abstract

Results of the follow-up investigation into the evaluation of contaminated nutritional supplements (NS) as source of adverse analytical findings (AAF) in doping control urine samples between 2014 and 2020 (7 years) are presented. Here, analytical data on NS, which were received in connection with AAFs between 2021 and 2024 (4 years), are summarized.

It could be shown that in 22 of 114 NS, doping substances not declared on the product labels were detected. Seventeen of these products were received with intact packaging seals. The test results of 5 unsealed open products could not be verified by the analysis of the corresponding originally packed, sealed, and independently obtained products. In 11 products, doping substances were detected in the products intact packaging seals as well as in the corresponding opened supplements. In approximately 40% of the follow-up investigations with regards to AAFs, the detection of prohibited substances in the analyzed NS was possible. The roughly estimated concentrations in the supplements cover a wide range, suggesting that some products were contaminated while others were most likely intentionally adulterated with prohibited substances. The detected substances belong to various classes of the WADA Prohibited List, with the substance class “other anabolic agents” being the most affected.

The number of doping cases with analyzed supplements increased from 5 to 10 cases per year. The affected substance classes have changed from anabolic androgenic steroids and stimulants to SARMS and diuretics.

## Introduction

According to the regulations of the World Anti-Doping Agency (WADA) International Standard for Laboratories (ISL) Annex A: Code of Ethics [1], laboratories may analyze commercial material or preparations (e.g. dietary or herbal supplements) if specifically requested by an Anti-Doping Organization or a hearing body as part of a Results Management or adjudication process. In 2021, the findings of contaminated nutritional supplements (NS), analyzed between 2014 and 2020 (7 years) in the context of investigations into adverse analytical findings (AAFs) were evaluated and published [2]. In total, 24 (22%) of 110 analyzed NS returned positive test results. Fourteen of the 24 positive NS were obtained as originally closed and sealed containers. The detected substances belong to various classes of the WADA Prohibited List [3]. Most of them are categorized as anabolic androgenic steroids (AAS), other anabolic agents, and specified stimulants, but also diuretics and glucocorticoids were found. As follow-up, a comparable evaluation of the results since 2021 was performed.

## Experimental

Evaluation of the results of the analysis of NS, which were received in connection with AAFs between 2021 and 2024.
The analysis was performed using gas chromatograhy-mass spectrometry and liquid chromatography-mass spectrometry according to methods described by Parr *et al.* [4] and Geyer *et al.* [5] with modifications adapted to the respective matrix.

## Results and Discussion

A total of 114 nutritional supplements was analyzed on request of the relevant RMA in connection with the investigation of doping cases between 2021 and 2024. For 22 cases, a total of 33 positive findings was recorded, seventeen results originating from originally closed and sealed products, and sixteen results from supplements that were already in use by athletes (Tables 1 and 2).
The detected substances belong to various classes of the WADA Prohibited List [3]. Most of them originate from the class other anabolic agents (SARMS), followed by the classes of anabolic androgenic steroids and specified stimulants (Table 2).
The roughly estimated concentrations in the supplements cover a wide range, suggesting that some products were contaminated while others were most likely intentionally adulterated with prohibited substances (Table 1).
In 40%, the adverse analytical findings of the doping control urine samples could be traced back with a high probability to the consumption of the investigated supplements. However, between 2014 and 2020, that share was found to be higher with 49%. The number of doping cases with analyzed supplements increased from 5 to 10 cases per year. The affected substance classes have changed from anabolic androgenic steroids and stimulants to SARMS and diuretics.

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| Year | Product | Production (*distribution) country | Pharmaceutical form | Detected doping substance | Positive result obtained from open container (used supplement) | Positive result obtained from originally closed and sealed container (closed supplement) |
|---|---|---|---|---|---|---|
| 2021 | Slimming product | Brasil | Tea | Hydrochlorothiazide ~ 30 µg/g | X | X |
| | Pre workout supplement | Russia | Powder | 4-Methylhexan-2-amine ~ 16 mg/g | X | X |
| 2022 | Pre workout supplement | Sweden | Powder | 5-Methylhexan-2-amine ~ 3 mg/g | X | |
| | Amino acids | USA | Powder | Metandienone ~ 954 µg/g | X | |
| 2023 | Ecdysterone | *Thailand | Capsules | S-23 ~ 3.7 ng/g | | X |
| | Testosterone booster | USA | Capsules | S-23 ~ 0.6 ng/g | X | X |
| | Ecdysterone | *Thailand | Capsules | S-23 ~ 1.7 ng/g | X | X |
| | Testosterone booster | USA | Capsules | S-23 ~ 0.8 ng/g | X | X |
| | Testosterone booster | USA | Capsules | S-23 ~ 0.6 ng/g | | X |
| | Ecdysterone | *Thailand | Capsules | S-23 ~ 2.0 ng/g | | X |
| | Ecdysterone | *Thailand | Capsules | S-23 ~ 12.4 ng/g | X | |
| | Testosterone booster | USA | Capsules | S-23 ~ 1.2 ng/g | X | X |
| | Ecdysterone | *Thailand | Capsules | S-23 ~ 1.1 ng/g | X | |
| | Testosterone booster | USA | Capsules | LGD-4033 ~ 47.1 ng/g | X | X |
| | Tribulus Terrestris | India | Capsules | Methasterone ~ 8.4 mg/g | | X |
| | Tribulus Terrestris | Bulgaria | Tablets | Methasterone ~ 184 mg/g | | X |
| | Nootropics | USA | Capsules | Ostarine ~ 20 µg/g | X | X |
| | Testosterone booster | USA | Capsules | DHCMT ~ 10.8 µg/g | X | |
| | Testosterone booster | USA | Capsules | Metandienone ~ 1.0 µg/g | X | X |
| 2024 | Pre workout supplement | UK | Powder | 5-Methylhexan-2-amine ~ 28.3 µg/g | | X |
| | Ecdysterone | *Thailand | Capsules | 5-Methylhexan-2-amine ~ 1175 µg/g | X | X |
| | Testosterone booster | USA | Capsules | 5-Methylhexan-2-amine ~ 162 µg/g | X | X |

**Table 1.** Nutritional supplements contaminated or intentionally adulterated with prohibited substances (2012-2024)

| | Total number of cases | Total number of analyzed supplements | Positive findings in used supplements | Positive findings in closed supplements |
|---|---|---|---|---|
| SARMS | 21 | 54 | 8 | 9 |
| Anabolic steroids | 7 | 25 | 3 | 3 |
| Diuretics | 5 | 15 | 1 | 1 |
| Stimulants | 6 | 9 | 4 | 4 |
| β2-Agonists | 2 | 7 | 0 | 0 |
| Aromatase inhibitors | 1 | 4 | 0 | 0 |
| **Total number** | **42** | **114** | **16** | **17** |

**Table 2.** The number of analyzed supplements and AAFs based - with high probability - on contaminated or intentionally adulterated nutritional supplements (2021-2024)

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## Conclusions

It could be shown that in 22 of 114 NS, analyzed in connection with AAFs, doping substances not declared on the product labels were detected. In such cases, it is important that the result management authority (RMA) conduct further investigations to assess the consistency between the application regimen of the NS (dose, frequency, duration, last application before doping control sample collection, etc.) and the estimated concentration of the detected substance in the doping control sample.

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# Black market products suspected to contain doping relevant ingredients - report for 2024

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## Abstract

Doping in popular and recreational sport has been reported for decades. The main goals of consumers are: a better appearance, health, and weight loss. The black market for performance enhancing drugs is a common source. The analysis of confiscated products, as well as products from test purchases is one aspect of monitoring the black market regarding emerging and established performance enhancing drugs [1-4]. The European Monitoring Center for Emerging Doping Agents (EuMoCEDA) analyzed a total of 41 products qualitatively and quantitatively in 2024. An outstanding finding was the detection of insulin instead of semaglutide in an Ozempic®-labelled product.

Anabolic agents, growth factors, as well as growth hormone releasing factors were determined by high performance liquid chromatography / high resolution mass spectrometry (HPLC-(HR)MS)-experiments. For gas chromatography / (high resolution) mass spectrometry (GC-(HR)MS) – experiments, analytes were derivatized and measured in full-scan mode. Included substances were anabolic agents, stimulants, beta-2-agonists and narcotics. For the analysis of peptides and proteins, aliquots were separated by polyacrylamide gel electrophoresis and subsequently stained with coomassie blue. Analytes included, but were not limited to human growth hormone (hGH), growth factors (e.g.: FGF, MGF, etc.), various erythropoietins (EPO), and growth hormone releasing factors [5].

In 2024, a total of 41 suspicious (illicit) black market products were analyzed at the Center for Preventive Doping Research. Doping-relevant findings accounted in 72 cases for 21 different drugs (multi-findings included), from which 53% of the ingredients were not or falsely declared. Anabolic agents were determined in 89% of all identified doping relevant compounds; 7% accounted for Peptide hormones, growth factors, related substances and mimetics, and 4% accounted for hormone and metabolic modulators. The analytes, which were currently not doping relevant, were sugars, fatty acids, and vitamins.

An outstanding finding was the detection of insulin in a product labelled to contain semaglutide (Ozempic®). Espacially in the light of the health risks associated with unknowingly administering insulins this product underlines once more the relevance of screening for peptide ingredients, and although these make up a rather small proportion of the total number of doping-relevant findings, they still repeatedly lead to unusual results.

## Introduction

The source of performance enhancing drugs for recreational/mass sport athletes is still the black market [1-3]. The assessment of confiscated products, as well as products from test purchases is an important

and essential part of monitoring the black market regarding developments to novel performance enhancing drugs. The European Monitoring Center for Emerging Doping Agents (EuMoCEDA) analyzed a total of 41 products qualitatively and quantitatively throughout 2024.

## Experimental

Depending on the formulation (oily solution, lyophilized, etc.), samples were solved or extracted with water, acetic acid (2% aq.), and/or acetonitrile (50:50 v/v) and subsequently diluted to yield an adequate concentration of labeled drug content. For gas chromatography, extracted and afterwards dried samples were reconstituted in ethyl acetate, derivatized with N-methyl-N-(trimethylsilyl)-trifluoro-acetamide (MSTFA), or a mixture of MSTFA / ethanethiol and ammonium iodide, respectively [1]. The samples were screened by HPLC-ESI-MS using an Acquity UPLC interfaced to a Xevo TQ-XS from Waters. For HRMS experiments, a Thermo Q-Exactive plus, a Thermo Exploris, and an Agilent 6550 iFunnel Q-TOF mass spectrometer were used. GC-MS experiments were performed on a Trace 1310 Gas Chromatograph in combination with a TSQ 8000 Evo Triple Quadrupole Mass Spectrometer from Thermo. To screen the most common target analytes in black market products, high performance liquid chromatography/mass spectrometry (HPLC-MS) experiments were conducted in multiple-reaction-monitoring (MRM) mode. Anabolic agents, stimulants, growth factors, natural and synthetic insulins, IGF-1 and synthetic analogs as well as growth hormone releasing factors could be determined by high performance liquid chromatography / high resolution mass spectrometry (HPLC-HRMS)-experiments in full-scan mode. Qualification and quantification of analytes were obtained by conducting product-ion scans with substance-specific fragmentation pathways. For gas chromatography / mass spectrometry (GC-MS) experiments, analytes were derivatized and measured in full-scan mode. Qualitative and quantitative analysis were accomplished by using reference substances and/or reference databases. Included substances were anabolic agents, stimulants, beta-2-agonists and narcotics [1]. For the analysis of proteins, aliquots were separated by polyacrylamide gel electrophoresis and subsequently stained with coomassie blue. Analytes included, but were not limited to human growth hormone (hGH), growth factors (e.g.: FGF, MGF, etc.), various erythropoietins (EPO), and growth hormone releasing factors [5].

## Results and Discussion

In 2024, a total of 41 suspicious (illicit) black market products were analyzed at the Center for Preventive Doping Research. Doping-relevant findings accounted in 72 cases for 21 different drugs (multi-findings included), from which 53% of the ingredients were not or falsely declared (Table 1).

| Drug | Finding | Labelled | Not labelled | |
|---|---|---|---|---|
| **S1. Anabolic agents** | | | | Doping relevant |
| Boldenone | 1 | | 1 | |
| -Undecylenate | 8 | 5 | 3 | |
| Clenbuterol | 1 | 1 | | |
| Drostanolone | | | | |
| - Propionate | 2 | 2 | | |
| Metandienone | 2 | 2 | | |
| Nandrolone | | | | |
| - Decanoate | 1 | 1 | | |
| Stanozolol | 4 | 2 | 2 | |
| Testosterone | | | | |
| - Cypionate | 1 | | 1 | |
| - Decanoate | 1 | | 1 | |
| - Enantate | 13 | 4 | 9 | |
| - Isocaproate | 3 | 1 | 2 | |
| - Phenylpropionate | 2 | 2 | | |
| - Propionate | 15 | 3 | 12 | |
| Trenbolone | | | | |
| - Acetate | 6 | 4 | 2 | |
| - Enantate | 4 | 1 | 3 | |
| **S2. Peptide hormones, growth factors, related substances, and mimetics** | | | | |
| EPO | 2 | 2 | | |
| GHRP-2 | 1 | | 1 | |
| GHRP-6 | 1 | 1 | | |
| hCG | 1 | 1 | | |
| **S4 Hormone and metabolic modulators** | | | | |
| Clomifene | 1 | 1 | | |
| Insulin | 2 | 1 | 1 | |
| **21 Doping relevant drugs** | **72** | **34** | **38** | |
| **Unspecific / other drugs** | | | | Currently not doping relevant |
| Fatty acids | 2 | | 2 | |
| Sugars | 2 | | 2 | |
| Vitamins | 3 | 3 | | |
| Citric acid | 1 | 1 | | |
| **In total 41 products with 25 analytes** | **80** | **38** | **42** | |

**Table 1.** Identified drugs in black market products

An outstanding finding was the detection of insulin in a product labelled to contain semaglutide (Ozempic®) (Figure 1 and 2). Espacially in the light of the health risks associated with unknowingly administering insulins this product underlines once more the relevance of screening for peptide ingredients, and although these make up a rather small proportion of the total number of doping-relevant findings, they still repeatedly lead to unusual results.


**Figure 1.** Injection pen labelled to contain semaglutide (Ozempic®) but containing insulin apidra

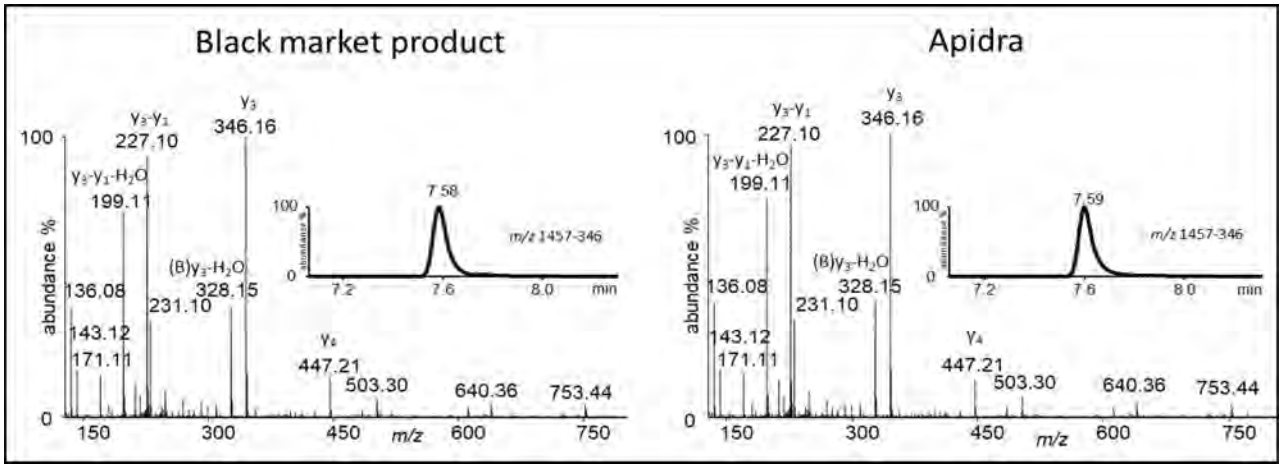


**Figure 2.** HESI-product ion spectra of insulin apidra in falsified injection pen (left) and positive control (right); XIC of ion transitoin m/z 1457-346 as insets.

As shown in Figure 3, 89% of the identified doping-relevant compounds accounted for anabolic agents; 7% accounted for peptide hormones, growth factors, related substances and mimetics, and 4% accounted for hormone and metabolic modulators. The analytes, which were currently not doping relevant, were sugars, fatty acids, and vitamins. The findings of 53% incorrectly labelled substances in 2024 confirm again the unknown health risks and the importance of continued monitoring of black market products.

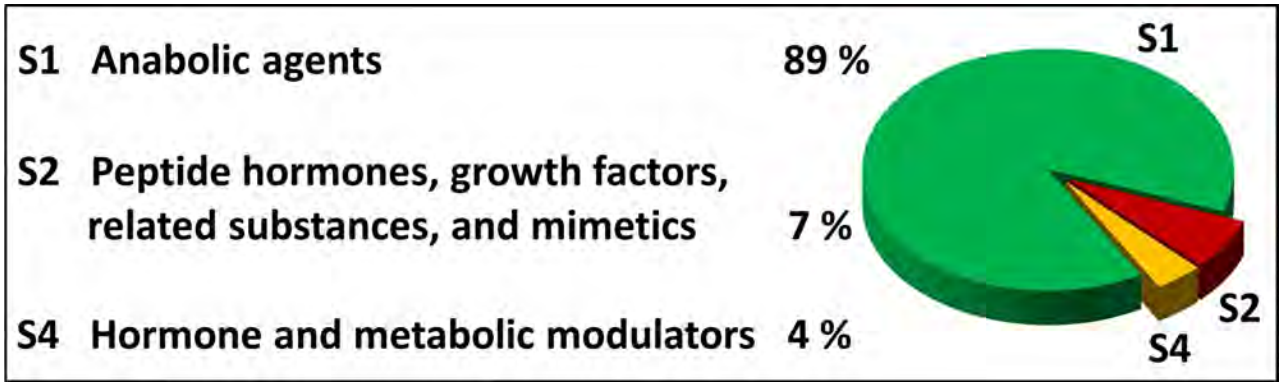


**Figure 3.** Apportionment of identified doping relevant drugs in analyzed black market products 2024

## Conclusions

The athletes of recreational/mass sport hazard their physical health by misusing black market products. Faked and falsely labeled preparations represent a spezific problem. Anabolic agents are still the most popular products to improve body shape. The Cologne Anti-Doping Laboratory´s commitment under the umbrella of EuMoCEDA, yielded the analysis of traded drugs, as well as the compilation of informations concerning availability, handling and forms of misuse of black market products. This shows again the requirement and relevance of monitoring the black market and the investigation of distributed products under the umbrella of EuMoCEDA.

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# Assessing the enzymatic hydrolysis of endogenous steroids

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## Abstract

According to the WADA technical document to detect endogenous steroids in urine (TD-EAAS), the enzymatic hydrolysis shall be carried out with purified β-glucuronidase from E. coli. The completeness of hydrolysis of the glucuroconjugated urinary steroids shall be assessed with isotopically labelled A-glucuronide (or an equivalent alternative). To our knowledge, the criteria to consider that hydrolysis was complete in a sample varies from laboratory to laboratory and, basically, depends on the expertise and/or on the results obtained during the periodic WADA EQAS, for example. The aim of the work was to study the enzymatic hydrolysis of urine samples after the incubation with β-glucuronidase varying the incubation time and the amount of A-D4-glucuronide and Etio-glucuronide used.
This work showed that (i) the hydrolysis yield is highly influenced by the urine sample content and its unique and specific features, (ii) there were no significant differences in the hydrolysis yields dependent on the concentration of A-D4 and Etio-D5, (iii) the acceptance of the hydrolysis in a sample based on the ratio A-D4/Etio-D5 should be carefully assessed (iv) no effect on the IRMS results was observed in the same sample with different hydrolysis yields and (v) the hydrolysis kinetics were not the same when samples with different isotopic content were analyzed.

## Introduction

According to the technical document TD-EAAS, the enzymatic hydrolysis shall be carried out with purified β-glucuronidase from *E. coli.* The completeness of hydrolysis of the glucuroconjugated urinary steroids shall be assessed with isotopically labelled A-glucuronide (or an equivalent scientifically recognized alternative). To our knowledge, the criteria to consider that the hydrolysis was complete in a sample varies from laboratory to laboratory and, basically, depends on the expertise and/or the results obtained on the External Quality Assessment Scheme, which positions the laboratory within the group of Laboratories depending on the *z-score* parameter. The aim of this work was to study the enzymatic hydrolysis yield (HY) of urine samples after the incubation with β-glucuronidase to assess the completeness of the process.

## Experimental

Samples

- Synthetic urine (**SU**) was prepared in-house by adding ammonium phosphate, creatinine, glycine, oxalic acid, alanine, glucose, albumin, urea and sodium chloride, in deionized water
- Negative urine (**NU**) sample was fresh urine collected from a male donor with no medication or supplementation declared.

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- Positive urine (**PU**) sample was a pool of samples collected in the first eight hours post-administration of a single oral dose of 4-androstenediol (50 mg) to the same volunteer.

Preparation

2 mL of each sample were hydrolyzed with 30 µL β-glucuronidase from *E. coli* at pH 7. Liquid-liquid extraction with tert-butyl methyl ether was done at pH 9-10 and the trimethylsilyl derivatives were analyzed by GC-MS/MS.

Protocol

- Samples were incubated (n=3) at 55°C for 15, 30, 45, 60, 75, 90 and 105 minutes.
- Internal standard (ISTD): Solution of A-D4-glucuronide and Etio-D5 were prepared in a ratio 1:1. Different solutions were prepared to reach concentrations from 100 to 1500 ng/mL in urine, by adding 25 µL of the ISTD mixture to each sample.
- Analytes: concentrations of androsterone (A), etiocholanolone (Etio), testosterone (T), epitestosterone (E), 5βAdiol and 5αAdiol were compared for each hydrolysis time incubation.
- The yield of hydrolysis was assessed considering the ratio between A-D4 and Etio-D5.

Instrument

GC-qTOF: Column: HP 1 (17 m, d: 200 µm, film: 0.11 µm), Gas: He (flow 0.9 mL/min); Injector temperature: 280°C; Injection Mode: Split 1:20; Injection volume: 1 µL; Source: EI (70 eV); Acquisition mode: Full scan (*m/z* 50 to 800). Temperature Program: 180°C hold 2.5 min; Ramp 1: 3°C/min to 211°C, hold 2 min; Ramp 2: 10°C/min to 238°C, hold 0 min; Ramp 3: 40°C/min to 320ºC, hold 3.2 min.

## Results and Discussion

Concentration *versus* time incubation: no significant differences were observed in the HY dependent on the concentration of A-D4 and Etio-D5 added in the samples. NU showed higher HY supporting the idea that matrix provides stability during the analysis [1,2]. At 60 minutes of hydrolysis, the HY for SU was observed between 76-85% meanwhile for NU it was observed between 93-105%. Figure 1 shows the results.

After 60 minutes of incubation, a sample that shows a HY of 80% (based on free A-D4 measured) may quantify with an accuracy of 85-92% for A, Etio, 5βAdiol and 5αAdiol, 100% for T and E, and 54-76% of 11-oxo-Et, OHA and OHE. Nevertheless, a sample that shows a HY higher than 90% may quantify with an accuracy higher than 96% all the profile parameters except for OHA and OHE (76% and 85% respectively). Since these compounds can be used as ERCs for IRMS analysis, the assay was carried out in NU and PU, and no differences were observed on delta values when HY was close to 80% and higher than 90%. It confirmed previous report on this regard [3].

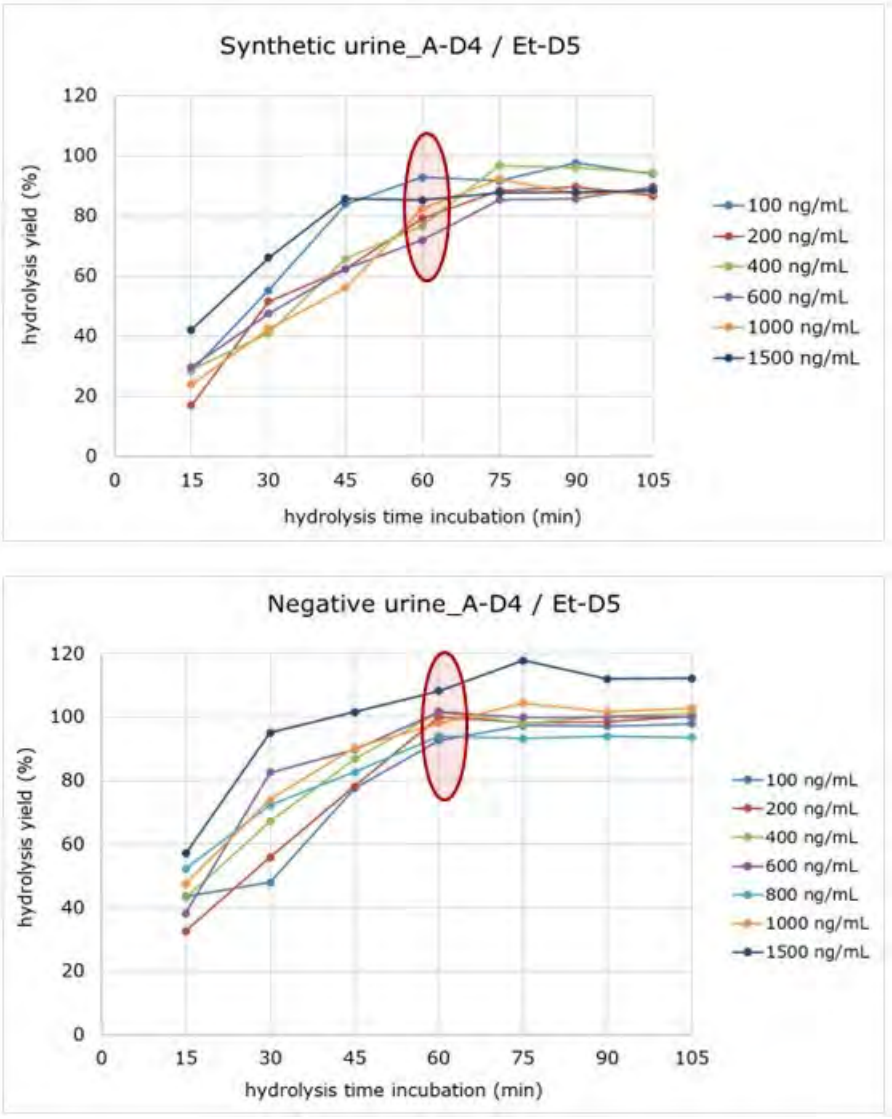


**Figure 1.** Kinetics of hydrolysis of Androsterone-D4-glucuronide (from 100 to 1500 ng/mL) in the synthetic urine (no matrix) and negative urine (physiological concentrations of endogenous steroids profile)

The HY assessed in samples with different concentrations of steroid metabolites showed the expected results for SU and NU. That is, the longer the incubation time, the higher the concentration of free analytes. Nevertheless, at 15 min of PU incubation, the isotopic contribution of the A present in urine affected the A-D4 intensity. The difference in the $^{2}H$ atoms in A-D4 and Etio-D5 also influenced the "false-assessment" of HY, because the Etio-D5 are less influenced by the Etio isotopic contribution in urine than A-D4 by A. Also, it has been described that enzymatic activity is more favored in an environment rich in $^{13}C$ than in an environment rich in $^{2}H$ [4].

Therefore, hydrolysis would be more favorable for A and Etio than for A-D4, which also justifies the results. Summarizing, the lower the concentration of A-D4 and Etio-D5, the greater the error in estimating the HY in a sample with high concentrations of A and Etio, due to their isotopic contribution. Figure 2 shows the results.

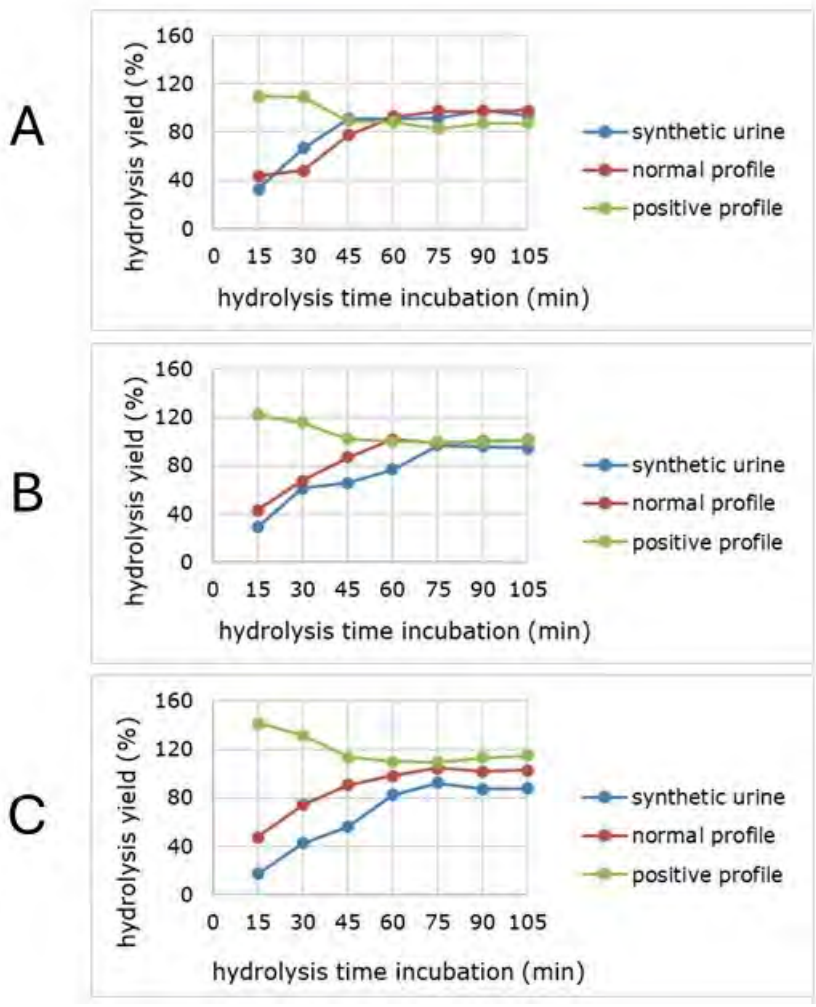


**Figure 2.** Kinetics of the hydrolysis of Androsterone-D4-glucuronide at (**A**) 100 ng/mL, (**B**) 400 ng/mL and (**C**) 1000 ng/mL, in the synthetic urine (no matrix), negative urine showing physiological concentrations of endogenous steroids profile (normal urine) and a sample collected after the administration of 4-androstenediol (positive urine)

## Conclusions

There were no significant differences in the HY dependent on the concentration of A-D4-glcuronide and Etio-D5. But the lower the concentration of A-D4 and Etio-D5, the greater the error in estimating the HY mainly in samples with high concentrations of A and Etio, due to their isotopic contribution.

At 60 minutes of incubation, a HY of 80% (based on free A-D4) showed greater variations in the recovery of profile parameters (from 54 to 92%). However, a sample with a HY greater than 90% is equivalent to a recovery higher than 96% for the main profile parameters. The acceptance criterion for hydrolysis ultimately may influence the behavior of ABP, hence the importance of homogenizing the acceptance criteria among the laboratories. Nevertheless, it should be mentioned that the results of the steroid profiles, obtained at interlaboratory studies do not reveal a problem with hydrolysis among the WADA laboratories so far.

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# Design of Experiments (DoE) as an alternative to One-Factor-At-a-Time (OFAT) approach in doping control method development – Preliminary results to miniaturize anabolic steroid analysis by GC-MS/MS

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## Abstract

Method optimization in doping control is typically carried out using the One-Factor-At-a-Time (OFAT) approach in which the impact of altering one variable is evaluated while the remaining factors are kept fixed. This work aims to promote the use of Design of Experiments (DoE) in WADA accredited laboratories to evaluate the statistical significance of experimental variables and the impact of the synergies between them. Compared to OFAT, this chemometric tool requires minimal experimental effort to determine the optimal combination of factor settings. Besides, the outcomes of DoE can be simply interpreted by visualizing several intuitive charts provided by some statistical software such as *Statgraphics*.

To illustrate the advantages of DoE, we investigated the influence of three variables (volume of extraction solvent, pH, stirring time) on the liquid-liquid extraction (LLE) procedure before gas chromatography-tandem mass spectrometry (GC-MS/MS) analysis of anabolic androgenic steroids (AAS). The optimal experimental settings obtained for stirring time (10 min) and pH (7) allowed an 80% reduction in *tert*-butyl methyl ether (TBME) consumption regarding the classic sample preparation conditions using 5 mL of organic solvent and alkaline pH. Preliminary results suggested that the proposed miniaturized methodology allows the detection of exogenous AAS at concentrations below or equal to 50% MRPL. Also, quality control urine samples containing representative low and high concentrations of the markers of the steroid profile were successfully quantified.

## Introduction

LLE is widely used in bioanalytical sample preparation. When extracting ionizable analytes, most protocols adjust the pH of the sample to achieve fully unionized compounds [1]. LLE of deconjugated AAS is traditionally performed with 5 mL of organic solvent at alkaline pH even though most free steroids are neutral compounds [2]. On the other hand, sample preparation is clearly moving towards the increasing application of the principles of green analytical chemistry (GAC) [3]. Therefore, it is imperative to reduce the use of organic solvents also in the antidoping context, especially considering the great number of samples analyzed each year by WADA accredited laboratories.

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## Experimental

Most of the compounds in the scope of our GC-MS/MS screening analytical method are AAS (72.8%), but it also includes other anabolic agents, aromatase inhibitors, antiestrogenic substances, diuretics, stimulants, narcotics and cannabinoids. In the sample preparation procedure, 50 µL of β-glucuronidase and 100 µL of phosphate buffer (pH 7) are added to 2 mL of urine and, after hydrolysis for 1 h at 55°C, sample is made alkaline (pH 10) with 300 µL of carbonate buffer and compounds are extracted with 5 mL of TBME (5 min stirring). After centrifugation, the organic layer is transferred and evaporated to dryness. The dry residue is derivatized with 50 µL of MSTFA-$NH_4$I-dithioerythritol (30 min, 65°C).
Analysis are carried out in a 7890 gas chromatograph equipped with a 7000C triple quadrupole mass spectrometer (Agilent Technologies). Compounds are separated in a HP-ULTRA 1 capillary Agilent J&W GC (25 m x 0.2 mm, 0.11 µm film thickness) column. Helium is used as carrier gas (constant pressure mode). Injector operates in the split mode (10:1) at 280°C. The GC oven program is: 177°C, rate 3°C/min to 245°C, rate 17°C/min to 320°C (held for 2.5 min), with a total run time of 29.58 min. The transfer line and source temperatures are 280°C and 230°C, respectively. Data are acquired in MRM mode.
DoE was applied to analyze the effect of the main parameters affecting the LLE step (see Table 1): TBME volume (3 levels), pH (2 levels) and stirring time (2 levels). Design of experiments analysis was performed with *Statgraphics 19 Centurion* software. The main attributes of the DoE were as follows:

- Design class: $3 \cdot 2^2$ Multilevel Factorial
- Number of experimental factors: 3
- Number of blocks: 1
- Number of runs: 12
- Degrees of freedom: 4
- Resolution: V

The order of the experiments (Table 1) was fully randomized.

| Factors | Nº levels evaluated | Levels | | |
|---|---|---|---|---|
| | | Low | Medium | High |
| Volume (mL) | 3 | 1 | 3 | 5 |
| pH | 2 | 7 | - | 10 |
| Stirring (min) | 2 | 5 | - | 10 |
| Matrix of experimental conditions | | | | |
| Experiment | Volume (mL) | pH | Stirring (min) | |
| 1 | 3 | 7 | 10 | |
| 2 | 1 | 10 | 5 | |
| 3 | 3 | 7 | 5 | |
| 4 | 1 | 7 | 5 | |
| 5 | 5 | 10 | 5 | |
| 6 | 5 | 7 | 10 | |
| 7 | 5 | 10 | 10 | |
| 8 | 3 | 10 | 5 | |
| 9 | 5 | 7 | 5 | |
| 10 | 1 | 10 | 10 | |
| 11 | 1 | 7 | 10 | |
| 12 | 3 | 10 | 10 | |

**Table 1.** Factors and levels considered in the DoE and matrix of experimental conditions

## Results and Discussion

*Statgraphics* generates several graphics helpful to understand the results of DoE. For practical reasons, only some representative examples are illustrated in Figure 1. In Pareto Charts the bar length is proportional to the influence of each variable or interaction. Those exceeding the vertical line (95% confidence interval) are statistically significant. The length of the lines in Main Effects Plots is proportional to the effect of each factor, and the slope sign indicates the level producing the highest response. In Interaction Plots, the more nonparallel the lines are, the greater the strength of the synergies between variables.

A pH of 7 led to better responses for all AAS (see, as an example, 4β-Hydroxy-stanozolol and Testosterone in Figure 1), except for 16β-Hydroxy-stanozolol, Furazabol M1 and Fluoxymesterone (PC, M1). Besides, pH was statistically significant for 42 AAS. Conversely, more efficient extractions were achieved at pH 10 for Clenbuterol (Figure 1), Zilpaterol, Ractopamine, Norfenefrine, p-Hydroxy-amphetamine and all narcotics (see Morphine in Figure 1) except Buprenorphine. These compounds could be also analyzed by LC-MS/MS and probably included in the scope of our dilute-and-shoot protocols.

10 min was the optimal stirring time for 97% of analytes and this variable was statistically significant for 36% of them, including 4β-Hydroxy-stanozolol and Testosterone (see Pareto Charts in Figure 1). Except for a score of compounds (mostly narcotics, e.g. Morphine), the influence of volume was not linear and the quadratic effect (AA interaction) was strong meaning that the optimal level is not in the extremes of the experimental region (1 mL or 5 mL) but inside it (≈3 mL). This is clearly displayed in Main Effect Plots of AAS (Figure 1). In fact, areas for this family of compounds (except 4) showed a clear increase (120-336%, mean 187%) in Exp.1 (3 mL, pH 7, 10 min) regarding those obtained under our current experimental settings (Exp. 5).

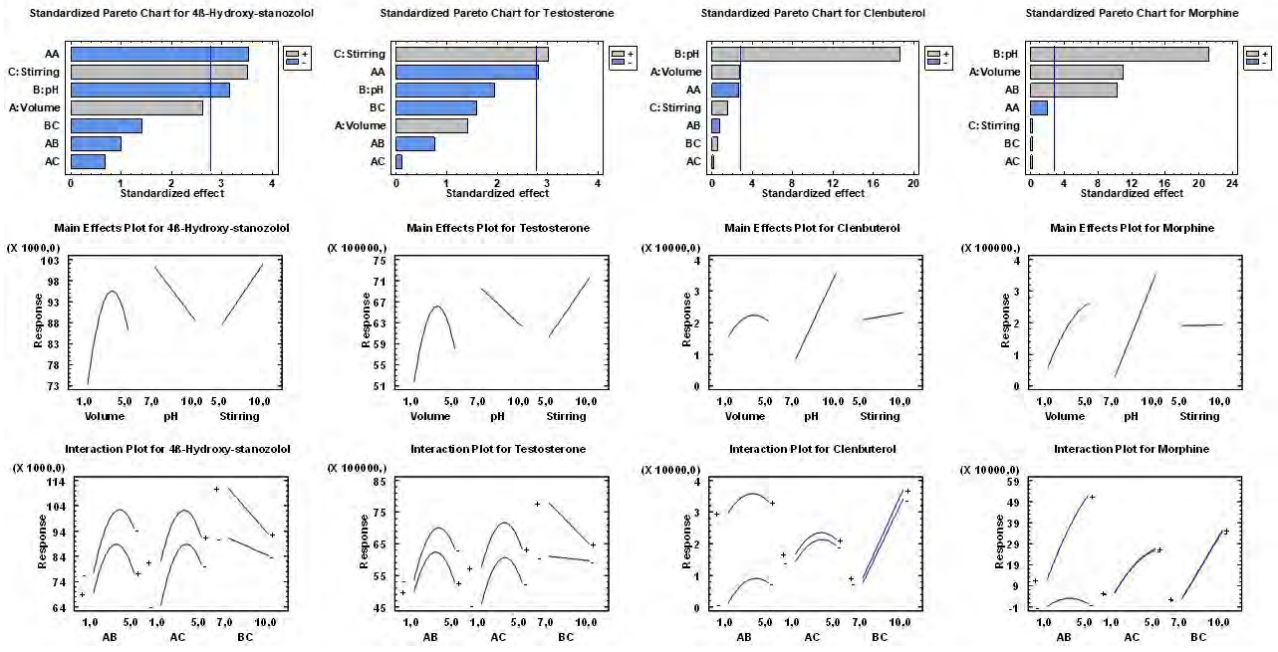


**Figure 1.** Pareto Charts, Main Effects Plots and Interaction Plots for 4β-Hydroxy-stanozolol, Testosterone, Clenbuterol and Morphine

Further studies are needed to study deeply the influence of volume, but preliminary experiments to reduce solvent consumption (1 mL instead of 5 mL) were performed using the AAS ideal settings for stirring time (10 min) and pH (7). All exogenous AAS were detected at 50% MRPL except 17α-Ethyl-18-methyl-5α-estrane-3α,17β-diol (Norboletone M1) and 9α-Fluoro-17α-methyl-4-androstene-3α,6β,11β,17β-tetra-ol (Fluoxymesterone M1) and satisfactory results were obtained after quantification of endogenous AAS markers of the steroid profile (Table 2).

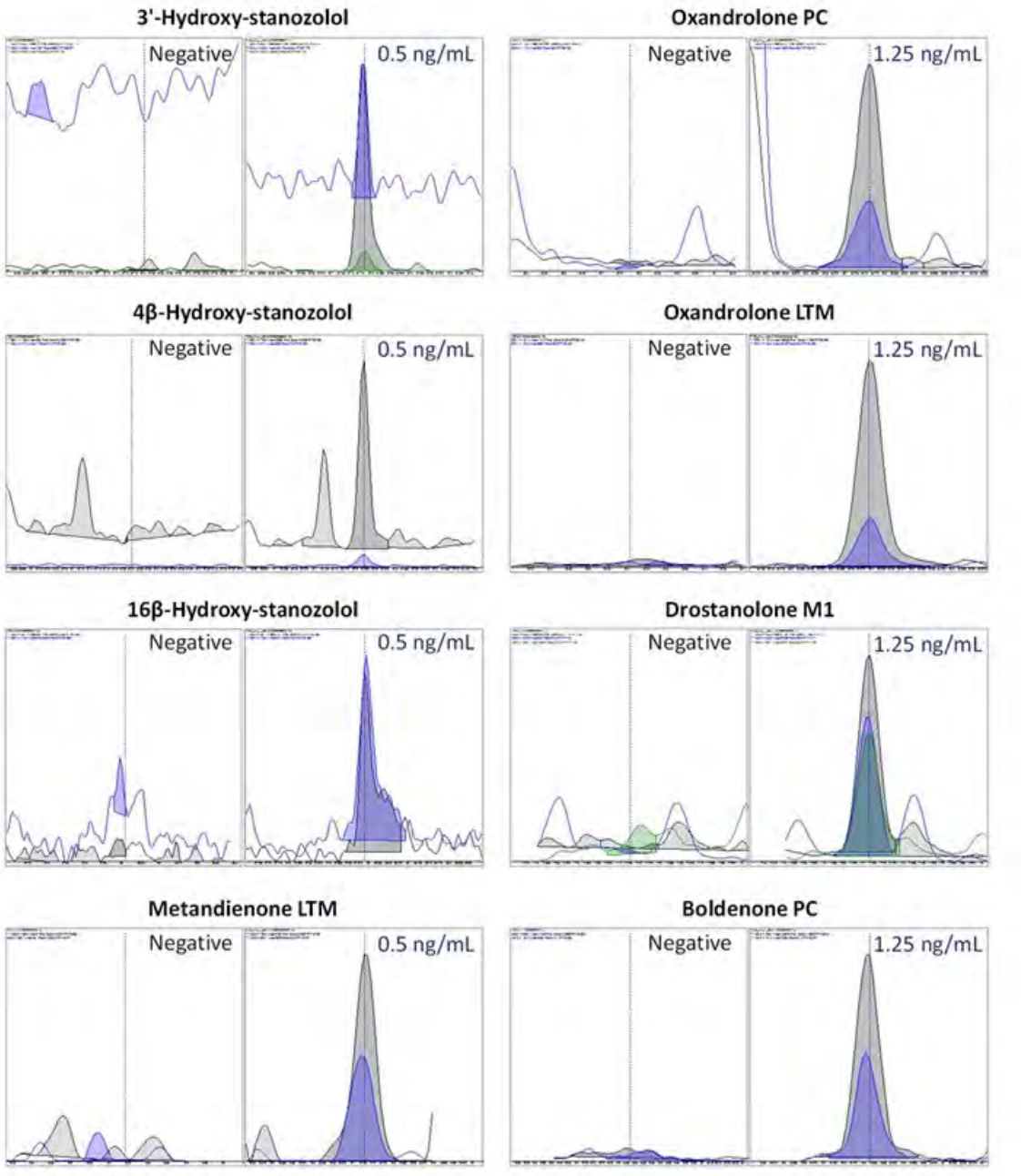


**Figure 2.** Chromatograms of some exogenous AAS at 50% MRPL (pH 7, 10 min stirring, 1 mL TBME)

| QC at representative low concentrations | | | | | | | |
|---|---|---|---|---|---|---|---|
| | T/E | [Androsterone] | [Etiocholanolone] | [5αAdiol] | [5βAdiol] | [Epitestosterone] | [Testosterone] |
| Determined (ng/mL) | 1.10 | 858 | 888 | 10.9 | 33.0 | 2.10 | 2.31 |
| Theoretical (ng/mL) | 1.01 | 881 | 940 | 11.7 | 33.8 | 2.15 | 2.16 |
| Accuracy (%) | 109 | 97.4 | 94.4 | 93.5 | 97.4 | 97.7 | 107 |
| QC at representative high concentrations | | | | | | | |
| | T/E | [Androsterone] | [Etiocholanolone] | [5αAdiol] | [5βAdiol] | [Epitestosterone] | [Testosterone] |
| Determined (ng/mL) | 1.53 | 2160 | 2510 | 59.6 | 156 | 14.7 | 22.5 |
| Theoretical (ng/mL) | 1.50 | 2480 | 2760 | 68.1 | 158 | 16.8 | 25.6 |
| Accuracy (%) | 102 | 87.3 | 91.0 | 87.5 | 98.4 | 87.2 | 87.9 |
| EQAS sample | | | | | | | |
| | T/E | [Androsterone] | [Etiocholanolone] | [5αAdiol] | [5βAdiol] | [Epitestosterone] | [Testosterone] |
| Determined (ng/mL) | 1.15 | 1030 | 741 | 35.3 | 100 | 18.3 | 21.1 |
| Theoretical (ng/mL) | 1.15 | 1110 | 796 | 36.7 | 101 | 19.3 | 22.1 |
| Accuracy (%) | 100 | 93.1 | 93.1 | 96.3 | 99.0 | 95.0 | 95.6 |

**Table 2.** Quantification of EAAS in low QC, high QC and EQAS samples (pH 7, 10 min stirring, 1 mL TBME)

## Conclusions

DoE has been applied to study the effect of three variables (volume of solvent, pH, stirring time) on the LLE step included in our GC-MS/MS screening analytical method. It was found that pH 7 leads to better recoveries for nearly all AAS. Moreover, the limits of detection (LOD) could be improved by increasing the stirring time from 5 min to 10 min.

The influence of volume should be investigated deeper but, as a preliminary trial to reduce solvent consumption, LLE experiments using 1 mL of TBME, 10 min stirring and pH 7 were performed. All AAS but two were detected at 50% MRPL and successful outcomes were obtained after quantification of EAAS in low QC, high QC and EQAS samples.

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# Detection of 17,17-dimethyl-18-nor-Δ13-stanozolol-1'N-glucuronide in post-administration urine samples

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## Abstract

A typical metabolic pathway of 17α-methylated steroids involves the epimerization of the 17α-methyl-17β-hydroxy-moiety, resulting in metabolites with 17β-methyl-17α-hydroxy-structure. Epimerization mechanism involves the departure of a 17β-hydroxy-sulfate, leading to the corresponding carbocation intermediate that, along with epimerization, is prone to Wagner-Meerwein rearrangement, forming products with a 17,17-dimethyl-18-nor-Δ13-structure. In some cases, steroid metabolites with this structure have been identified as long-lasting metabolites. Stanozolol, a commonly abused 17α-methylated steroid, is particularly prevalent among athletes using performance-enhancing drugs. Recently, 17-epistanozolol-1'N-glucuronide has been identified as the primary metabolite for monitoring stanozolol abuse due to its longer excretion period compared to other stanozolol metabolites. Based on the epimerization mechanism described above, Wagner-Meerwein rearrangement products are also expected to be formed from stanozolol (Figure 1).

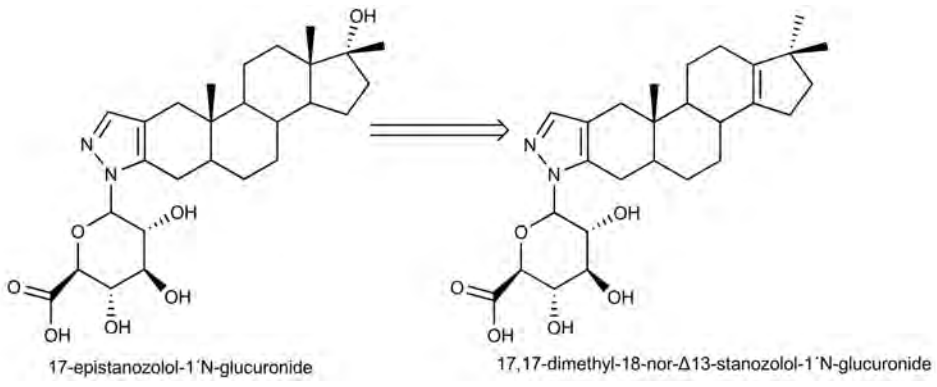


**Figure 1.** Conversion of 17-epistanozolol-1'N-glucuronide to 17,17-dimethyl-18-nor-Δ13-stanozolol-1'N-glucuronide by Wagner-Meerwein rearrangement

The aim of the current study was to determine whether such metabolites are present in stanozolol metabolism and evaluate their potential for retrospective detection. To investigate this, 17,17-dimethyl-18-nor-Δ13-stanozolol-1'N-glucuronide was synthesized, and stanozolol excretion study samples were analyzed. Additionally, six stanozolol-positive urine samples (3 female, 3 male) were included in the current study as proof of concept. The novel 17,17-dimethyl-18-nor-Δ13-stanozolol-1'N-glucuronide metabolite could be detected up to 16 days in the post-administration samples. In the positive samples, 17,17-dimethyl-18-nor-Δ13-stanozolol-glucuronide could be detected together with a second peak, which probably corresponds to the 2'N analog. Therefore, 17,17-dimethyl-18-nor-Δ13-stanozolol-glucuronide

represents a novel, potentially long-term excreted metabolite of stanozolol, expanding our understanding of its metabolism and offering an additional marker to strengthen the certainty of Adverse Analytical Findings (AAFs).

## Introduction

Stanozolol, a commonly abused 17α-methylated anabolic-androgenic steroid, is particularly prevalent among athletes using performance-enhancing drugs. According to the anti-doping testing figures of the World Anti-Doping Agency (WADA) for 2023, 168 cases of AAFs for stanozolol were reported, making up 14% of all occurrences in the S1.1 Drug Class [1]. According to the literature [2-4], Wagner-Meerwein rearrangement products are also expected to be formed from stanozolol. The aim of the current study was to determine whether such metabolites are present in stanozolol metabolism and evaluate their post-administration detection time window. To investigate this hypothesis, 17,17-dimethyl-18-nor-Δ13-stanozolol-1'N-glucuronide was synthesized and characterized by LC-HRMS/(MS) and the synthesized standard was analyzed against post-administration samples from a stanozolol excretion study, together with stanozolol-positive samples following a targeted metabolic investigation approach [5].

## Experimental

Synthesis of the analytical standard

100 ng of stanozolol-1'N-glucuronide in 500 µL of ACN were heated with 500 µL HCl 3N at 80°C for 2 h. The reaction mixture was then neutralized and diluted with 4 ml of water. Products were extracted with SPE C18 cartridges (500 mg, 6 mL).

Urine samples

In the current study, excretion study samples already used to describe and confirm the long-term 17-epistanozolol-1'N-glucuronide metabolite [4,5] were analyzed. A single oral dose of 5 mg stanozolol (Winstrol®) was administered to a healthy male volunteer and samples were collected up to 28 days after administration. Additionally, six stanozolol-positive urine samples (from 3 female and 3 male athletes), previously analyzed in Seibersdorf Laboratory, with confirmed stanozolol metabolite concentrations between 2.5 ng and 6 µg were included as proof of concept. The samples were anonymized and approved for research.

LC-HRMS analysis

An online-SPE-LC-HRMS method described earlier in detail was used for this work [6]. Briefly, 3 µL internal standard (D3-Testosterone-glucuronide, 1 µg/mL) were added to 125 µL urine sample and injected directly. An Accucore Phenyl-Hexyl, 10 x 3 mm column was used for the online extraction (Fischer Scientific, Loughborough, UK) and separation was carried out on a Kinetex®-EVO C-18, 100 x 2.1 mm column (Phenomenex, Aschaffenburg, Germany). For chromatography, mobile phases containing water with 0.2 % v/v formic acid (solvent A) and methanol with 0.1 % v/v (solvent B), constant flow of 0.4 mL/min, and an injection volume of 25 µL were used. The following gradient was carried out: 10% solvent B for 2 min, 10% solvent B up to 100% over 7 minutes, hold 100% B for 2 min and again 10% B for 2 min. A Q-Exactive Orbitrap system (Thermo Fisher, Austin, Texas, USA) in positive electrospray

ionization mode (ESI+) in parallel reaction monitoring (PRM) mode was applied using the transitions indicated in Table 1.

| Substance | Formula | Precursor ion [*m/z*] | Species | Product ion 1 [*m/z*] / [NCE] | Product ion 2 [*m/z*] / [NCE] |
|---|---|---|---|---|---|
| 18nor-stan-g | $C_{27}H_{38}N_2O_6$ | 487.2803 | $[M+H]^+$ | 311.2482 / 35 | 81.0447 / 35 |
| 17epi-stan-g | $C_{27}H_{40}N_2O_7$ | 505.2908 | $[M+H]^+$ | 329.2587 / 60 | |
| D3-testo-g | $C_{25}H_{32}D_3O_8$ | 468.2671 | $[M+H]^+$ | 109.0645 / 35 | |

NCE: Normalized Collision Energy

**Table 1.** Mass transitions for 17,17-dimethyl-18-nor-Δ13-stanozolol-1'N-glucuronide (18nor-stan-g), 17-epistanozolol-1'N-glucuronide (17epi-stan-g) and internal standard (ISTD) D3-Testosterone-glucuronide (D3-testo-g)

## Results and Discussion

Excretion study samples

Post-administration urine samples already used for the identification of 17-epistanozolol-1'N-glucuronide [5] were used in this study, and the obtained results were used to compare the detection time windows of 17,17-dimethyl-18-nor-Δ13-stanozolol-1'N-glucuronide against 17-epistanozolol-1'N-glucuronide. 17-epistanozolol-1'N-glucuronide remains the stanozolol metabolite with the longest known detection window to date, lasting in post-administration samples for up to 28 days [5,7], while 17,17-dimethyl-18-nor-Δ13-stanozolol-1'N-glucuronide was detectable for 16 days. To ensure better comparability of the results, peak areas were corrected for specific gravity and the results are presented on a logarithmic scale (Figure 2).

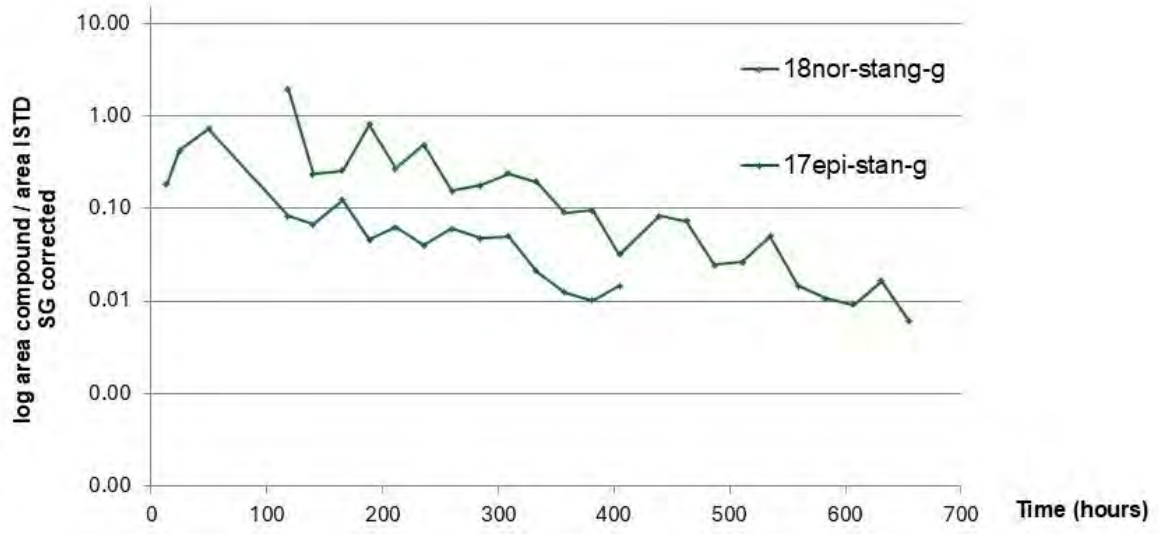


**Figure 2.** Excretion curves of 17-epistanozolol-1'N-glucuronide (17epi-stan-g) and 17,17-dimethyl-18-nor-Δ13-stanozolol-1'N-glucuronide (18nor-stang-g); y axis: Concentrations in nanograms per milliliter on logarithmic scale, x axis: Time in hours

Proof of concept samples

17,17-dimethyl-18-nor-Δ13-stanozolol-1'N-glucuronide was detectable in all six stanozolol-positive samples. Chromatograms and corresponding mass spectra of the synthesized standard (**A**), a positive sample (**B**) and blank urine sample (**C**) are presented in Figure 3. In the positive samples, 17,17-dimethyl-18-nor-Δ13-stanozolol-glucuronide (RT 9.51) could be detected together with a second peak, which probably corresponds to the 2'N analog (RT 9.69).

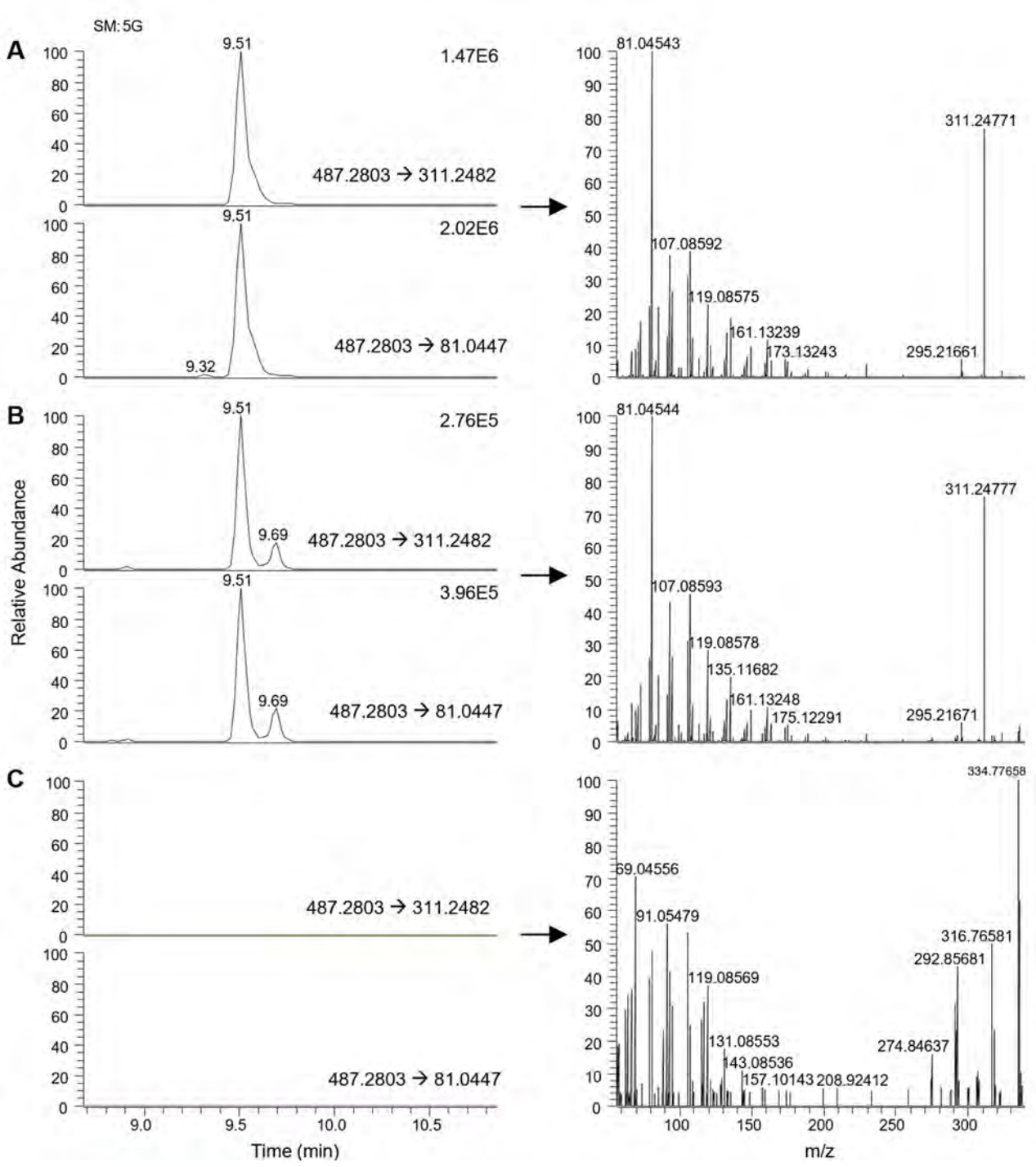


**Figure 3.** PRM chromatograms and corresponding mass spectra of the synthesized standard (**A**), a positive urine sample (**B**) and a blank urine sample (**C**)

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The peak areas were corrected with the internal standard and adjusted for the sg of the respective urine samples. Graphs were plotted on a logarithmic scale. 17,17-dimethyl-18-nor-Δ13-stanozolol-1'N-glucuronide was detected in lower concentrations and for a shorter time than the 17epi-glucuronide, however this new metabolite has the potential for long-term detection that could increase the metabolic certainty of the stanozolol findings as it can be detected for longer times in comparison with stanozolol-1'N-glucuronide [5]. An important advantage of its implementation in CP methods for stanozolol is that it can be detected in the same analytical method as 17-epistanozolol-1'N-glucuronide. An optimized method with a more elaborate sample preparation could lead to better results and longer detection windows.

## Conclusions

The current study describes a novel 17,17-dimethyl-18-nor-Δ13 metabolite of the anabolic androgenic steroid stanozolol. This metabolite could be detected in post-administration urine samples for up to 16 days. Its detection expands our understanding of stanozolol metabolism and provides an additional marker that can be incorporated into the same confirmation method of anti-doping laboratories to strengthen the certainty of adverse analytical findings (AAFs). Furthermore, it demonstrates that there is more to learn about the metabolism of stanozolol in the non-hydrolyzed glucuronide fraction, and more research is needed in this direction.

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Tsivou M, Athanasiadou I, Gmeiner G

# Monitoring microbial activity in sport urine samples: The Seibersdorf experience

Doping Control Laboratory, Seibersdorf Labor GmbH, Seibersdorf, Austria

## Abstract

Microbial contamination of doping-control urine samples, being transported in most cases at ambient temperatures, compromises sample integrity and affects the endogenous steroid profile, especially during the warm months of the year. WADA-accredited laboratories monitor microbial activity using markers of 3α-hydroxysteroid dehydrogenase (HSD) activity (5αAND/A, 5βAND/Etio ratios ≥ 0.1; free T/ total T > 0.05), and samples meeting those criteria are reported as invalid in ADAMS. Nevertheless, those markers do not cover all enzymatic activities in urine samples. For this reason, Seibersdorf Laboratory has implemented the substance 3β-ethoxy-5α-androstane-17β-ol in the initial testing procedure for endogenous steroids as a marker to detect 17-keto reductase activity. Since 2022, in accordance with TDEPO in force, WADA-accredited Laboratories include any information about “signs of microbial and/or proteolytic activity which may have affected the stability of the ERA(s) detected” or the “absence of electrophoretic ERA band signals” in ADAMS test report. The objective of the current study was to get an overview of the occurrence of microbially degraded urine samples analysed in Seibersdorf Laboratory during the period 2016 – 2024. The average percentage of invalid samples exhibiting HSD activity was 1.1% with 53% corresponding to male athletes. During the 2024 summer months, 0.27% of the analyzed urine samples indicated detectable 17-keto reductase activity with conversion of 3β-ethoxy-5α-androstane-17-one to 3β-ethoxy-5α-androstane-17β-ol. Data from samples tested for ERAs revealed that 9.0% of the samples showed signs of microbial and/or proteolytic activity, while 3.9% lacked electrophoretic ERA band signals.

## Introduction

According to WADA Guidelines for sample collection, urine must be transported in a way minimizing any sample degradation risk due to delays and high temperatures [1]. While some urine samples are refrigerated or frozen during storage by TA/SCA, most samples are transported at ambient temperatures. Even if small numbers of microorganisms (environmental species, normal microbial flora and pathogens from urinary tract infections) are present, they multiply exponentially under favourable conditions [2]. Microbial activity is monitored in urine samples using markers described in WADA’s TD2021EAAS [3]. In addition, comments such as “signs of microbial and/or proteolytic activity which may have affected the stability of the ERA(s) detected” or “absence of electrophoretic ERA band signals” are entered in ADAMS according to TD2024EPO [4]. The current study provides an insight of microbially contaminated samples analysed in Seibersdorf Laboratory.

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## Experimental

Data were extracted from the LIMS database between 2016 and 2024. An total of 156,138 doping control urine samples from national and international federations were analyzed according to the validated initial testing (ITP) and confirmation procedures (CP) of Seibersdorf Laboratory. Factors such as transportation period, gender, and pH values were also taken into consideration.

## Results and Discussion

### 3α-Hydroxysteroid dehydrogenase activity

Based on the criteria of microbial contamination for ITP (5αAND/A, 5βAND/Etio ratios ≥ 0.1) [3], the average percentage of invalid samples over the past nine years has been 1.1%, ranging from 0.6 to 1.5 %. July was the month with the highest number of invalid samples (Figure 1a). A greater proportion of invalid samples came from male athletes (53% vs. 47%) (Figure 1b).

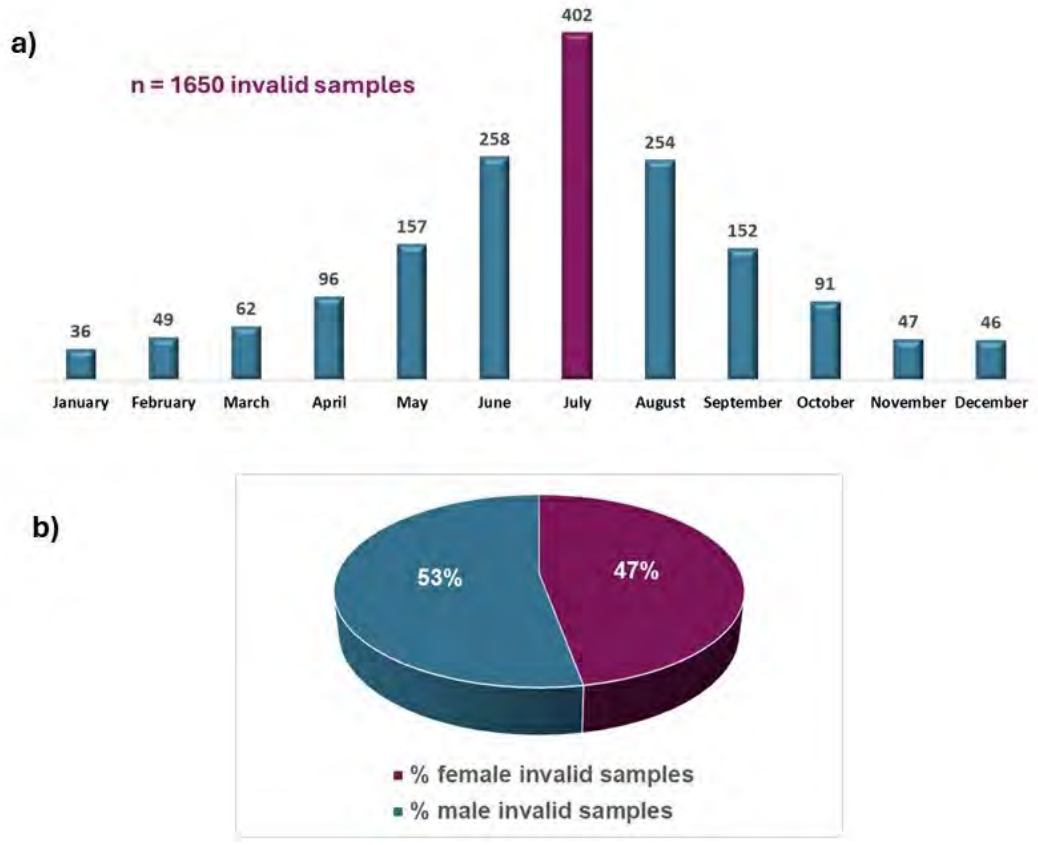


**Figure 1.** a) Average monthly distribution of invalid samples due to signs of microbial HSD activity (5αAND/A, 5βAND/Etio ratios ≥ 0.1) (2016-2024); b) Repartition of microbially invalid samples between males and females

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24% of invalid samples had pH values above 8.5. The average transportation time for invalid samples from the collection sites to Seibersdorf Laboratory was 7.7 days. A few rare incidences with unusually long transportation times (over 50 days) were also recorded but these were linked to specific issues (Figure 2).

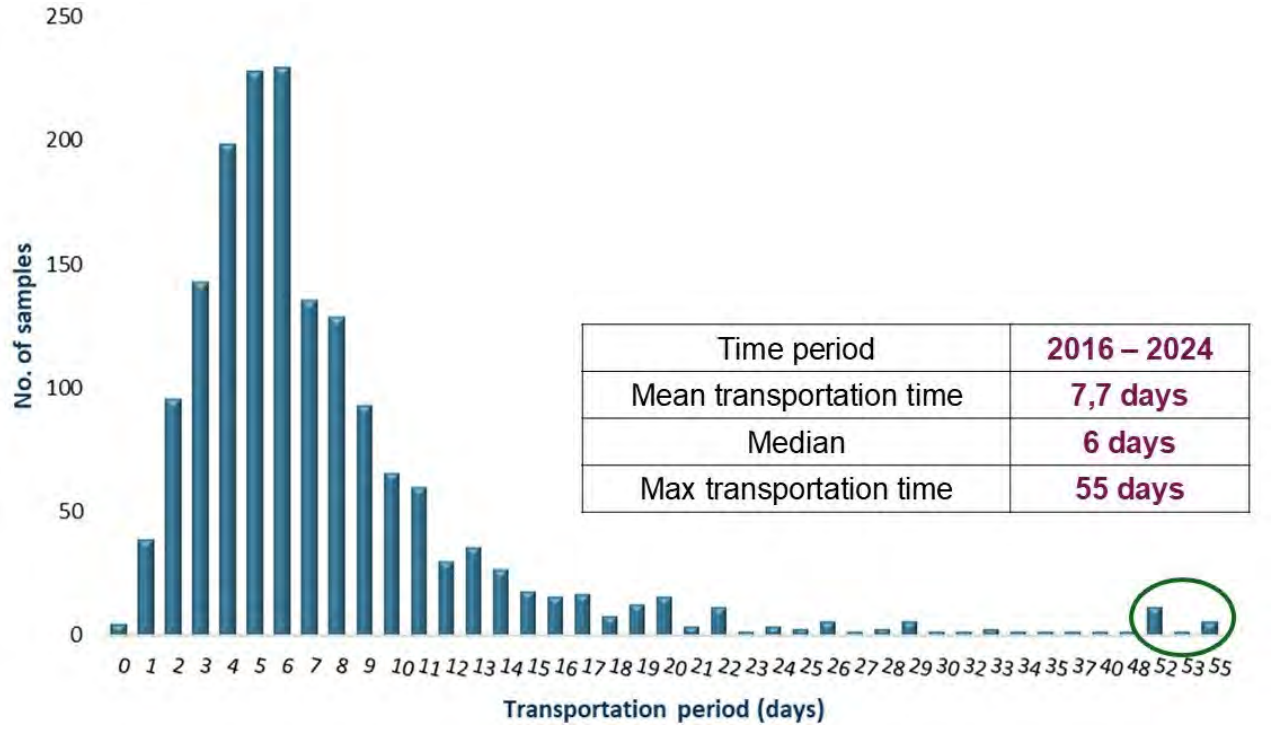


**Figure 2**: Histogram depicting the transportation period of urine samples to Seibersdorf

**17-Keto reductase activity**

During the 2024 summer months, 0.27% of the analyzed urine samples (n = 6,761) indicated detectable 17-keto reductase activity with conversion of 3β-ethoxy-5α-androstane-17-one (included in the ISTD mixture) to 3β-ethoxy-5α-androstane-17β-ol; an example of such a sample with free T/total T = 0.89, pH 8.2 is depicted in Figure 3. In this sample, the conversion of 3β-ethoxy-5α-androstane-17-one into 3β-ethoxy-5α-androstane-17β-ol was prevented during the CP. In the period between ITP and CP, samples are placed at -20 °C. As previously reported in [5], no 17-keto activity could be observed in samples prepared after freezing.

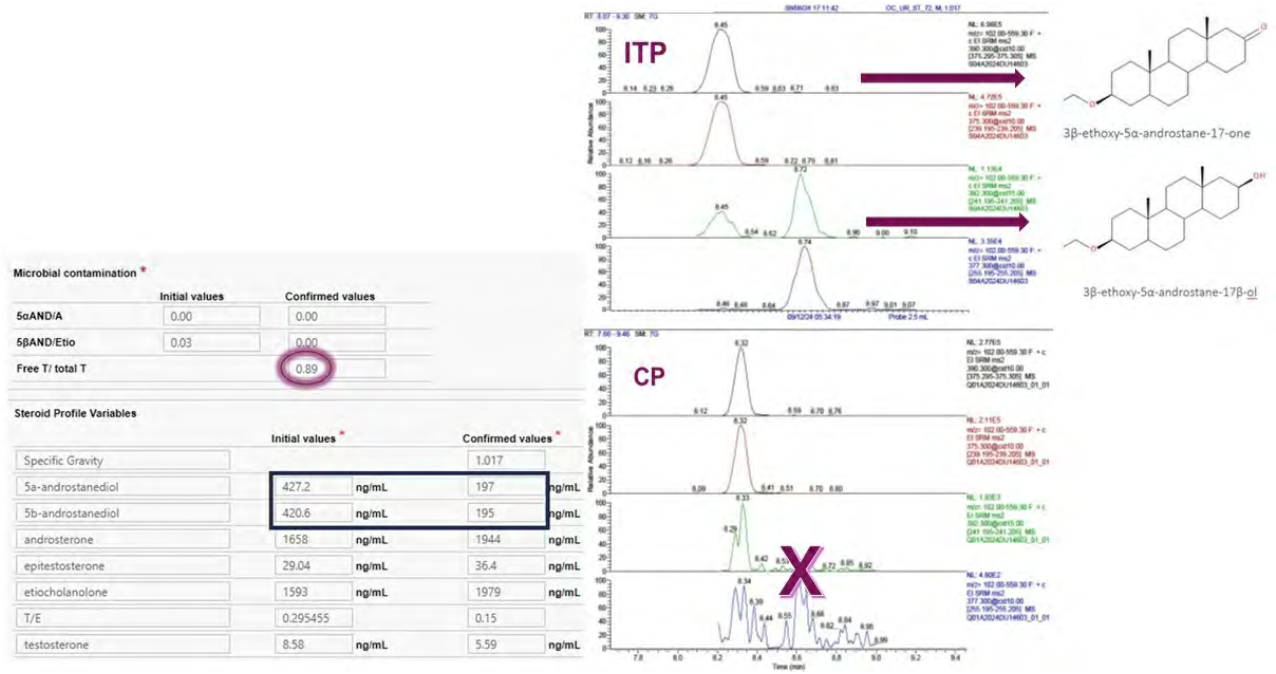


**Figure 3.** Sample with 17-keto reductase activity detected during ITP but not during CP through conversion of the marker substance 3β-ethoxy-5α-androstane-17-one to 3β-ethoxy-5α-androstane-17β-ol

The confirmed levels of 5α-androstane-3α,17β-diol and 5β-androstane-3α,17β-diol decreased, while the respective concentrations of androsterone and etiocholanolone increased proportionally [6]. It is worth mentioning that 17-keto reductase activity was also observed in samples not invalidated according to the criteria of microbial contamination [3].

**Microbial / proteolytic activity and absence of electrophoretic ERA band signals**

Data from the 2022-2024 period (n = 15,648) on samples tested for ERAs revealed, that 9.0% of the samples showed signs of microbial and/or proteolytic activity, while 3.9% lacked electrophoretic ERA band signals (Figure 4).

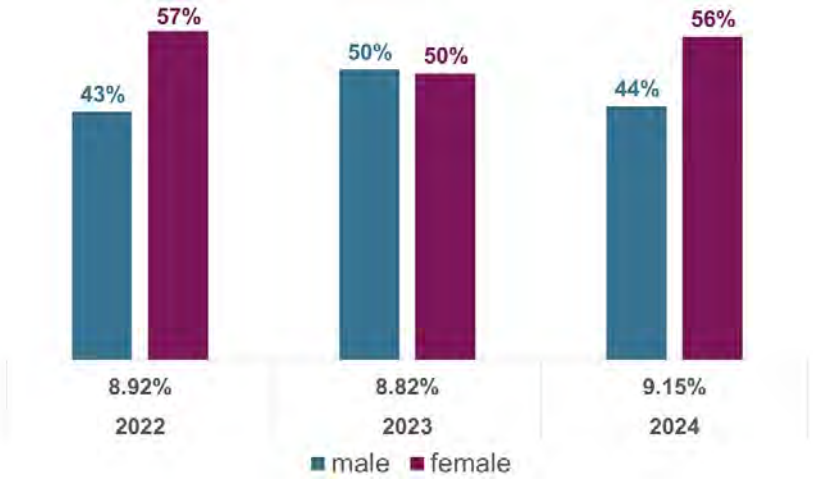


**Figure 4.** Yearly distribution (%) of urine samples per gender tested for ERAs with signs of microbial and/or proteolytic activity

The average monthly distribution indicated a peak in June for both microbial/proteolytic activity and the lack of ERA band signals. Among the samples showing microbial and/or proteolytic activity, 7.3% had pH values above 8.5, compared to just 1.8% of the samples with no ERA band signals with higher pH values. The mean transportation time for the affected samples was 6 days.

## Conclusions

In the current study, data were extracted from the LIMS database to gather information about urine samples affected by microbial activity, as analyzed in Seibersdorf Laboratory. Data are considered representative due to the large sample size, which includes samples from IFs, ADOs, and major events across European, African, and Asian countries. The average percentage of invalid samples exhibiting HSD activity over a period of nine years was 1.1%. The systematic monitoring of 17-keto activity could be a valuable tool to prevent unnecessary steroid profile confirmation analyses in cases of flagged or suspicious ABP passports. 9.0% of the samples tested for ERAs showed evidence of microbial and/or proteolytic activity. Absence of electrophoretic ERA band signals was recorded in 3.9% of the samples.

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# *In vitro* investigation of the masking efficiency of various cyclodextrins on doping substances and alteration of the steroid profile

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## Abstract

In this preliminary study, the masking efficiency of different classes of cyclodextrins [α-cyclodextrin, β-cyclodextrin, γ-cyclodextrin, methyl-β-cyclodextrin, (2-hydroxypropyl)-β-cyclodextrin, (2-hydroxypropyl)-γ-cyclodextrin] is evaluated on doping substances, especially threshold substances, non-threshold substances (NTS) with MRL, and steroid profile. Initially, the cyclodextrins masking efficiency on drostanolone metabolite, 19-norandrosterone, 19-noretiocholanolone, boldenone metabolite and parent, clenbuterol, zilpaterol, salbutamol, THC, morphine, and on the steroid profile was evaluated. Each cyclodextrin (6 mg/mL) was added externally (as a tampering agent) to different positive urine samples and kept at 2°C to 6°C (refrigerator) for one day. After being processed using the routine Initial Testing Procedure (ITP) method, prohibited substance concentrations were compared with and without cyclodextrin in positive urine samples. γ-Cyclodextrin showed a significant 70 to 75% concentration decrease for the 19-NA, boldenone metabolite, drostanolone metabolite, 25 to 30% concentration decrease for the marijuana metabolite (THC), and boldenone parent. Regarding the steroid profile, alpha metabolites like androsterone (A) and 5α-androstane-3α,17β-diol (5αAdiol) showed a 5 to 6-fold and 2 to 3-fold decrease in concentration, respectively. Due to this, the A/T ratio and 5αAdiol/E ratio have been altered, which is part of the steroidal module in APMU.

## Introduction

Cyclodextrins (CD) are cyclic oligosaccharides consisting of α-cyclodextrin, β-cyclodextrin, γ-cyclo-dextrin or more glucopyranose units linked by α-(1,4) bonds. Glucopyranose units form a conical cylinder with a hydrophilic outer surface and a lipophilic central cavity. This central cavity can encapsulate hydrophobic compounds [1]. One of the most popular areas of research in antidoping is the study of "masking agents," substances/techniques that can "hide" other illegal substances, thereby decreasing the effectiveness of experimental strategies used to detect doping agent abuse through biological fluid analysis. FMSI (Italy) conducted research on investigating the masking capacity of liposomes on anabolic androgenic steroids. It was found that liposomes could be used as masking agents for prohibited drugs, without having any direct effect on enhancing performance [2].

## Experimental

### Sample preparation

2 mL of positive urine were taken with (12 mg) and without cyclodextrin and kept at 2-6°C for one day. 50 µL of Internal Standard mixture, 1 mL of 0.2 M phosphate buffer (pH – 7.0) and 50 µL of β-

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glucuronidase (E. coli) were added and incubated at 60°C for 60 minutes. Then, the mixture was kept at room temperature for cooling. For solid phase extraction, the Inert-sep HLB cartridges were conditioned with 2 mL methanol followed by 2 mL water. After that, 2 mL of the samples were loaded on the cartridge. Matrix cleanup was done with 1 mL of water, followed by 0.5 mL 25% methanol in water, and the final wash was performed with 1 mL hexane. Substances were eluted into a labelled glass tubes with 2 mL of 5% methanol in ethyl acetate. The elute(s) were dried using the nitrogen evaporator at 60°C for 20 minutes. Tubes were kept on a block heater for 10-15 minutes to remove the residual moisture from the tube. The dried elute were dissolved with a 50 µL of MSTFA/IODO –TMS/DTE mixture (1000/2/2: *v/v/w*) and the tubes were kept at 60°C for half an hour.

**GC-MS/MS Analysis**

The GC–MS/MS analysis chromatographic separation was performed on an HP Ultra-1 capillary column (length: 17 m; diameter: 0.2 mm; film-thickness: 0.11 µm). The GC oven was programmed for an initial temperature of 180°C for 1 minute, followed by a 3°C/min ramp up to 229°C, and the final ramp of 40°C/min was applied up to 300°C. This final temperature was maintained for 4 minutes; the total run time was 23.1 minutes. Helium as the carrier gas at a constant pressure mode (121 kPa). The GC/MS inlet and interface temperatures were kept at 280°C and 280°C. EI source and quadrupole analyzers temperatures were maintained at 230°C and 150°C, respectively. The mass spectrometer was operated in multiple reaction mode.

## Results and Discussion

The FMSI (Italy) study also showed that adding liposomes to urine samples containing steroids caused a masking effect, leading to a reduced analytical recovery of both the parent compound and the glucuronide metabolites [2]. Cyclodextrin exhibits liposome-like characteristics as well. Its structure and capacity to form inclusion complexes with pharmaceuticals make it a popular complexing agent [3]. It is also widely utilised in drug delivery systems. It is an excellent agent for making any drug more soluble, making it an excellent choice for use as a masking agent. Botre *et al.* evaluated the masking efficiency of different types of β-cyclodextrins on testosterone and epitrenbolone [4]. Owing to all of these we made the decision to investigate various cyclodextrins masking efficiency on doping substances in the current study.

| Name of the cyclodextrins used | Name of the Drugs Investigated |
|---|---|
| Alpha (α) | 19-NA, 19-NE |
| Beta (β) | Boldenone Metabolite & P |
| Gama (γ) | Drostanolone Metabolite |
| Methyl-β-Cyclodextrin | Clenbuterol, Zilpaterol |
| (2-Hydroxypropyl)-β-Cyclodextrin | Salbutamol, Morphine, THC |
| (2-Hydroxypropyl)-γ-Cyclodextrin | Steroid Profile markers |

**Table 1.** Drugs investigated with various types of cyclodextrins

Prohibited substance concentrations were compared with cyclodextrin (6 mg/mL) and without cyclodextrin in positive urine samples. γ-Cyclodextrin has shown a significant 70 to 75% concentration decrease for the 19-NA, boldenone metabolite (Figure 1) and drostanolone metabolite, and 25 to 30% concentration decrease for the marijuana metabolite (THC) and boldenone parent (Figure 2). Regarding the steroid profile, alpha metabolites like androsterone (A) and 5α-androstane-3α,17β-diol (5αAdiol) have shown a 5 to 6 fold and 2 to 3 fold decrease in concentration, respectively. Due to this, the A/T ratio and 5αAdiol/E ratios have been altered, which are part of the steroidal module in APMU (Table 2).

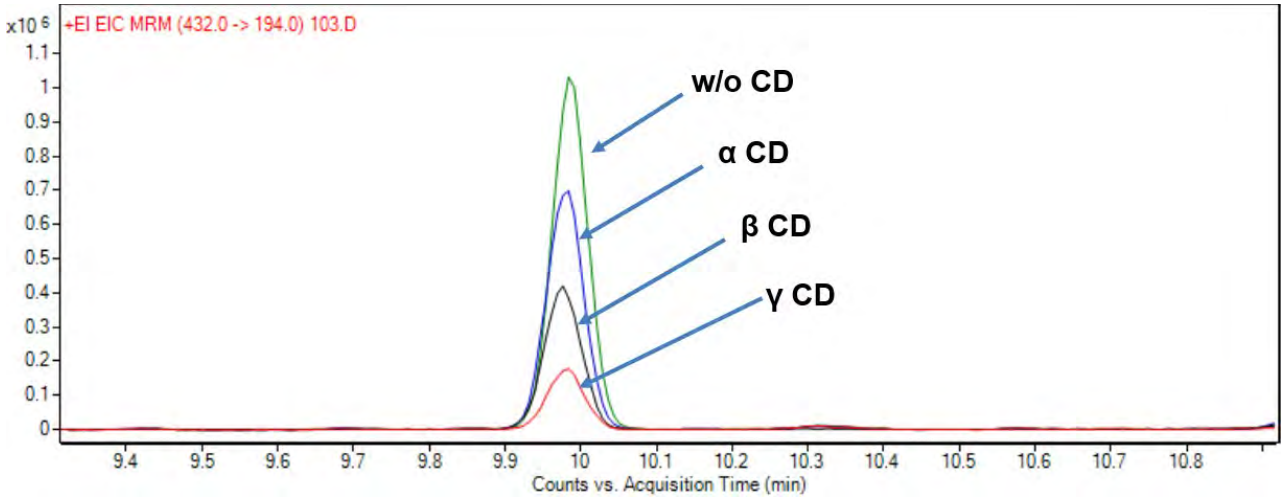


**Figure 1.** MRM chromatograms of boldenone met without & with CD

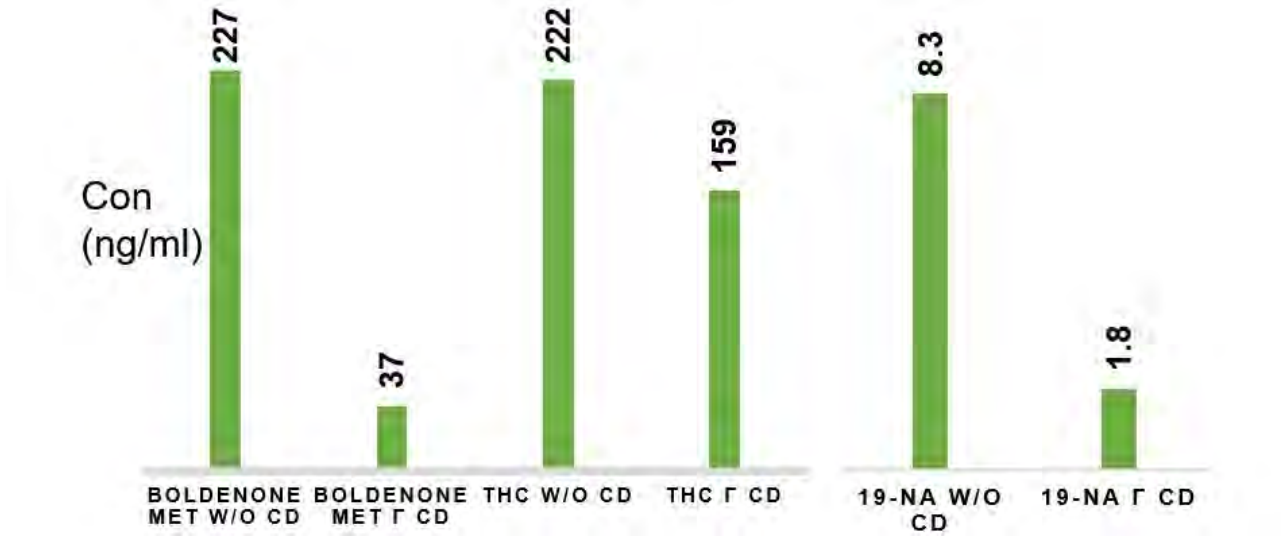


**Figure 2.** Concentrations of substances in positive urine w/o CD and with γ CD

| Sample | ANDRO | ETIO | 5A3B DIOL | 5B 3A DIOL | EPI | TESTO | T/E | A/T | A/ETIO | 5A/5B | 5A/E | A/E | 5A/T |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Without CD | 2327 | 2204 | 47.6 | 314 | 19.2 | 14.6 | 0.76 | 159.52 | 1.06 | 0.15 | 2.48 | 121 | 3.3 |
| Alpha(α) CD | 2096 | 2088 | 39.4 | 271 | 18.5 | 13.5 | 0.73 | 155.73 | 1.00 | 0.15 | 2.13 | 113.44 | 2.93 |
| Beta (β) CD | 614 | 760 | 24.8 | 21.1 | 17.6 | 14.1 | 0.80 | 43.49 | 0.81 | 1.18 | 1.41 | 34.89 | 1.76 |
| Gama (γ) CD | 335.84 | 418.51 | 14.98 | 30.31 | 19.1 | 14.5 | 0.76 | 23.09 | 0.80 | 0.49 | 0.78 | 17.57 | 1.03 |
| Methyl Beta CD | 1000 | 1307 | 35.7 | 39.9 | 19.1 | 14.6 | 0.76 | 68.64 | 0.77 | 0.90 | 1.87 | 52.38 | 2.45 |
| Hydroxy Beta CD | 1174 | 1454 | 33.7 | 67.4 | 20.7 | 13.8 | 0.67 | 85.15 | 0.81 | 0.50 | 1.63 | 56.66 | 2.44 |
| Hydroxy Gama CD | 216 | 353 | 13.6 | 5.8 | 19.1 | 14.4 | 0.75 | 15.0 | 0.6 | 2.34 | 0.7 | 11.28 | 0.95 |

**Table 2.** Steroid profile of a sample without cyclodextrin and with various types of cyclodextrins

## Conclusions

Six different cyclodextrins were investigated in order to confirm the masking efficacy for doping substances. Among the six cyclodextrins that were investigated, Gamma (γ) cyclodextrin has shown the highest capacity to mask studied doping substances and endogenous anabolic androgenic steroids. False negative test findings will result from manipulating a urine sample with Gamma (γ) cyclodextrin, particularly when non-threshold substances with MRL and threshold substances are involved.

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Ahi S, Wolff K

# Salbutamol elimination study in urine: Case studies and experience from the Drug Control Centre, King's College London

Drug Control Centre King's College, London, Great Britain

## Abstract

Salbutamol is a short-acting β2-adrenergic agonist widely prescribed for the management of asthma and exercise-induced bronchoconstriction. Its use via inhalation is permitted under World Anti-Doping Agency (WADA) regulations; however, urinary concentrations exceeding the decision limit of 1200 ng/mL are classified as an Adverse Analytical Finding (AAF) unless supported by appropriate pharmacokinetic (PK) data. This distinction is critical in anti-doping science, as distinguishing between legitimate therapeutic use and potential misuse poses ongoing challenges.

In 2024, the Drug Control Centre at King's College London undertook two controlled salbutamol elimination studies in athletes who had previously produced AAFs. Both studies involved carefully monitored inhaled administrations under therapeutic conditions, with serial urine collections to establish elimination patterns. The results demonstrated that urinary concentrations of salbutamol following standard inhaled dosing remained consistently below the WADA decision limit. These findings strengthen the evidence that therapeutic inhalation is highly unlikely to generate concentrations above the regulatory threshold.

Nevertheless, the studies also highlighted individual variability in elimination profiles. Factors such as dosing frequency, hydration status, renal function, and the use of concomitant medications may influence urinary concentrations and clearance rates. This paper presents the methodology, results, and broader implications of these case studies, emphasizing the importance of robust PK investigations in supporting fair and scientifically sound anti-doping policy.

## Introduction

Salbutamol (albuterol) is a short-acting β2-adrenergic agonist (SABA) commonly prescribed for asthma and exercise-induced bronchoconstriction (EIB). By relaxing bronchial smooth muscle, it provides rapid relief of airway obstruction. In sport, however, salbutamol poses regulatory challenges. While therapeutic inhalation is permitted under World Anti-Doping Agency (WADA) rules, excessive use may enhance performance through bronchodilation and metabolic effects [1,2]. To balance treatment and misuse, WADA allows inhaled doses up to 1600 µg per 24 hours, with urinary concentrations above 1200 ng/mL classified as an Adverse Analytical Finding (AAF), unless justified by a controlled pharmacokinetic (PK) elimination study. This paper reports two such studies conducted at the Drug Control Centre, King's College London, in 2024, following AAFs in athletes.

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## Experimental

Participant Characteristics

Both participants were adult competitive athletes, females of European ethnicity, with BMI values of 23.4 and 26.1 kg/m², respectively. Both were in good general health and had well-documented histories of asthma managed with inhaled β2-agonists and corticosteroids. No renal or hepatic impairments were reported. These details are included to contextualize interindividual variability in elimination kinetics.

Case 1: Salbutamol AAF

An athlete presented with a urinary salbutamol concentration of 1.80 µg/mL (1800 ng/mL) in a sample with a specific gravity (SG) of 1.026, exceeding the WADA threshold of 1200 ng/mL. The athlete reported the use of Ventolin™ 100 µg metered-dose inhaler (MDI), administered as four puffs (total 400 µg). To replicate reported therapeutic use, a witnessed administration of four puffs was conducted under controlled conditions. Five urine samples, including a baseline blank prior to inhalation, were collected over an 8-hour period to monitor excretion and peak urinary concentrations. The athlete's concomitant medications included Levothyroxine, Vitamin D, Omeprazole, Nortriptyline, Paracetamol, Ibuprofen, and Seretide inhaler, which were documented to consider potential interactions with salbutamol pharmacokinetics.

Case 2: Salbutamol AAF

Another athlete provided a urine sample with a salbutamol concentration of 1.55 µg/mL (1550 ng/mL) and SG 1.016. The athlete reported regular use of Salamol CFC-free 100 µg MDI. A witnessed administration replicating the athlete's reported inhalation regimen was performed. Six urine samples, including a baseline blank, were collected over an 8-hour period to determine the urinary elimination profile. Concomitant medications included Montelukast 10 mg, Codeine 30 mg, Ventolin 100 µg, Fexofenadine 180 mg, and Zopiclone 7.5 mg.

Analytical Method

All urine samples were analyzed using a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method employing a dilute-and-shoot approach, targeting free salbutamol. The method demonstrated a relative combined standard uncertainty (Uc) of 6.1% at 1000 ng/mL, which is well below the maximum permissible uncertainty of 10% specified in WADA Technical Document TD2022DL, ensuring reliable quantification of salbutamol in the athlete samples.

## Results and Discussion

### Results

In Case 1, the salbutamol elimination study showed no detectable drug in the baseline blank sample. Following inhalation, urinary concentrations peaked at 2–3 hours post-administration, reaching 210 ng/mL, and then declined progressively to 40 ng/mL by 7.5 hours (full dataset presented in Table 1).

In Case 2, a low level of salbutamol (23 ng/mL) was detected in the baseline sample. Concentrations gradually increased over the first 2–3 hours, reaching a peak of 423 ng/mL at 4–5 hours, and although they declined slightly thereafter, levels remained elevated at 392 ng/mL at 7.5 hours (Table 2).

In both studies, urinary concentrations observed during the controlled elimination experiments were well below the 1000 ng/mL threshold set for pharmacokinetic evaluations, despite the athletes' initial routine doping control samples exceeding the WADA AAF limit of 1200 ng/mL. High interindividual variability was observed between the two athletes, particularly in peak concentration and elimination rate. Possible explanations include differences in lung deposition efficiency, metabolic rate, renal clearance capacity, body composition, and hydration level at the time of sampling. These findings indicate that, under controlled therapeutic inhalation, salbutamol concentrations remain substantially below levels associated with performance enhancement.

| Samples | SG | Time since first urine sample (hr:min) | Urine Volume (mL) | Mean Salbutamol concentration (ng/mL) |
|---|---|---|---|---|
| Sample 1 | 1.006 | 00:00 | 80 | 0 |
| Sample 2 | 1.013 | 02:49 | 80 | 210 |
| Sample 3 | 1.009 | 05:06 | 90 | 140 |
| Sample 4 | 1.004 | 06:36 | 90 | 50 |
| Sample 5 | 1.004 | 07:46 | 75 | 40 |

**Table 1.** Case 1: Urinary salbutamol elimination study results

| Samples | SG | Time from first collection (hr:min) | Urine Volume (mL) | Mean Salbutamol concentration (ng/mL) |
|---|---|---|---|---|
| Sample 1 | 1.020 | 00:00 | 80 | 23 |
| Sample 2 | 1.004 | 00:50 | 90 | 3 |
| Sample 3 | 1.004 | 01:35 | 90 | 13 |
| Sample 4 | 1.005 | 02:45 | 90 | 59 |
| Sample 5 | 1.016 | 04:38 | 80 | 423 |
| Sample 6 | 1.022 | 07:25 | 90 | 392 |

**Table 2.** Case 2: Urinary salbutamol elimination study results

**Discussion**

These findings reinforce existing evidence that therapeutic inhaled doses of salbutamol rarely exceed the urinary decision limit set by WADA (Figure 1 & 2). Both athletes' elimination profiles demonstrated rapid absorption followed by clearance within expected ranges, consistent with published pharmacokinetic studies [3,4].

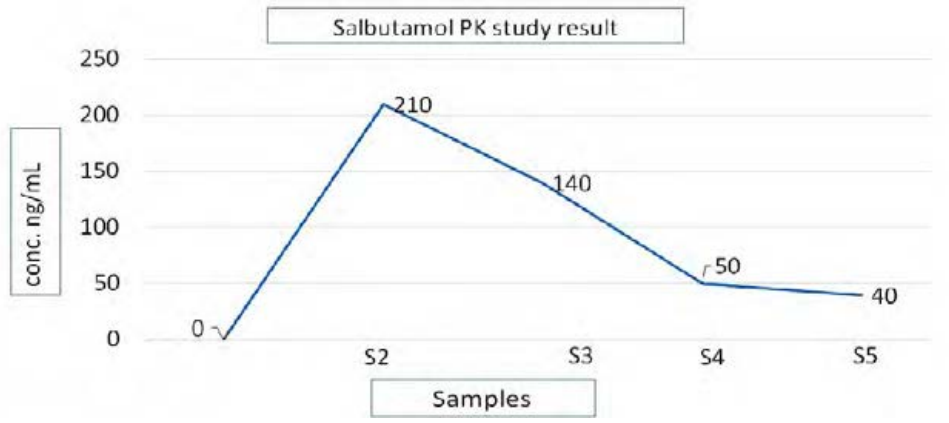


**Figure 1.** Case 1: Graph showing results of salbutamol elimination in athlete's urine

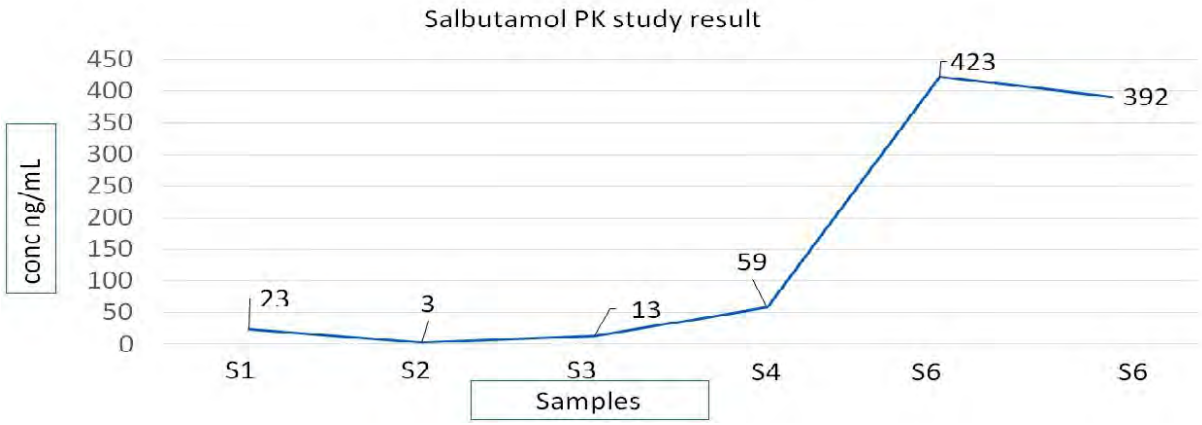


**Figure 2.** Case 2: Graph showing results of salbutamol elimination in athlete's urine

Some key points emerge from these cases:

1. Variability in Peak Concentrations
    - Case 1 peaked early at 210 ng/mL, while Case 2 peaked later and higher at 423 ng/mL.
    - Potential contributors include differences in inhalation technique, airway deposition, BMI, renal function, sex-related metabolism, and hydration status
2. Influence of Concomitant Medications
    - Both athletes were prescribed multiple medications, including corticosteroids and bronchodilators, which may affect metabolism or renal excretion. However, none are known to directly interfere with salbutamol clearance.
3. Hydration and Specific Gravity
    - Differences in urine SG (1.026 vs. 1.016) may partly explain concentration variability. Dehydration can elevate urinary concentrations independent of dosing.
4. AAF vs. Elimination Study
    - Despite initial AAFs (>1200 ng/mL), elimination studies showed values below 1000 ng/mL, supporting therapeutic use. This underscores the importance of PK studies.

5. Regulatory Implications
    - These results support WADA's current threshold, but they also highlight the need for continued evaluation. The observed findings are consistent with foundational studies that informed WADA's current decision limit, including those by Dickinson et al. (1989) and Hemmersbach et al. (1990), which first demonstrated that therapeutic inhalation produces urinary concentrations typically below 1000 ng/mL [5,6].

## Conclusions

The two case studies presented here demonstrate that controlled therapeutic inhalation of salbutamol results in urinary concentrations well below the WADA decision limit of 1200 ng/mL, even in athletes who initially returned Adverse Analytical Findings. The observed variability in elimination profiles that we observed underscores the role of individual physiological factors, hydration status, and concomitant medications in influencing urinary excretion. These findings reinforce the validity of the current WADA framework for therapeutic use exemptions, while highlighting the importance of conducting controlled pharmacokinetic evaluations to distinguish permitted use from potential misuse. Further research is warranted to investigate the impact of repetitive dosing, dehydration, and polypharmacy on urinary salbutamol concentrations. The Drug Control Centre remains committed to generating robust PK data to support fair adjudication of AAFs and uphold the integrity of competitive sport.

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## Acknowledgements

The LC team at the Drug Control Centre is gratefully acknowledged for their valuable support in this project.

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# Identification of a phase II metabolite of the selective androgen receptor modulator AC-262536 during routine doping control

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## Abstract

The class of selective androgen receptor modulators (SARMs) has been the subject of intense and dedicated clinical research over the past two decades. Selective androgen receptor modulators (SARMs) are a class of new emerging 'designer' steroid compounds gaining popularity over more well established anabolic-androgenic steroids (AAS) amongst both non-professional and elite athletes. They were first proposed in the late 1990s as potential new treatments for a range of human diseases, e.g. benign prostatic hyperplasia, androgen-deficiency related disorders and conditions involving muscle and bone wasting.
SARM AC-262536 is a substance prohibited in sport at all times and included in section S1.2 of the WADA Prohibited List 2025. This work presents a metabolite of SARM AC-262536, which was detected in a urine sample and which may be considered by laboratories in routine doping control.

## Introduction

Selective androgen receptor modulators (SARMs) are an emerging class of drug compounds first proposed in the late 1990s [1] as potential new treatments for a range of human diseases, e.g. benign prostatic hyperplasia, androgen- deficiency related disorders and conditions involving muscle and bone wasting [1,2]. Due to the enormous muscle- and bone-anabolic properties of SARMs, as evidenced in numerous preclinical and clinical studies, the potential for misuse in the context of amateur and elite sport has been recognized and led to the inclusion of SARMs into the WADA Prohibited List in 2008 [3].
SARM AC-262536 is a substance prohibited in sport at all times and included in section S1.2 of the WADA Prohibited List 2025 [4]. According to the WADA Technical Document (TD2022MRPL), identification of SARM AC-262536 in urine at any concentration constitutes an Adverse Analytical Finding (AAF) [5].
This work presents a metabolite of SARM AC-262536, which was detected in a urine sample by means of ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS). Monitoring of this metabolite, along with the parent compound, could be considered by laboratories in routine doping control.

## Experimental

### Sample preparation

SARM AC-262536 is detected in the routine doping control using the two protocols.

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In the DaS method, 200 µL of urine was transferred to the Eppendorf 1.5 mL tubes, added the internal standards and diluted with 800 µL of water. Samples were then vortexed and centrifuged (10 min/ 14000 rpm). Next, 200 µL of supernatant was transferred to the vial. Injection volume was fixed at 10 µL.
In the second method, 3 mL of urine was spiked with internal standards. After an addition of 1.1 mL of 0.8 M phosphate buffer (pH 6.5) and β-glucuronidase (from *E.coli*), the samples were incubated at 50°C for 1 hour. Afterwards, samples were cooled down to room temperature. Next, after an addition of 1 mL of 20% $K_2CO_3$/$KHCO_3$ (1:1, v/v), the extraction with 6 mL of methyltert-butyl ether was performed for 20 minutes. Samples were then centrifuged (5 min/16495 RCF), and the ether phase was recovered and evaporated under a nitrogen flow at 55°C. The dry residue was reconstituted in 100 µL of acetonitrile/ $H_2O$ mixture (1:1, v/v).

**Instrumental analysis**

Liquid chromatography

Analyses were performed on a UPLC Acquity chromatograph (Waters, Milford Massachusetts, USA) equipped with HSST3 (1.8 µm, 2.1 × 100 mm) and BEH C18 (1.7 µm, 2.1 × 100 mm) columns.
The mobile phases consisted of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B) and the LC gradient was employed at the constant flow rate of 300 µL/min at 45°C.
For the Das method, the concentration of acetonitrile was gradually increased in a linear manner: from 0% to 15% within the first 6 minutes, from 15% to 100% in 1 minute. Then it was kept constant for an additional 2 minutes. Finally, the column was re-equilibrated for 2 minutes with the mobile phase of initial composition. In turn, in the second method, it was changed from 5% to 35% within the first 2 min and then from 35% to 50% in 6 min. Next, the concentration of acetonitrile was increased from 50% to 100% in 1 min and then kept constant for an additional minute. Finally, the column was re-equilibrated for 1 min with the mobile phase of the initial composition.
Samples were stored at 10°C in the autosampler prior to analysis.

Mass spectrometry

SARM AC-262536 was traced in a multiple reaction monitoring (MRM) mode with Xevo TQ-S (Waters, Milford Massachusetts, USA) and Xevo TQ-XS (Waters, Milford Massachusetts, USA) mass spectrometers equipped in an electrospray ionization (ESI) source and with a new atmospheric pressure ionization source, commercialized as **UniSpray™**.
AC-262536 was traced at the cone voltage (CV) set at 10 V with the following selected precursor ion–product ion transitions at their respective collision energies (CE): m/z 279.15 > 169.05, CE 20 eV; 279.15 >193.08, CE 38 eV; 279.15 > 195.09, CE 25 eV; 279.15 > 235.12, CE 20 eV. In turn, AC-262536 glucuronide was traced at the cone voltage (CV) set at 30 V with the following selected precursor ion–product ion transitions at their respective collision energies (CE): m/z 455 > 159, CE 20 eV; 455 > 261, CE 20 eV; 455 > 279, CE 20 eV.

## Results and Discussion

The results obtained from the ITP of the athlete's urine sample for the dilute-and-shoot approach are shown in Figure 1.

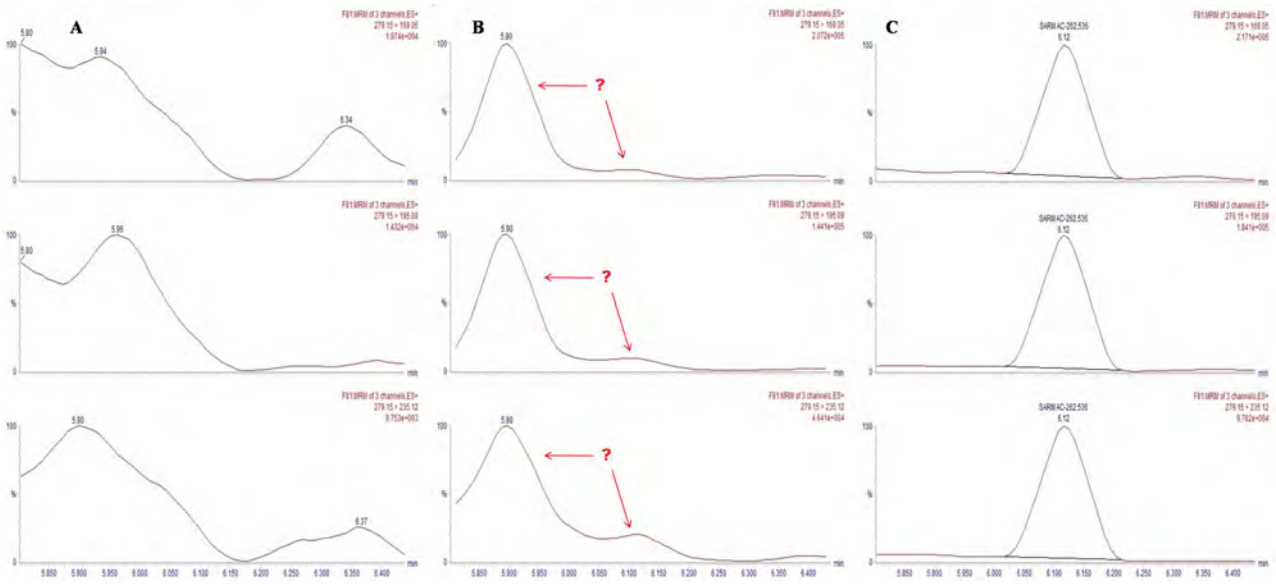


**Figure 1.** Chromatograms of the samples from ITP of SARM AC-262536: (A) blank urine, (B) athlete's sample, (C) QC sample (0.5 ng/mL)

The RT for the SARM AC-262536 in the QC sample was 6.12. In turn, in the athlete's sample, no significant signal specific to the SARM AC-262536 was observed. Furthermore, the earlier one "unknown peak" (RT 5.9) was recorded. The MS scan analysis of the sample showed the **other** significant peak (RT 4.76). Its "Parent scan" analysis revealed a mass 176 Da higher than that of SARM AC-262536 (465 + $H^+$) (Figure 2).

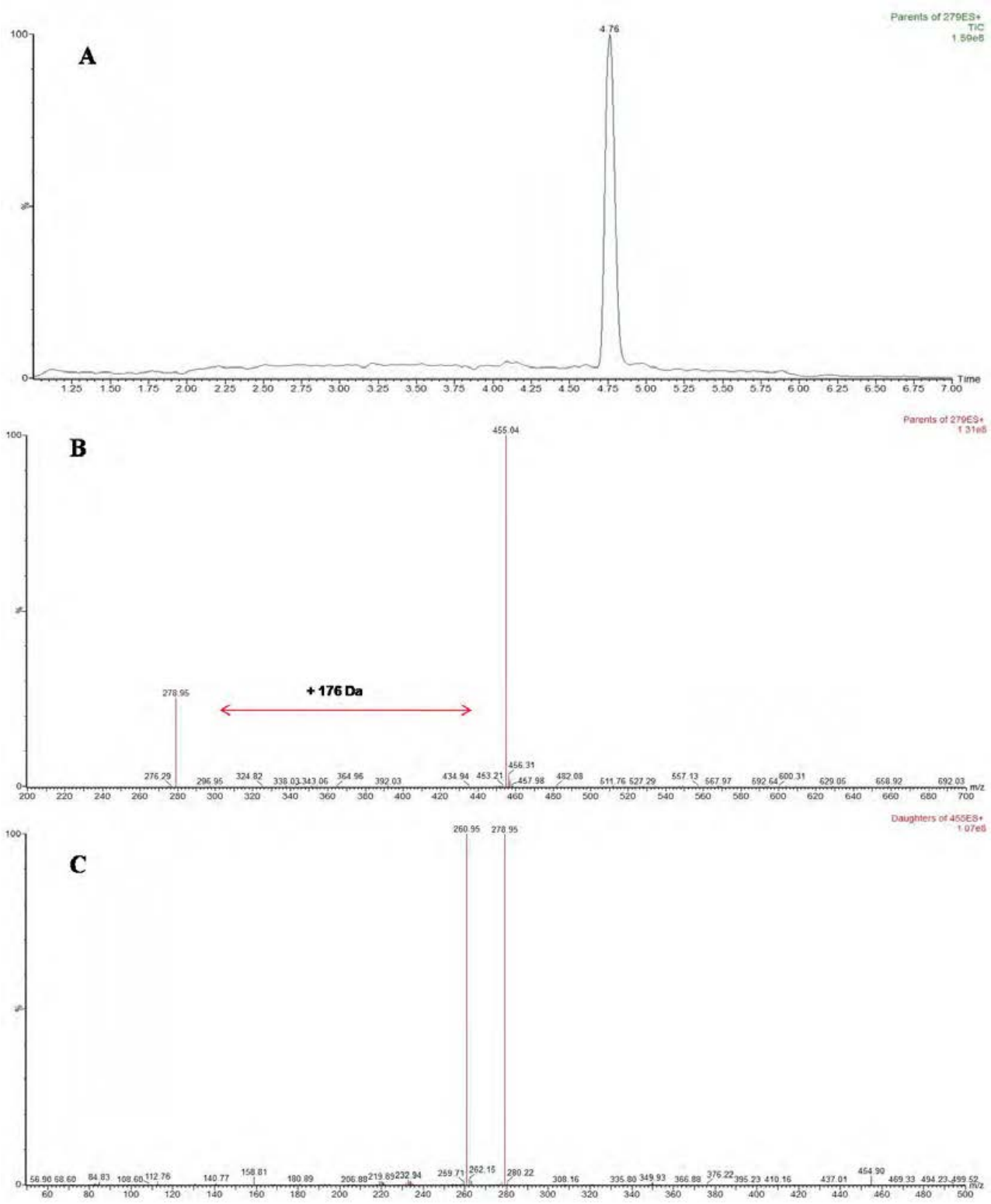


**Figure 2.** Athlete's sample analysis: (A, B) parent scan and (C) daughter scan

Due to the lack of certified reference material, the MRMs for the SARM AC-262536 glucuronide were obtained experimentally. Next, the sample was re-analyzed, including the settings for glucuronide

conjugate (**Figure 3**). Visual inspection of the chromatogram of the blank urine sample revealed no significant interferences in the RT for the MRMs of the SARM AC-262536 glucuronide.

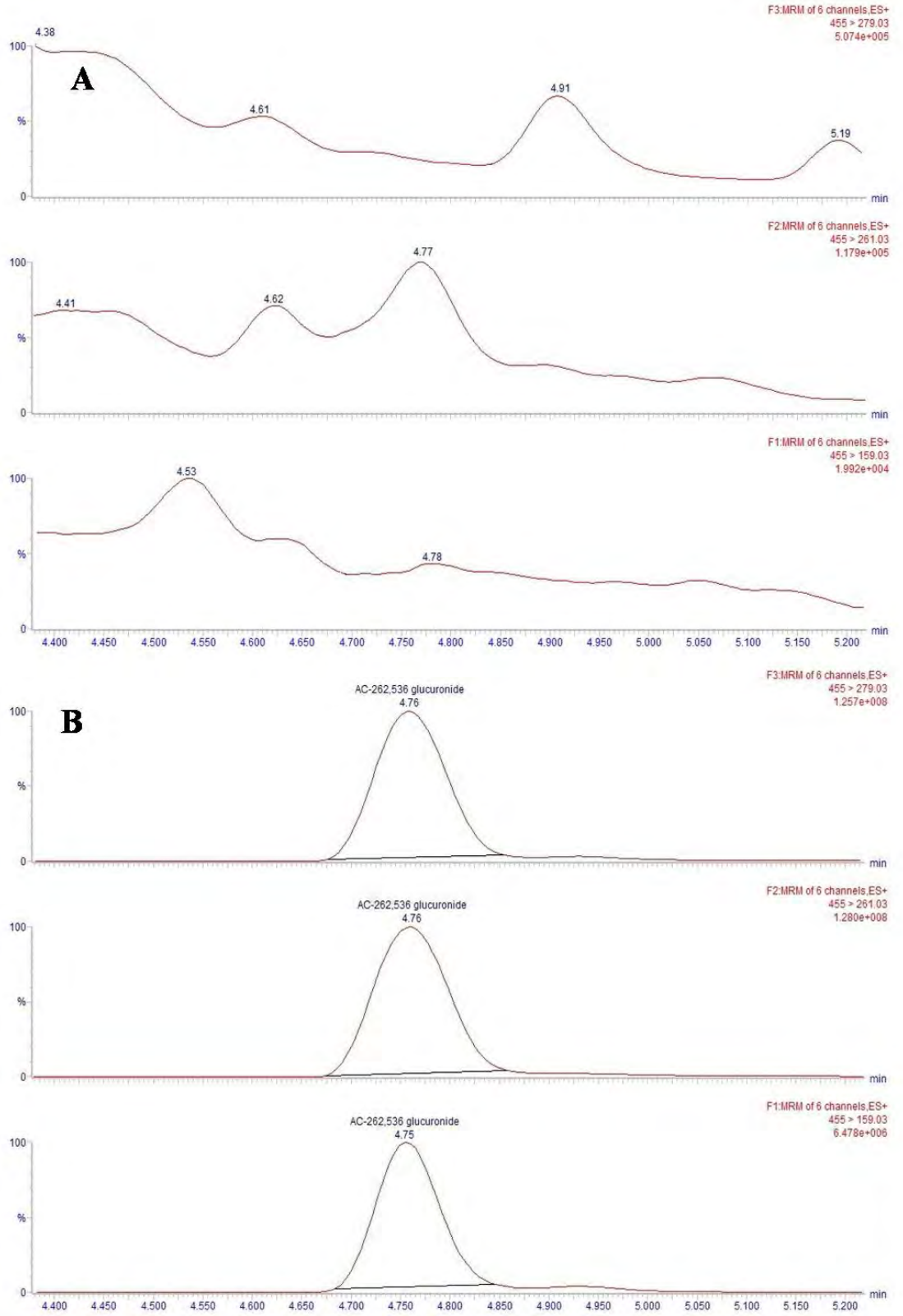


**Figure 3.** Chromatograms of the sample of SARM AC-262536 glucuronide analysis: (A) blank urine and (B) athlete's sample

Additionally, the tested sample was prepared in accordance with the protocol involving enzymatic hydrolysis and extraction. The results for this are shown in **Figure 4**. The RT for the SARM AC-262536 in the athlete's sample and QC sample was 7.17 and 7.18, respectively. A significant difference in the peak area of the SARM AC-262536 in comparison with the dilute-and-shoot approach was observed.

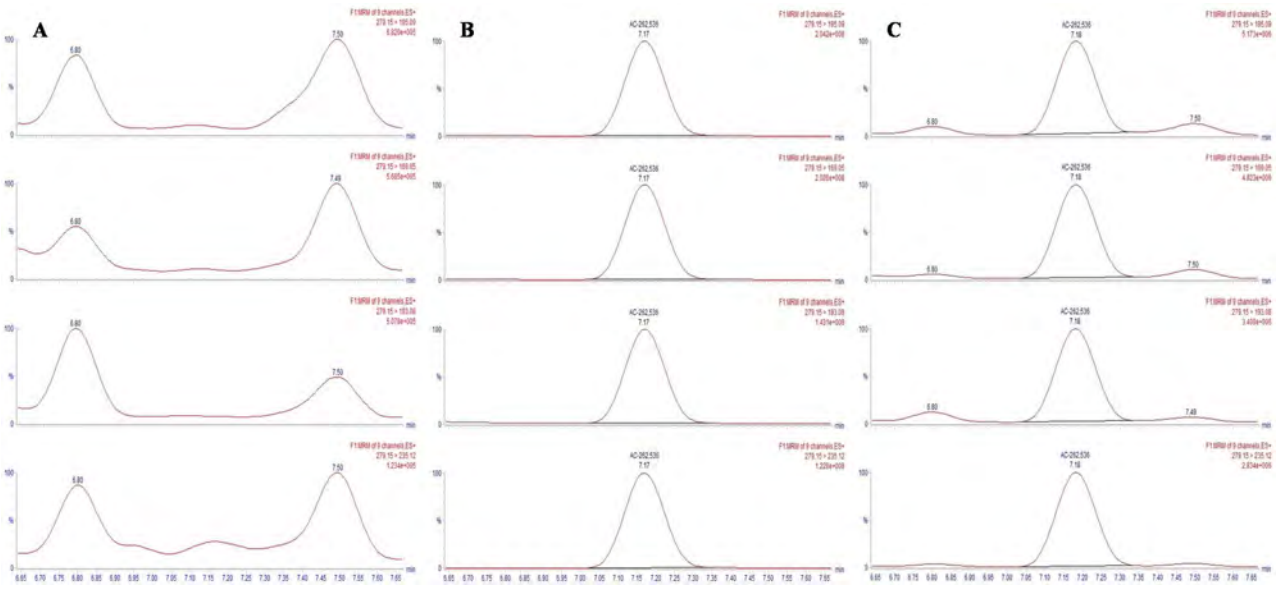


**Figure 4.** Chromatograms of the samples after performing enzymatic hydrolysis: (A) blank urine, (A) athlete's sample and (C) QC sample (1 ng/mL)

Identification of the metabolites of the prohibited substances and their monitoring in routine analysis has a meaningful character for doping control. Furthermore, these activities are also conducted in doping control in animals, such as horses. Cuttler et al.[6] presented the metabolism of the SARM AC-262536 in vitro and in urine, plasma, and hair in horses. According to this paper, the nine phase I metabolites were identified in vitro. Some of them were detected in urine and plasma, as well. Furthermore, the most valuable target with the longest detection window (Epi-AC-262536) in both urine and plasma was proposed. Results from our case also confirm the results of Cuttler et al. study, that SARM AC-262536 is excreted as a free form and as a glucuronide conjugate in human urine, as well.

## Conclusions

A metabolite of SARM AC-262536 was identified in an athlete's sample and could be taken into account by laboratories in their dilute-and-shoot approach. In turn, due to the specificity of the metabolism of the SARM AC-262536, the Confirmation Procedure involving enzymatic hydrolysis of the sample should be considered by the laboratories.

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## Acknowledgements

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# Measurement of the void volume of a column: A harmonized method

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## Abstract

Accurate determination of the hold-up time ($t_M$) in liquid chromatography ensures that early-eluting peaks are not due to unretained analytes. The hold-up time is the sum of extra-column time ($t_{ext}$) and dead time ($t_0$), where $t_0$ relates to the interstitial (void) volume ($V_0$) and the mobile phase flow rate ($F_c$) by $t_0 = V_0/F_c$. True $t_0$ values are also essential for identifying analytes excluded from stationary phase pores. Conventional approaches, such as uracil injection or baseline disturbance, produce $t_0$ values dependent on mobile phase composition. In 2023, F. Gritti and K. Smith introduced a composition-independent method using isotope-labelled acetonitrile ($CD_3CN$) with pure acetonitrile as the mobile phase. This approach measures the true interstitial volume, encompassing both the interparticle voids and the pore volume within packing beads. In this study, $t_M$ was determined for five columns and $t_{ext}$ for a Shimadzu 8030 LC-MS by injecting 20 µL of pure $CD_3CN$ into a mobile phase of 0.01 % formic acid in pure acetonitrile at 0.50 mL $min^{-1}$. Detection was performed in positive-ion mode at *m/z* 45.0. The simplicity and precision of this method support the use of isotope-labelled mobile-phase components as a harmonized approach for composition-independent determination of column hold-up time.

## Introduction

Determining the hold-up time ($t_M$) in liquid chromatography is essential for confirming that early-eluting peaks are not from unretained analytes. The hold-up time $t_M$ is the sum of extra-column time ($t_{ext}$) and dead time ($t_0$), with $t_0 = V_0/F_c$, where $V_0$ is the interstitial volume and $F_c$ is the mobile phase flow rate. True $t_0$ values are critical for identifying analytes too large to access stationary phase pores [1]. Conventional dead-time measurements, such as using uracil or baseline disturbances, depend on mobile phase composition. Dolan [1] proposed estimating $V_0$ from column dimensions, while Gritti and Smith [2] developed a composition-independent method using isotope-labelled acetonitrile ($CD_3CN$) with pure acetonitrile to determine the true interstitial volume.

## Experimental

### Chemicals and columns

Formic acid and acetonitrile (HPLC grade) were obtained from Fisher Chemicals (Germany). Acetonitrile-$d_3$ ($CD_3CN$) was purchased from Cambridge Isotope Laboratories, Inc. (MA, USA). Four C18 reversed-phase columns (CORTECS T3 (Waters), Zorbax Eclipse Plus (Agilent), LUNA (Phenomenex) and ODS Hypersil (Thermo Scientific)) and one pure silica column (Poroshell 120, Agilent) were used (see Table 1 for column dimensions).

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**Instrumentation and method**

Column hold-up volume measurements were performed using a Shimadzu 8030 triple quadrupole LC-MS/MS system, operated at a potential of +4500 V in single-ion monitoring (SIM) mode at *m/z* 45. The mobile phase consisted of 0.01% formic acid in acetonitrile, delivered at a flow rate of 0.5 mL $min^{-1}$. A 20 µL aliquot of $CD_3CN$ was injected for analysis. The extra-column time was determined by replacing the column with a zero-volume union.

## Results and Discussion

Figure 1 shows the single-ion ( *m/z* = 45.0) chromatograms obtained for $CD_3CN$ using the zero-volume union (a) and each of the five tested columns (b – f). The retention time for the zero-volume union corresponds to the extra-column time ($t_{ext}$ = 0.104 min), from which the extra-column volume ( $V_{ext}$) was calculated as 0.052 mL. Chromatograms from the five columns exhibit distinct hold-up times, arising from variations in column length, internal diameter, and particle size . Table 1 lists the column dimensions, experimentally determined void volumes ($V_0$), and calculated empty volumes ($V_{emp}$). $V_0$values were obtained by subtracting $V_{ext}$ from the measured hold-up volume. Columns with wider internal diameters generally showed larger hold-up volumes than narrower columns of the same length.

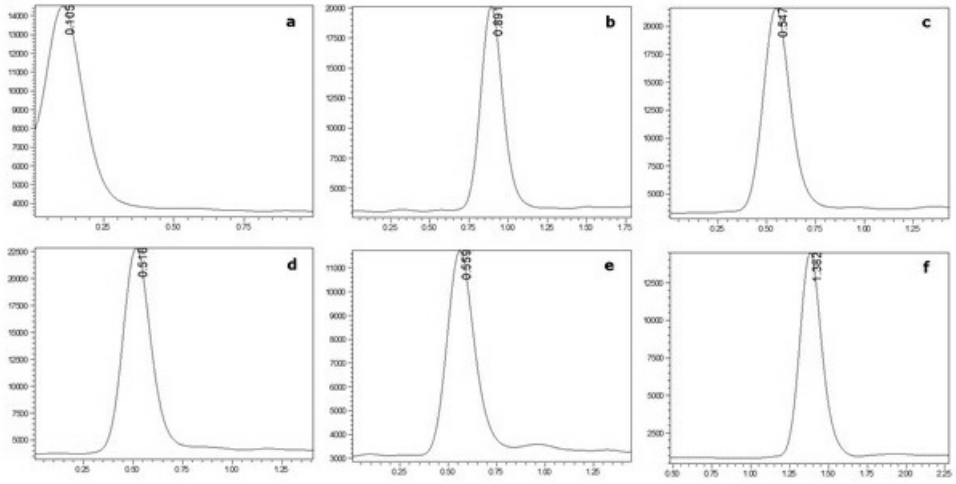


**Figure 1.** Single-ion (*m/z* = 45.0) chromatograms of CD?CN for (a) zero-volume union; (b) CORTECS T3 C18 (100 × 3.0 mm, 2.7 µm); (c) Zorbax Eclipse Plus C18 (100 × 2.1 mm, 1.8 µm); (d) Poroshell 120 (100 × 2.1 mm, 2.7 µm); (e) LUNA C18 (100 × 2.0 mm, 3 µm); and (f) ODS Hypersil (60 × 4.6 mm, 3 µm) columns. Mobile phase: 0.01 % formic acid in acetonitrile; flow rate: 0.5 mL/min; injection volume: 20 µL.

| Column | $V_M$ (mL) | $V_0$ (mL) | $V_{emp}$* (mL) | % ($V_0/V_{emp}$) |
|---|---|---|---|---|
| CORTECS T3 C18: 100 × 3mm × 2.7 µm (120 Å) | 0.446 | 0.394 | 0.707 | 56 |
| Zorbax Eclipse Plus C18: 100 × 2.1 mm × 1.8 µm (95 Å) | 0.274 | 0.222 | 0.346 | 64 |
| Poroshell 120: 100 × 2.1 mm × 2.7 µm (120 Å) | 0.259 | 0.207 | 0.346 | 60 |
| LUNA C18: 100 × 2 mm × 3 µm (100 Å) | 0.281 | 0.229 | 0.314 | 73 |
| ODS Hypersil: 60 × 4.6 mm × 3µm (120 Å) | 0.691 | 0.639 | 0.997 | 64 |

* $V_{emp}$: volume of empty column $= \pi d^2 l/4$, where $d$ is the i.d. of column and $l$ is the length of column.

**Table 1.** Dimensions and measured void volumes of the tested columns

Dolan's equation ($V_0$ = 0.5 / $d^2$/1000, where / is column length and $d$ is internal diameter in mm) assumes that 64 % of the column volume is occupied by the mobile phase [1]. This assumption held true for only two of the five columns, highlighting the need to experimentally determine $t_0$, particularly when calculating retention factors ($k = (t_R - t_0)/ t_0$) for mechanistic studies.

Random close packing of solid spheres results in a maximum packing density of approximately 64%, leaving about 36% interstitial space for the mobile phase [3]. The relatively high $V_0/V_{emp}$ ratios observed for some columns may be attributed to the intrinsic porosity of the packing material, which increases the mobile phase accessible volume beyond that of solid spheres.

The addition of 0.01% formic acid to the acetonitrile mobile phase enhanced protonation of $CD_3CN$ during electrospray ionization, ensuring sufficient *m/z* 45 ion intensity for reliable void volume determination.

## Conclusions

The described LC-MS/MS method, using $CD_3CN$ as a non-retained marker, enables accurate determination of hold-up volumes across columns of different geometries and stationary phases. Owing to its simplicity and precision, this approach is recommended as a harmonized protocol for determining hold-up time ($t_M$) in chromatographic studies.

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

# A screening method for detecting Voxelotor in doping control analysis

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## Abstract

Voxelotor is a haemoglobin S polymerization inhibitor that has been developed for the treatment of patients with sickle cell disease (SCD). Voxelotor is prohibited in sports and is included in World Anti-Doping Agency (WADA) prohibited list, class *M1. Manipulation Of Blood and Blood Components.*

The aim of the current study was to develop and validate a method for the detection of Voxelotor, using a triple quadrupole UHPLC/MS-MS method with liquid-liquid extraction (LLE) at a basic pH of 9.6, based on hydrolysis, which is able to detect concentrations as low as 2.5 ng/mL. The present method was validated according to WADA technical documents for matrix effect, limit of detection (LOD), carryover, stability and reproducibility.

Our method was proven to be suitable for the identification of Voxelotor according to WADA technical documents in force, TD2023IDCR and TD2022MRPL, due to the specificity of the chromatographic method, which ensures reliable analytical results without interferences.

## Introduction

Voxelotor is prohibited in sports and is included in World Anti-Doping Agency (WADA) prohibited list, *class M1. Manipulation Of Blood and Blood Components* [1]. Voxelotor is a haemoglobin S polymerization inhibitor that has been developed for the treatment of patients with sickle cell disease (SCD). Clinically, Voxelotor has been shown to significantly improve anemia by reducing hemolysis and increasing blood Hb levels [2]. In the specialized literature, Voxelotor and its metabolites were detected using solid phase extraction (SPE)[3]. This study proposes a method for the detection of Voxelotor in urine samples by liquid-liquid extraction (LLE) and detection by liquid chromatography - tandem mass spectrometry (UHPLC-MS/MS).

## Experimental

Voxelotor was purchased from LGC Standards, Wessel, Germany. Tert-butyl methyl ether, acetonitrile, potassium bicarbonate and potassium carbonate were purchased from Merck (Redox Romania). Ultrapure water was obtain using a Milli-Q Q-POD equipment from Merck. Sample preparation method is presented in Figure 1. Equipment used was an UHPLC-MS/MS systems EXION LC AD/AB SCIEX 6500+. The chromatographic column was Zorbax SB-C18, 2.1 x 50 mm, 5 microns. The mobile phase was solvent A: $H_2O$ (0.1% HCOOH and 5 mM Ammonium formate), solvent B: ACN/$H_2O$ (9/1, *v/v*) (0.1% HCOOH and 5 mM Ammonium formate), injection volume 2 µL, flow rate 0.25 mL/min. The gradient started at 0% B, held for 1 min, went from 0% to 100% in 14 min, stay at 100% B 2 min and equilibrate 0% B in 3 min.

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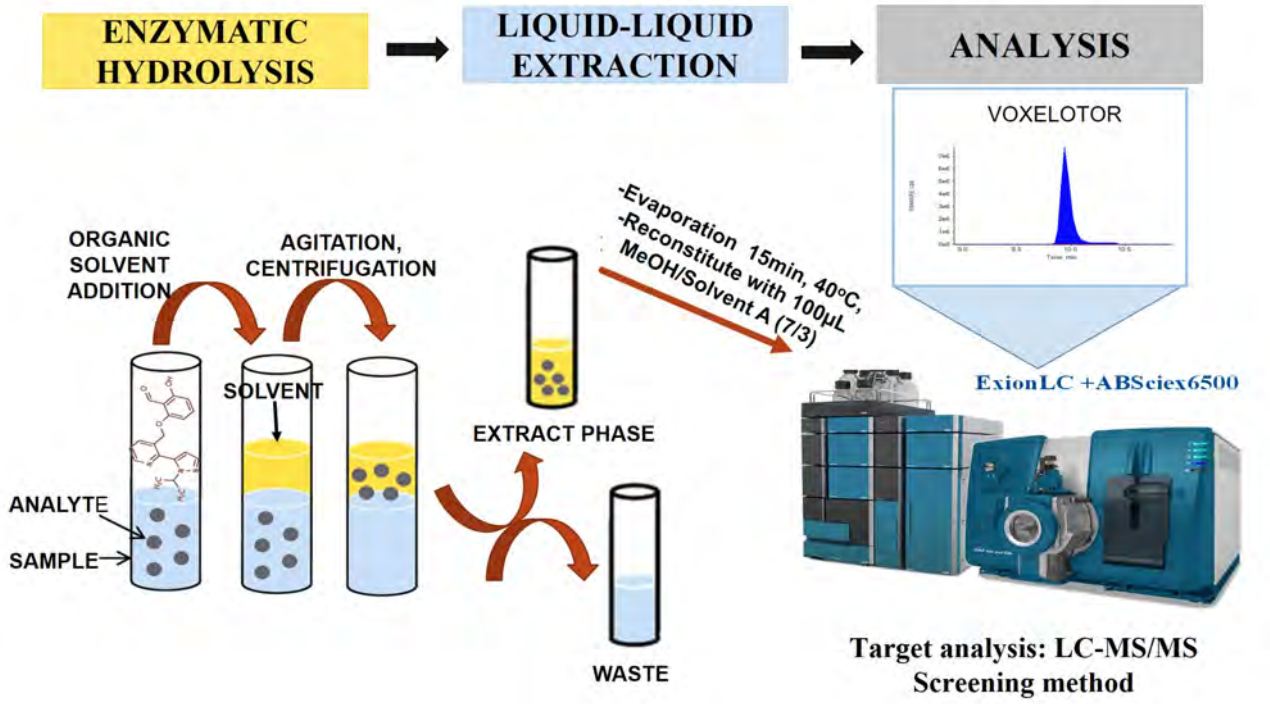


**Figure 1.** Sample preparation scheme

Mass Spectrometer AB SCIEX 6500+, Ionization source ESI positive, Source Temperature 500°C, Capillary voltage 5500V, nebulizer gas (GS1) 50 psi, auxiliary gas (GS2) 50 psi, curtain gas (CUR) 35 psi, collision cell gas (CAD) 12. The acquisition mode was multiple reaction monitoring (MRM), with following transitions 338.08>158.1, 338.08>130.1.

Validation of Voxelotor detection method in urine by triple quadrupole UHPLC-MS/MS, combined with LLE sample preparation was performed in accordance to technical note for WADA laboratories and included the following parameters: selectivity, limit of detection (LOD), reliability of detection, carryover. and sample extract stability (Table 1).

| Validation Parameter | Experimental Conditions | Acceptance Criteria |
|---|---|---|
| Selectivity (blank samples) | 10 blank urines prepared according to method presented | Absence of interfering signals at retention time of the analyte |
| LOD for Non-Threshold Substances without a MRL | 10 samples spiked at 5ng/mL<br>10 samples spiked at 2.5ng/mL | S/N >3 in all samples for all monitored transitions |
| Reliability of detection | 2 batches of 10 samples prepared in 2 days by 2 different analysts | 100% Detection rate (10/10 samples) |
| Carryover | a urine spiked at 25ng/mL was prepared and injected preceded by 1 blank urine and followed by 3 blank injections | Carryover not observed in the third injected blank |
| Sample Extract Stability | after 3 and 7 days of storage in the autosampler at 4°C | 100% Detection rate (10/10 samples) |

**Table 1.** Criteria for evaluating the validation parameter

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## Results and Discussion

The method proved specific for the target analyte with no interferences present on monitored transitions at the retention time of the analyte (a urine blank is presented in Figure 3A). LOD was found to be 2.5 ng/mL, all replicates had S/N ratios greater than 3 for all monitored transitions (a urine spiked at 2.5 ng/mL is presented in Figure 3B). The method was found to be reliable; both batches of samples analysed had an S/N >3 for all transitions. No carry-over was observed in the blank injections after the sample spiked at 25 ng/mL. Sample extracts proved stable Voxelotor being detected after 7 days of storage at 4°C.

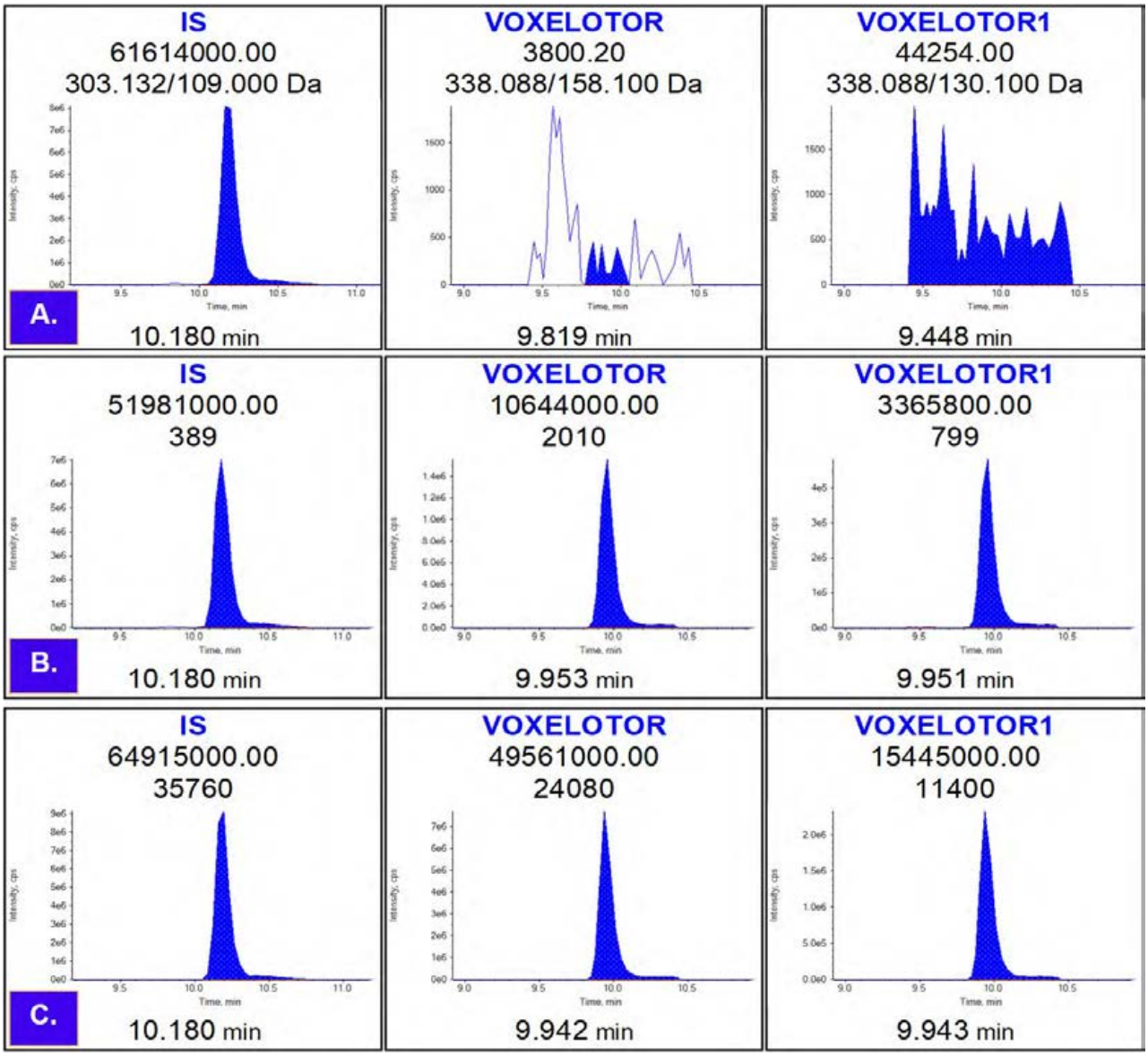


**Figure 2.** Multiple reaction monitoring chromatograms of Voxelotor: A. blank of a urine sample, B. in a urine sample containing target analyte at a concentration of 2.5 ng/mL, and C. in a solution without matrix effect (ultrapure water) containing target analyte at a concentration of 5 ng/mL

## Conclusions

According to our results, the developed methods, instrumental and sample preparation have proved to be suitable for Voxelotor identification and were validated according to WADA technical documents TDMRPL, TDIDCR. The method demonstrates the capability to detect the compound as low as 2.5 ng/mL. LLE is simple, less expensive than SPE and saves time for analysis.

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Barber A, Naud J, Ayotte C, Lalonde K, Couture M

# Increased recovery for sulfoconjugated 7-keto-DHEA metabolites using acid catalyzed hydrolysis with THF

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## Abstract

Although several analytical methods targeting sulfo-conjugated steroid metabolites exist in the literature, method artifacts affecting specific androgenic anabolic steroids and their metabolites may have led to an under representation of the importance of the sulfo-conjugated steroid fraction in the context of doping control. An acid catalyzed hydrolysis using tetrahydrofuran developed in our laboratory was compared to a similar acid catalyzed hydrolysis involving a mixture of ethyl acetate/methanol. For the majority of compounds investigated, no statistically significant difference in recovery between the two methods was observed. However, some metabolites of 7-keto-DHEA were shown to have practically a 100-fold increase in recovery using the acid catalyzed hydrolysis with THF compared to the ethyl acetate/ methanol method. This is of particular interest for the detection of 7-keto-DHEA doping and has possible ramifications on which target compound are most suited for stable isotope analysis to determine whether the analytes of interest come from either an exogenous origin or and endogenous origin through in situ microbial reworking. Interestingly, different isomers were seen to have large differences in recovery from one to another. For example, we observed a more drastic increase in recovery for 7α-OH-DHEA relative to 7β-OH-DHEA. The increased recovery for 7α-OH-DHEA-sulfate in particular may make it a more interesting target compound during GC/C/IRMS confirmation.

## Introduction

Published analytical methods for confirmation of 7-keto-DHEA (3β-Hydroxy-androst-5-ene-7,17-dione) doping using GC/C/IRMS have attempted to detect this misuse through either the endogenous steroid profile [1,2] or targeting the acetylated parent compound and several of metabolites including 7β-hydroxy-DHEA and 5α-androstane-3β,7β-diol-17-one [3,4]. The relative importance of these markers has been investigated as both sulfoconjugated and glucuronidated metabolites with contradictory information regarding the excretion of 7α-hydroxy-DHEA.

Using an optimized acid catalyzed hydrolysis involving THF and methanol method published by our laboratory [5] we observed concentrations of 7α-hydroxy-DHEA which ought to be measurable by GC/C/IRMS. We performed a comparison of the method involving an acidified ethyl acetate methanol mixture with the THF/methanol method.

## Experimental

Urine samples are first treated using our routine ITP analysis of the free and glucuronidated steroid fractions involving solid phase extraction, deglucuronidation via enzymatic hydrolysis, liquid liquid

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extraction (LLE), derivatization and subsequent analysis by GC/MSMS. The aqueous extract remaining after the LLE, containing sulfo-conjugated urine components, was adjusted to neutral pH and purified via SPE with SepPak C18 cartidges (Waters). The extract was treated using two different procedures to compare their relative efficiencies at extracting 7-keto-DHEA and metabolites (7α-OH-DHEA and 7β-OH-DHEA). For the acid catalyzed hydrolysis involving THF, the extract is treated with 1 mL of $H_2SO_4$ acidified THF, incubated at 50°C for 1 hour, neutralized with $NaHCO_3$ a double LLE and finally MSTFA/TMS derivatization. For comparison, the aqueous extracts after C18 SPE purification were solubilized in a mixture of ethyl acetate (70%) in methanol (30%). The solubilized extract was then treated with 1 mL of $H_2SO_4$ acidified ethyl acetate, incubated for 1 hour at 50°C, neutralized with ammonium hydroxide before performing and MSTFA/TMS derivatization.

Three (3) different anonymized athlete urine samples, previously having been found to not contain any substances on the prohibited list, were prepared using the above methods for the purpose of comparison. 1 µL of the urine extracts were analyzed in split mode (1:5) on an Agilent 7000GC with a DB 5MS column (25 m, 0.2 mm ID, 0.33 micron film thickness) with a constant helium flow of 1.2 mL/min and an injector temperature of 270°C.

## Results and Discussion

Molecules such as dehydroepiandrosterone, dehydroandrosterone, epiandrosterone, androsterone and etiocholanolone showed no difference in recovery between the different tested methods (Figure 1).

However, several modified DHEA structures showed a statistically significant increase in recovery using the acidified THF hydrolysis method (Figure 2). Two of the known 7-keto-DHEA metabolites, 7α-OH-DHEA and 7β-OH-DHEA, showed an increase in recovery of up to 99% and 88% respectively allowing for the $\delta^{13}C$ signature of 7α-OH-DHEA to be measurable by GC/C/IRMS.

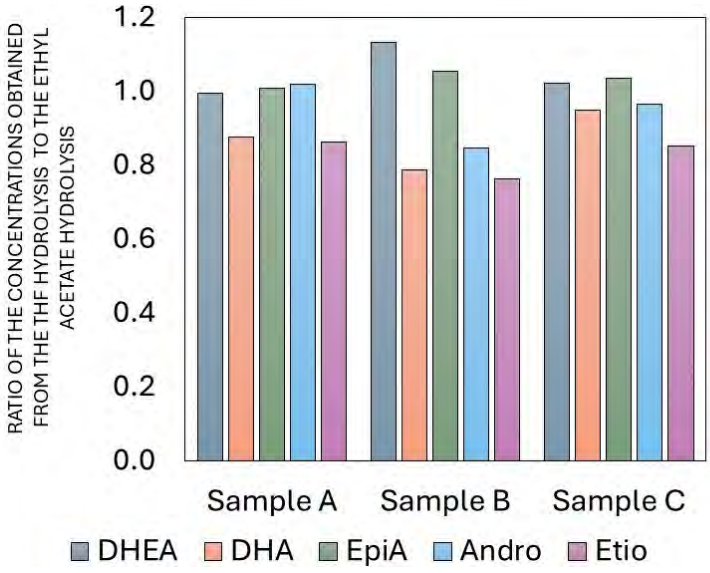


**Figure 1.** Comparison of the ratio of the concentration obtained from the THF hydrolysis relative to the ethyl acetate hydrolysis for 5 steroids (dehydroepiandrosterone, dehydroandrosterone, epiandrosterone, androsterone and etiocholanolone)

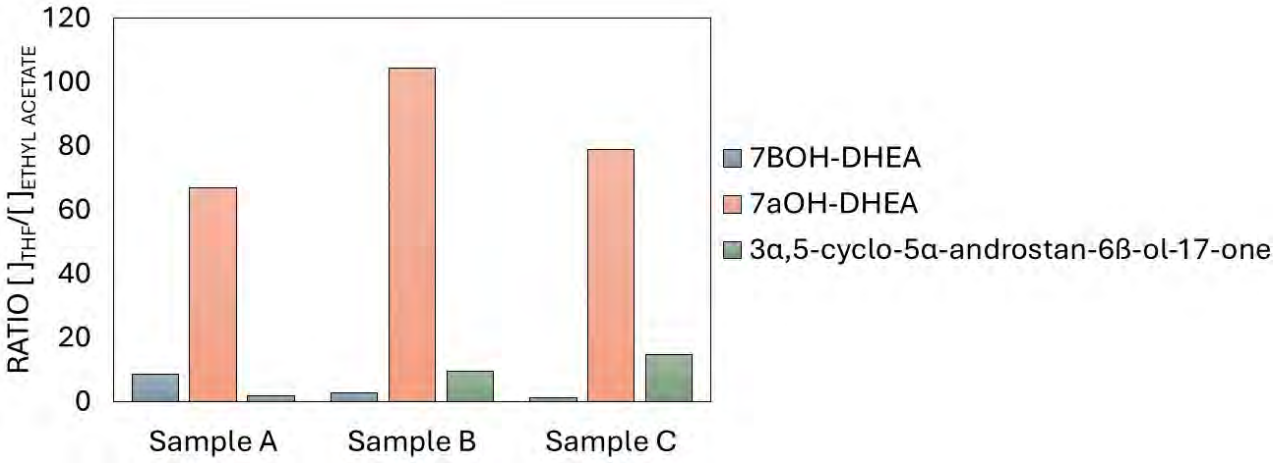


**Figure 2.** Comparison of the ratio of the concentration obtained from the THF extraction relative to the ethyl acetate extraction several modified DHEA structures

In addition to the increase in recovery, the extracts obtained using THF were found to contain fewer impurities/byproducts when analyzed by GC-MSMS (Figure 3). This is particularly beneficial if this acid catalyzed hydrolysis is to be used in GC/C/IRMS confirmation procedures, as the cleaner extracts are less likely to lead to contaminated target compound peaks when analyzed on the GC/C/IRMS.

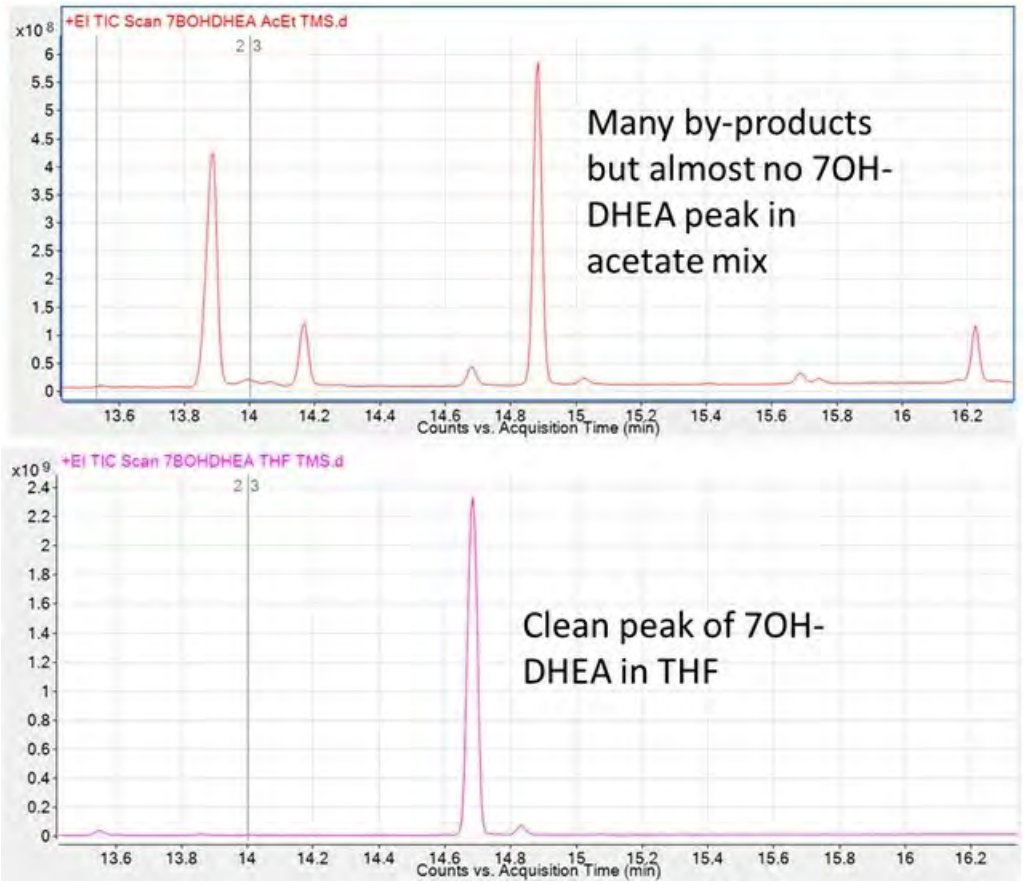


**Figure 3.** TIC scan for 7β-OH-DHEA obtained using the acidified ethyl acetate (top) and acidified THF (bottom) solvolysis

To determine which samples ought to be confirmed by the previously described method, a reference population for both 7α-OH-DHEA and 7β-OH-DHEA were generated using out initial testing procedure results. Although the corresponding IRMS results have not been compiled, based purely on the ITP results, we preliminarily suggest that samples containing more than 50 ng/mL of 7α-OH-DHEA and greater than 200 ng/mL of 7β-OH-DHEA be treated as presumptive analytical findings and the origin of 7-keto-DHEA should be evaluated using GC/C/IRMS (Figure 4).

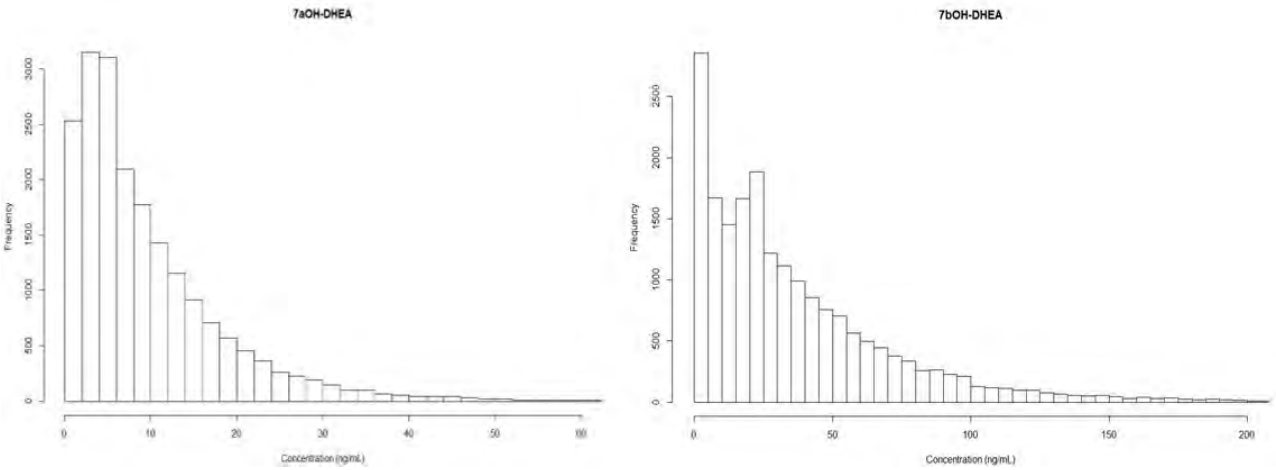


**Figure 4.** Reference population results for 7α-OH-DHEA (left) and 7β-OH-DHEA (right) obtained during from initial testing procedure results (N= 25622)

## Conclusions

The increased recovery using a THF methanol mixture instead of ethyl acetate/methanol for DHEA, 7-keto-DHEA and metabolites matches with the literature from 1958 [6]. In addition to the increased recovery, fewer byproducts and possible contaminants which may interfere with future GC/C/IRMS confirmations were observed when THF/methanol is used for this acidic hydrolysis. This opens the door for GC/C/IRMS confirmation of metabolites such as 7α-OH-DHEA. Other sulfate cleaving chemical and enzymatic reactions are still useful for the vast majority of steroids tested and should still be considered when no impact on recovery is observed, and the associated confirmation procedures remain fit for purpose and meet the criteria specified in their relevant normative documents.

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We would like to acknowledge Natacha Lo Vullo and Nicolas Le Berre for their contribution to this project.

Edey C, Salvacion S, Brooker L, Agon V

# Steviol can interfere in the GC-C-IRMS analysis of steroids in urine

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## Abstract

The GC-C-IRMS analysis of steroids in urine requires extensive cleanup of the biological matrix, typically including HPLC fraction collection. Ensuring the purity of each HPLC fraction forms a critical part of the method development process so that each steroid compound can be analysed without isotopic fractionation and free from potential interfering substances. The work described here shows that steviol is one such interfering compound, and its removal should be considered, especially when analysing steroids without derivatisation.

## Introduction

Stevia products contain a mixture of purified steviol glycosides derived from the Stevia Rebaudiana plant. Purified steviol glycosides are used as a food additive that is 200-300 times sweeter than sugar but contains no calories [1,2]. They were initially approved for use in Australia in 2008 in limited foods and drinks, though the scope of approved use has since expanded and it is now very common in processed foods [3].

When consumed, these glycosides are cleaved in the large intestine and the resulting free steviol is metabolised in the liver to form steviol glucuronide, which is subsequently excreted in urine [1,2]. If steviol is not removed during HPLC cleanup, it may adversely impact the chromatography, reactor performance and delta values during GC-C-IRMS analysis.

## Experimental

Samples were extracted according to ASDTL's routine GC-C-IRMS procedure using a 1D-HPLC cleanup on a Thermo Ultimate3000 HPLC system. A Phenomenex Kinetix 5 µm C18 100 Å column (250 mm x 4.6 mm) was used to collect a total of eight fractions per sample. Diol and PD fractions (as well as A/Et and 16en) were combined prior to analysis. All fractions were analysed as free steroids, first by GCMS then by GC-C-IRMS. GCMS data was assessed prior to GC-C-IRMS analysis to ensure that analytes were within the linear range of the IRMS instrument and to check for interferences. Large peaks observed in the GCMS chromatograms were noted and, if possible, identified through library matching. A standard of steviol was purchased from Sigma-Aldrich and used to confirm the identity of a peak that was increasingly observed in routine samples.

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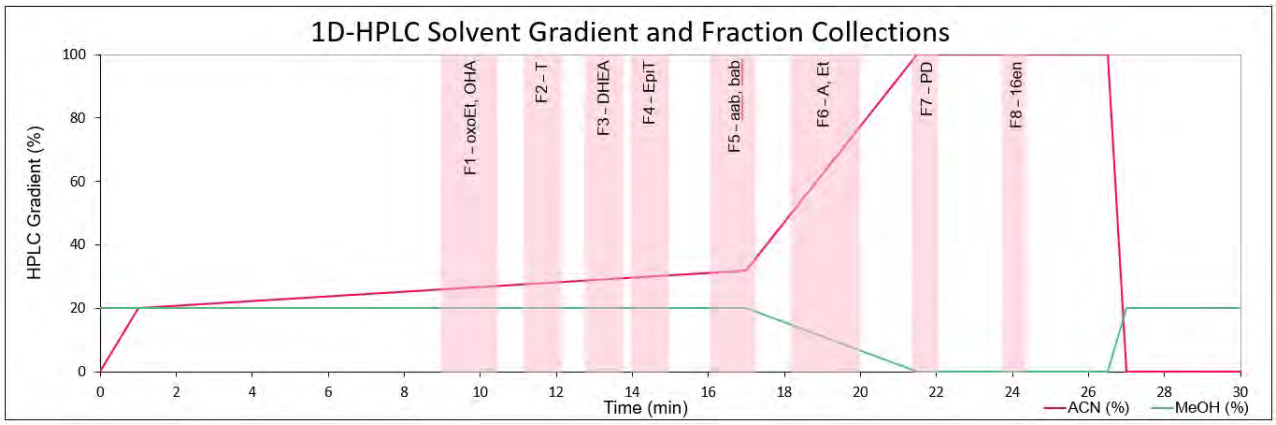


**Figure 1.** HPLC solvent gradient and collection windows for the 1D cleanup procedure. Column: Phenomenex Kinetix 5 µm C18 100 Å, 250 mm x 4.6 mm – 1.2 mL/min
Abbreviations: oxoEt: 11-oxoetiocholanolone, OHA: 11β-hydroxyandrosterone, T: testosterone, DHEA: dehydroepiandrosterone, EpiT: epitestosterone, aab: 5α-androstane-3α,17β-diol, bab: 5β-androstane-3α,17β-diol, A: androsterone, Et: etiocholanolone, PD: pregnanediol, 16en: 16(5α)-androsten-3α-ol

The extraction methodology on the Thermo Ultimate3000 was updated to improve the HPLC purification step by using a 2D-HPLC separation. After validation, this 2D purification replaced the aforementioned 1D purification for routine GC-C-IRMS analysis in ASDTL. A Phenomenex Kinetix 2.6 µm Phenyl-Hexyl 100 Å column (150 mm x 4.6 mm) is used in the 1$^{st}$ dimension with fractions for A/Et and 16en collected. The remaining analytes are diverted via a switching valve, at the appropriate retention time, to five separate 1 mL holding loops. Analytes are held in these loops until the completion of the 1$^{st}$ dimension run time when they are analysed sequentially, with fraction collection, on the 2$^{nd}$ dimension column – a Phenomenex Onyx Monolithic HD-C18 130 Å column (100 mm x 4.6 mm) (4). All fractions are then analysed as free steroids, first by GCMS then by GC-C-IRMS.

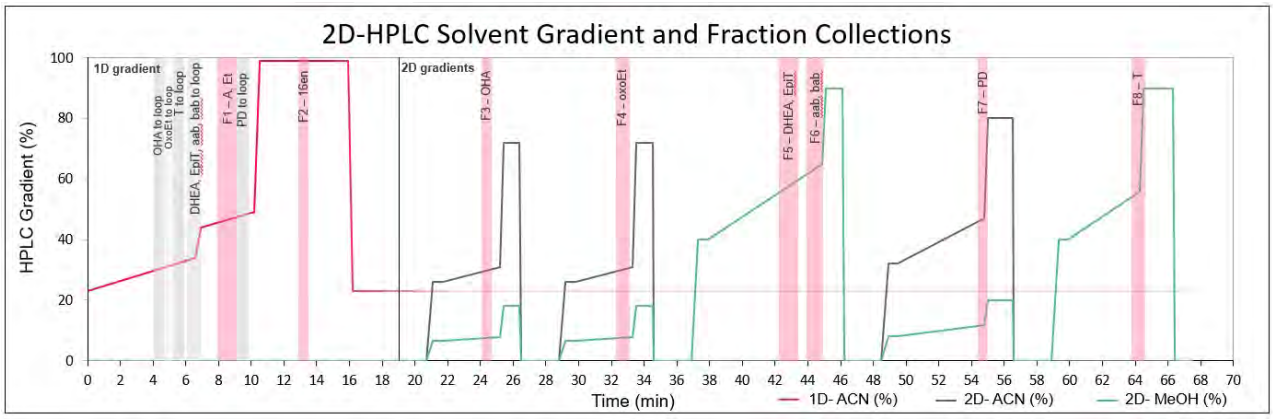


**Figure 2.** HPLC solvent gradient and collection windows for the 2D cleanup procedure. Column 1: Phenomenex Kinetix 2.6 µm Phenyl-Hexyl 100 Å, 150 mm x 4.6 mm – 1 mL/min; Column 2: Phenomenex Onyx Monolithic HD-C18 130 Å, 100 mm x 4.6 mm – 3 mL/min

## Results and Discussion

Using the 1D-HPLC cleanup procedure, a large peak eluting before PD was observed in several routine samples that adversely affected the GC-C-IRMS analysis as follows:

- Depleted delta values for PD (-27 to -30‰)
- Poor chromatography for PD
- Poor chromatography in subsequent injections
- Reduced performance of the combustion reactor in subsequent injections

This peak was presumptively identified as steviol through a library match using the NIST database. The identity of the peak was confirmed by comparison to a steviol standard.

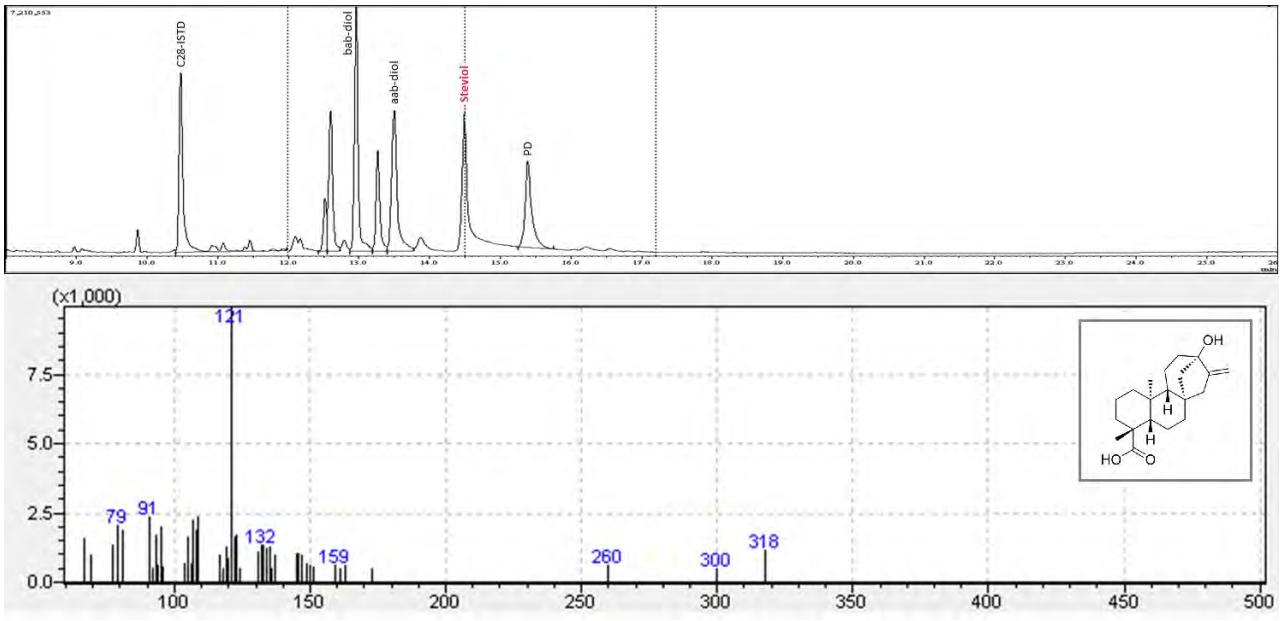


**Figure 3.** Top: Chromatogram showing RT of steviol in combined diol/PD fraction (1D-HPLC cleanup). Bottom: Mass spectrum and structure of steviol

The structure of steviol contains a carboxyl group and does not chromatograph well by GC underivatised, showing significant peak tailing. At high concentrations, this tailing clearly extends into the PD peak. At low concentrations, the retention times of steviol and PD appear sufficiently separated, however, it was noted that samples containing trace amounts of steviol could still exhibit depleted delta values and poor chromatography for PD.

The effect of steviol on the PD delta value was investigated through a spiking experiment. A blank, steviol free, urine was extracted according to the 1D-HPLC procedure. The collected PD fraction was split across four vials each containing PD at approximately 25 ng/µL. The vials were spiked with steviol at 0, 20, 50 and 100 ng/µL. Steviol was barely detectable in the chromatogram when spiked at 100 ng/µL and was not detected at lower concentrations. PD delta values were found to be affected in both the 50 and 100 ng/µL spikes.

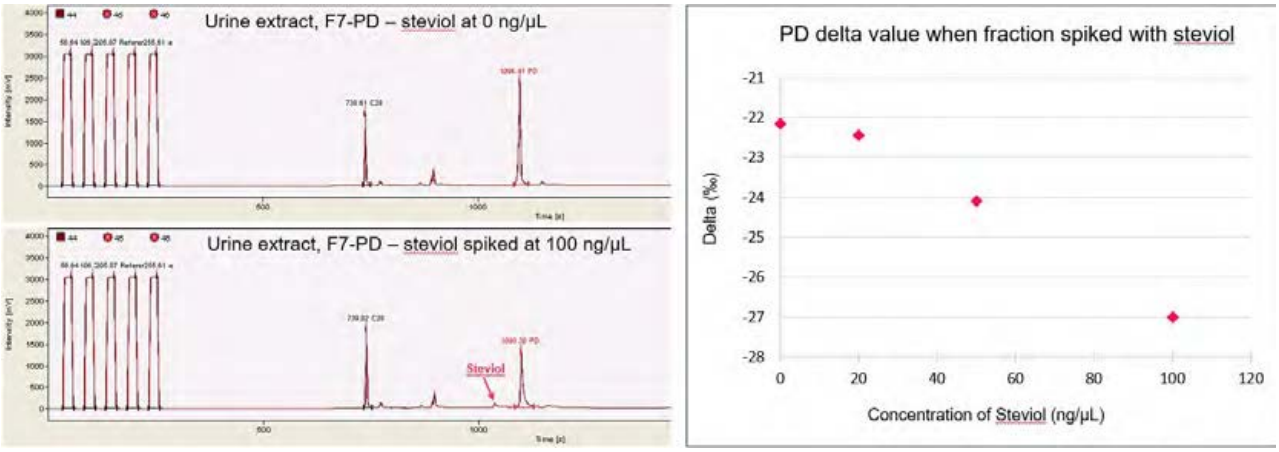


**Figure 4.** Spiking experiment demonstrating impact of steviol on chromatography and delta values

ASDTL has since validated and implemented a 2D-HPLC cleanup procedure that ensures that steviol is removed from any collected fractions. Several routine samples with high concentrations of steviol that had previously been analysed using the 1D-HPLC procedure were subsequently analysed using the 2D-HPLC procedure. Delta values for PD using the 2D-HPLC procedure were consistent with other ERCs in the same sample.

## Conclusions

Steviol was found to be a significant interference in the GC-C-IRMS analysis of PD using ASDTL's 1D-HPLC extraction procedure. Due to its increasing prevalence in urine samples, it should be considered as a potential interference when undertaking method development using HPLC fraction collection for the analysis of steroids in urine by GC-C-IRMS. This is particularly important if steroids are analysed without derivatisation. In ASDTL this interference was eliminated through the use of a 2D-HPLC cleanup procedure.

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# Analysis of small peptides by LC-MS using a 2D configuration with alternating analytical and regeneration columns

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Division of Medicine, University College London, Centre for Metabolism and Inflammation, London, UK[2]

## Abstract

The analysis of small peptides by liquid chromatography-mass spectrometry (LC-MS) often requires advanced methodologies to achieve high sensitivity, resolution, and reproducibility. In this study, a two-dimensional (2D) LC-MS configuration is employed, utilizing dual-column operation for growth hormone releasing peptide (GHRP), and gonadotropin-releasing hormone (GnRH) for urine samples. One column is dedicated to the analytical separation, while the second column undergoes regeneration, operating in alternating cycles. This setup ensures continuous operation, minimizes downtime, and improves system efficiency.

The method was optimized for the detection and analysis of small peptides, addressing challenges such as sample complexity and column saturation. Key parameters, including gradient elution and column switching timing, were fine-tuned to ensure operation and consistent performance. The 2D configuration significantly improved peak resolution and analytical throughput compared to single-column setups configuration. The 2D configuration highlights the potential of 2D LC-MS for high-performance of small peptide analysis. This approach demonstrates how alternating column use can efficiently enhance system longevity and optimize the use of time and resources. It allows for continuous sample analysis while minimizing downtime for column maintenance or reconditioning.

## Introduction

The analysis of small peptides by liquid chromatography-mass spectrometry (LC-MS) often requires advanced methodologies to achieve high sensitivity, resolution, and reproducibility. In this study, a two-dimensional (2D) LC-MS configuration is employed, utilizing dual-column operation for small peptides analysis. One column is dedicated to the analytical separation, while the second column undergoes regeneration, operating in alternating cycles. This setup ensures continuous operation, minimizes downtime, and improves system efficiency.

For a parallel regeneration setup, the chromatography pump and regeneration pump must be assigned specific names. The column manager is configured with two valve positions. Two inlet methods are used to operate this mode:

1. The first method sets the valve position to 1-1, meaning Column 1 is used for chromatography while Column 2 undergoes regeneration.
2. The second method sets the valve position to 2-2, reversing the roles—Column 2 is used for chromatography while Column 1 regenerates (Figure 1)

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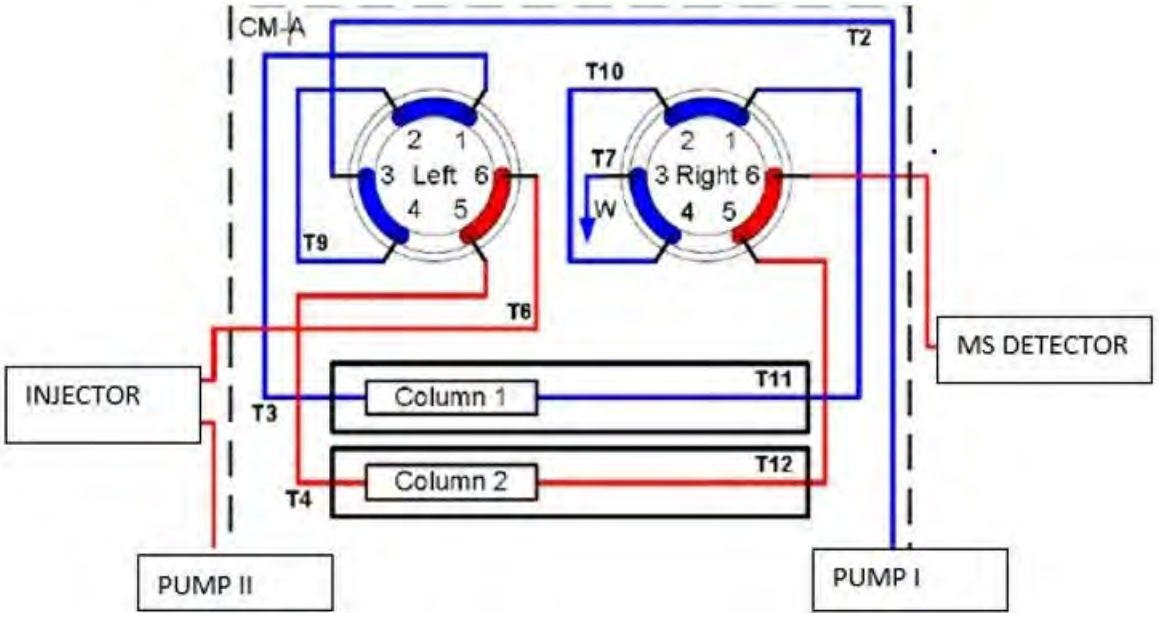


**Figure 1.** Parallel columns configuration (Alternate)

The sample sequence should alternate between these two methods: the first line should use position 1-1, the second line should use position 2-2, and this pattern should continue until the sequence is completed. Additionally, the user has the option to operate the system in 1D mode by setting the regeneration pump flow to zero and using the same method throughout the sequence.

**Figure 2.** 2D UPLC Hardware Configuration

## Experimental

### Sample Preparation

An automated SPE with the Biotage Extrahera was used for the sample preparation wherein 1 mL of urine was centrifuged at 4000 rpm/8 min and loaded into a preconditioned 1cc 30 mg WCX cartridge after the addition of ISTD, and washed with 1 mL of deionized water, eluted with 1 mL of elution solvent (5% formic acid in methanol). Then the eluates were evaporated in a vacuum centrifuge at 45°C for 1-2 hours. After evaporation, 100 µL of $H_2O$ was added to reduce the acidity of the sample, which was then transferred to vials and injected into the 2D-UPLC-HRMS**.**

### Instrumentation

An electrospray liquid chromatography tandem-mass spectrometry system (ESI-LC-MS-MS) for gradient chromatography was used. The instrumentation consisted of an Acquity, Ultra Performance Liquid Chromatographic system (UPLC), equipped with a solvent manager, a sample manager and a column manager for handling of two columns (Waters, Milford, MA).

### Chromatographic condition

UPLC™ Acquity I Class (Waters, Milford, MA, USA) was used for this study, UPLC consisted of Waters ACQUITY UPLC system with an Agilent Zorbax 300SB-C18 (2.1×150 mm, 1.8 µm) column. Mobile phases were water and ACN, both containing 0.2% formic acid. Gradient elution: 90% B for 1 min, 80% in 0.5 min, 50% in 6 min, held for 0.8 min, 10% in 0.7 min, held for 3 min, increased to 90% in 1 min and held for 4 min. Flow rate was 0.11 mL/min and sample volume was 10 µL.

### Mass spectrometry

The LC-system was coupled with a Thermo Scientific Q-Exactive™ benchtop Orbitrap-based mass spectrometer with parameters: spray voltage 4 kV, Source temperature 350°C, sheath gas 40, auxiliary gas 10. The instrument operated in positive full scan mode and Targeted MS/MS mode by PRM. Full scan mode resolution was 35,000, scan range 100-1200 *m/z*, Capillary voltage: 4.0 kV, ionization mode: ESI positive.

## Results and Discussion

The alternating-column 2D configuration (one column in analysis while the other regenerates) produced equivalent analytical performance for small peptides analysis. Across all QC levels and study samples, retention times (RTs) matched within ±0.30 min between the analytical and the regeneration columns, with no systematic drift. Peak shape (width at half height, tailing factor) remain within the acceptance criteria (Figure 3).

The 2D column-switching approach offers significant benefits in continuous workflow without downtime. While one column is being regenerated, the other is actively analyzing samples, eliminating the delays associated with column washing or equilibration in traditional 1D setups. This ensures maximized sample throughput, which is crucial for large-volume screening during major sporting events.

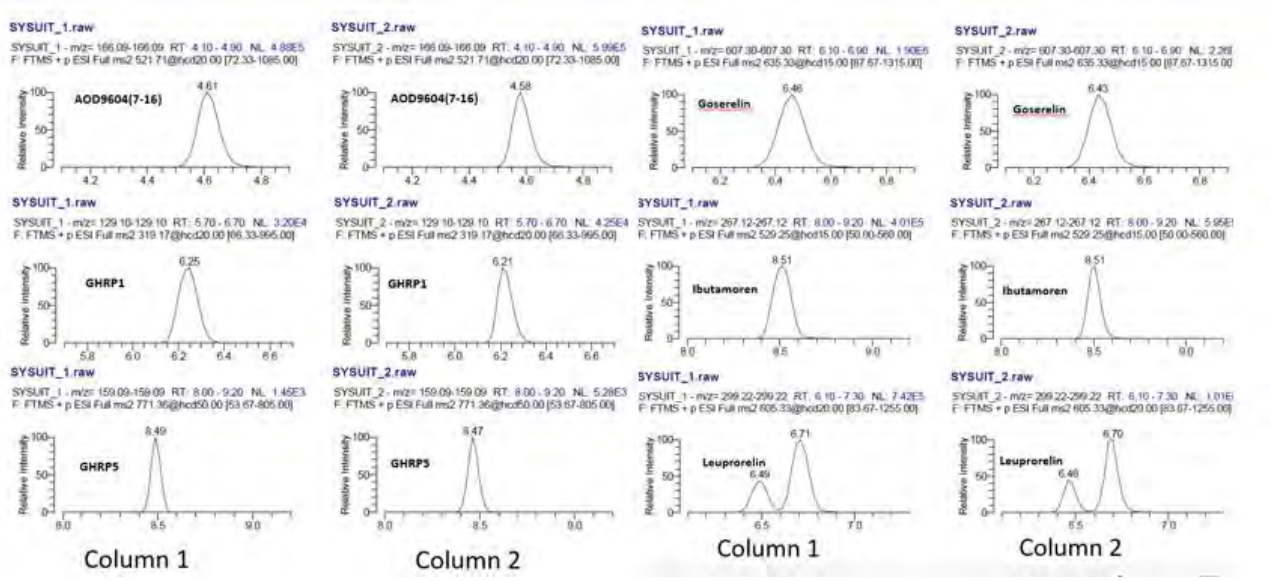


**Figure 3.** Extracted chromatograms of selected compounds eluted from column 1 and column 2 of the system suitability

## Conclusions

The method was optimized for the detection and analysis of small peptides, addressing challenges such as sample complexity and column saturation. The key parameters, including gradient elution and column switching timing, were fine-tuned to ensure improved operation and consistent performance. The 2D configuration significantly improved peak resolution and analytical throughput compared to single-column setups configuration. The work of Croft *et al.* utilized a 2D configuration for the small molecules to improve ITP efficiency,using two different columns, whereas the present work deals with the use of 2D technology with the same column and the same gradient for both columns. The difference in the retention times between the two columns did not exceed 0.3 min. The work shows how alternating column use can enhance efficiency and e.g. change to continuous sample analysis while minimizing downtime for column maintenance or reconditioning, combined with reduced inject-to-inject time. The adoption of this dual-column approach provides greater throughput, system reliability, and robustness, offering a clear advantage for routine antidoping analysis.

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# Comparison of the validation of two different derivatization methods for the confirmation of 2-fluoroamphetamine, 3-fluoroamphetamine and 4-fluoroamphetamine by gas chromatography-mass spectrometry

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## Abstract

Fluoroamphetamines (FA) are amphetamine derivatives which are not expressly listed in class S6 of the World Anti-Doping Agency (WADA) Prohibited List [1]. However, they have a similar structure to those in that class, so that an anti-doping laboratory may include them in its testing procedures. The current laboratory Initial Testing Procedure (ITP) for stimulants by gas chromatography-mass spectrometry (GC-MS) is not able to discriminate between isomers 2-fluoroamphetamine (2FA), 3-fluoroamphetamine (3FA) and 4-fluoroamphetamine (4FA).

In a previous work presented at the 39th MDI Workshop [2], it was concluded that Schiff base derivatization enables an optimal separation between the pair 2FA/3FA (with a resolution of 2FA/3FA around 1) and 4FA. However, there were not enough diagnostic ions for a reliable identification. At the same time, trifluoroacetyl derivatives (TFA) showed an excellent separation between 2FA and the pair 3FA/4FA (resolution of 3FA/4FA around 1), allowing, in addition, the identification with at least three diagnostic ions.

In this work, a study of the validation parameters for both derivatization methods has been performed to compare them and determine the more suitable analytical confirmation procedure. The results obtained show that the two derivatization agents allow the separation of the isomers, presenting good robustness in specificity, selectivity, the same limit of identification, LOI (5 times below the Minimum reporting level of WADA, MRL) and a similar and robust resolution (around 1.2) between the pairs of isomers that appear closer. It is concluded that TFA are more appropriate to confirm FAs instead of Schiff base derivatives.

## Introduction

To preserve the health of athletes and achieve cleaner sports, we need to develop new confirmation methods that, as in this case, allow us to correctly separate 2FA, 3FA, and 4FA (Figure 1). The separation of FAs isomers by GC-MS with our current methodology, which consists of extraction with tert-butyl methyl ether at pH 13, no derivatization and an Ultra 1 column, is difficult. This leads to the need to develop a confirmation method that allows their separation and identification. Based on the previous work presented at the 39th MDI Workshop [2], improvements were made to the chromatographic ramp to achieve a better separation between pairs of isomers. Robustness, selectivity, specificity, LOI, carryover, extract stability and recovery were studied on two derivatization methods to decide the better confirmation procedure analytical method.

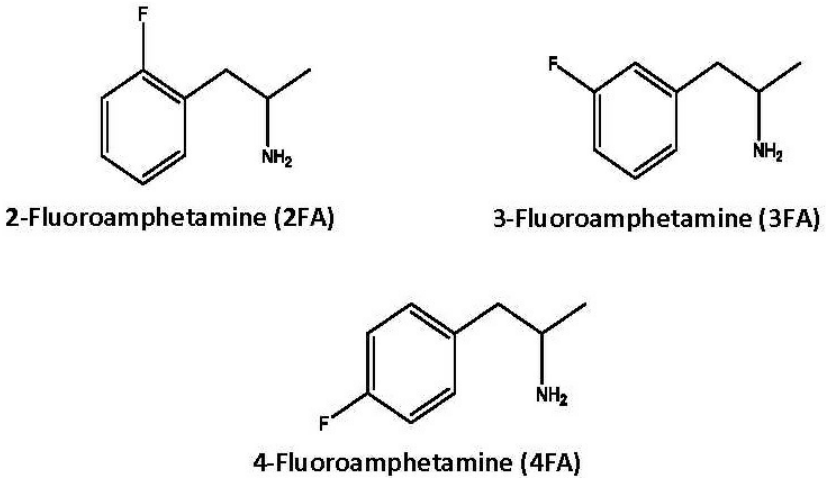



**Figure 1.** 2FA, 3FA and 4FA structures

## Experimental

2FA and 4FA, Cerilliant; 3FA, NMI; Amphetamine-D5 (ISTD), Supelco; Tert-Butyl methyl ether (TBME), sodium hydroxide, sodium sulfate and acetone, Merck; MBTFA (N-methyl-bis(trifluoroacetamide)) and MSTFA (N-methyl-N-trimethylsilyltrifluoroacetamide), Macherey Nagel. Column Ultra 2 (5%-phenyl-methyl-polysiloxane, 25 m, 0.2 mm, 0.33 µm), Agilent.

Extraction

5 mL of urine samples, 25 µL ISTD (10 µg/mL), 2 drops NaOH (pH 13), 2 mL TBME and 1 g of $Na_2SO_4$. Mixed in a shaker (20 min), centrifuged (5 min) and frozen. Samples were transferred to a vial and brought to dryness under a stream of $N_2$. Different derivatization procedures were carried out:

1. 50 µL TBME + 50 µL acetone + centrifugation (5 min) (Schiff base derivatives)
2. 100 µL MSTFA (80°C 10 min) + 10 µL MBTFA (80°C 10 min) [3] (acylated derivatives)

Analysis

Agilent 6890GC-5975MSD instruments.

Injection volume 3 µL; split, SIM acquisition mode.

Ramp: Initial temperature 70°C, Initial time (1.00 min); Rate 1 (1.5°C/min), Final temp (105°C), Final time (0.00 min); Rate 2 (30°C/min), Final temp (250°C), Final time (0.00 min); Rate 3 (100°C/min), Final temp (300°C), Final time (4.00 min), Run time 33.67 min.

Validation procedure

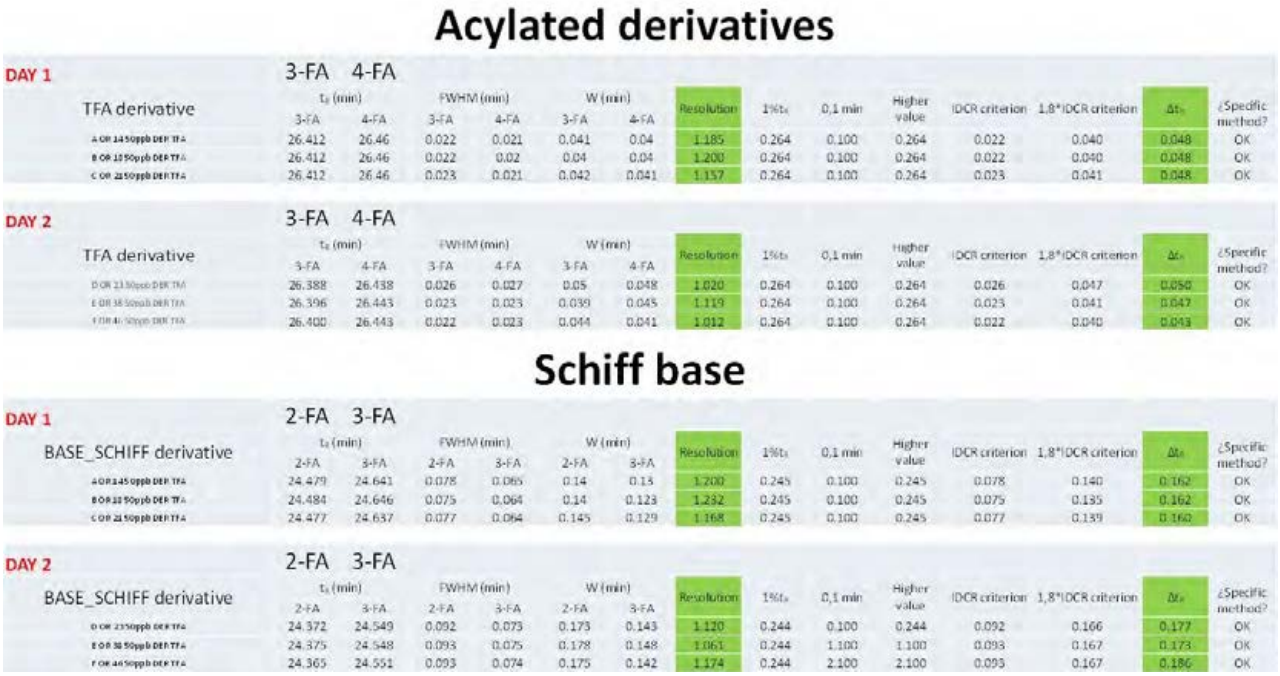
The validation procedure was performed by several analysts on different days using various instruments and representative urine samples, in accordance with WADA regulations.

- The resolution robustness between FA isomer peaks was studied. Urine samples spiked at MRPL (Minimum required performance level)-MRL (50 ng/mL) were extracted and analyzed.
- For LOI and selectivity, urine samples spiked at MRPL and at decreasing concentrations, as well as blank urine samples, were extracted and analyzed.

- Urine samples spiked with substances included in the laboratory scope were analyzed to identify potential interferences due to similarities in retention times and/or ion coincidences.
- Carryover was performed using a sample spiked at 5xMRPL, along with blank urine samples.
- The stability of the sample extracts was studied over several days at room temperature.
- Recovery was calculated after analyzing pre-doped and post-doped urine samples.

## Results and Discussion

In the resolution robustness experiment, acylated derivatives showed an excellent separation between the peaks for the adjacent pair 3FA/4FA [4] >1.00. For Schiff base derivatives [5], a similar resolution was achieved for the pair 2FA/3FA. â^†t$_R$ between the peaks of the pairs was greater than the maximum tolerance value of the TD2023IDCR (FWHM)[6] in more than 1.8 times (Figure 2).



**Acylated derivatives**

| DAY 1 | TFA derivative | 3-FA | 4-FA | $t_R$ (min) | | FWHM (min) | | W (min) | | Resolution | 1%$t_R$ | 0,1 min | Higher value | IDCR criterion | 1,8*IDCR criterion | $\Delta t_R$ | ¿Specific method? |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 3-FA | 4-FA | 3-FA | 4-FA | 3-FA | 4-FA | 3-FA | 4-FA | | | | | | | | |
| | A OR 14 50ppb DER TFA | 26.412 | 26.46 | 0.022 | 0.021 | 0.041 | 0.04 | 1.185 | 0.264 | 0.100 | 0.264 | 0.022 | 0.040 | 0.048 | OK |
| | B OR 18 50ppb DER TFA | 26.412 | 26.46 | 0.022 | 0.02 | 0.04 | 0.04 | 1.200 | 0.264 | 0.100 | 0.264 | 0.022 | 0.040 | 0.048 | OK |
| | C OR 21 50ppb DER TFA | 26.412 | 26.46 | 0.023 | 0.021 | 0.042 | 0.041 | 1.157 | 0.264 | 0.100 | 0.264 | 0.023 | 0.041 | 0.048 | OK |

| DAY 2 | TFA derivative | 3-FA | 4-FA | $t_R$ (min) | | FWHM (min) | | W (min) | | Resolution | 1%$t_R$ | 0,1 min | Higher value | IDCR criterion | 1,8*IDCR criterion | $\Delta t_R$ | ¿Specific method? |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 3-FA | 4-FA | 3-FA | 4-FA | 3-FA | 4-FA | 3-FA | 4-FA | | | | | | | | |
| | D OR 23 50ppb DER TFA | 26.388 | 26.438 | 0.026 | 0.027 | 0.05 | 0.048 | 1.020 | 0.264 | 0.100 | 0.264 | 0.026 | 0.047 | 0.050 | OK |
| | E OR 38 50ppb DER TFA | 26.396 | 26.443 | 0.023 | 0.023 | 0.039 | 0.045 | 1.119 | 0.264 | 0.100 | 0.264 | 0.023 | 0.041 | 0.047 | OK |
| | F OR 46 50ppb DER TFA | 26.400 | 26.443 | 0.022 | 0.023 | 0.044 | 0.041 | 1.012 | 0.264 | 0.100 | 0.264 | 0.022 | 0.040 | 0.043 | OK |

**Schiff base**

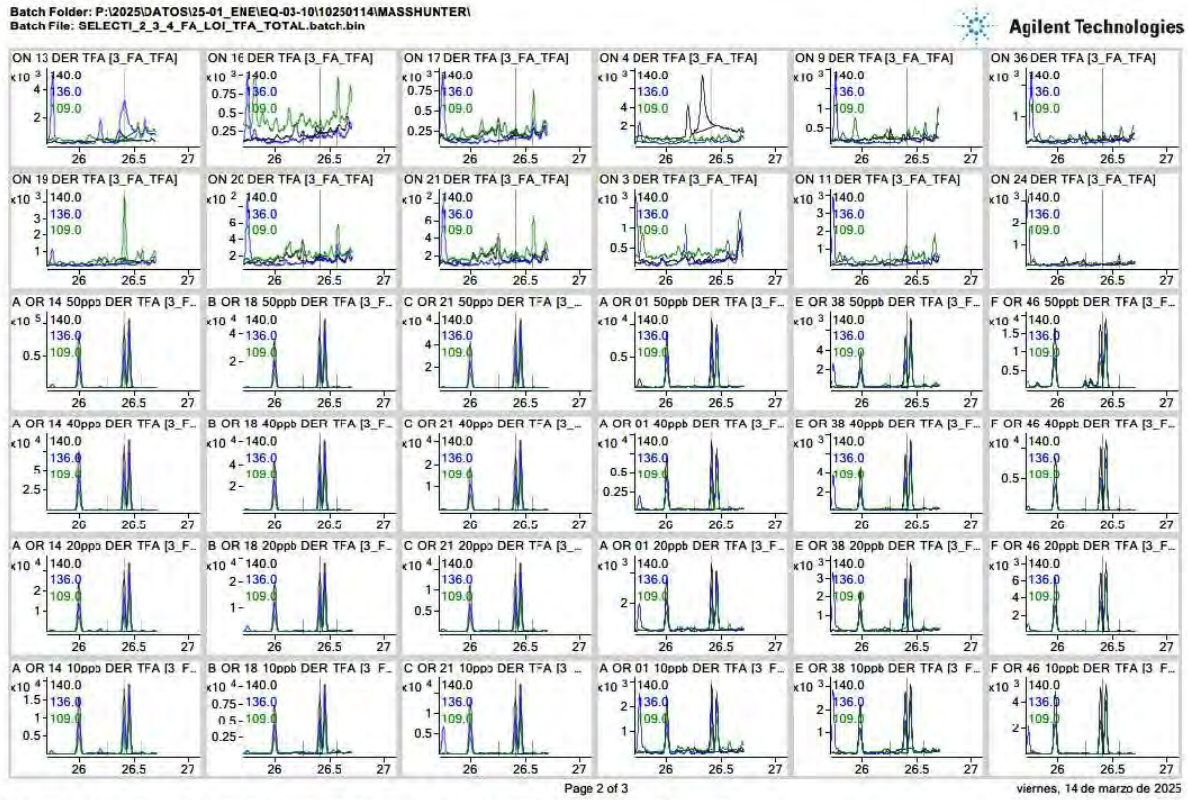
| DAY 1 | BASE_SCHIFF derivative | 2-FA | 3-FA | $t_R$ (min) | | FWHM (min) | | W (min) | | Resolution | 1%$t_R$ | 0,1 min | Higher value | IDCR criterion | 1,8*IDCR criterion | $\Delta t_R$ | ¿Specific method? |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2-FA | 3-FA | 2-FA | 3-FA | 2-FA | 3-FA | 2-FA | 3-FA | | | | | | | | |
| | A OR 245 50ppb DER TFA | 24.479 | 24.641 | 0.078 | 0.065 | 0.14 | 0.13 | 1.200 | 0.245 | 0.100 | 0.245 | 0.078 | 0.140 | 0.162 | OK |
| | B OR 38 50ppb DER TFA | 24.484 | 24.646 | 0.075 | 0.064 | 0.14 | 0.123 | 1.232 | 0.245 | 0.100 | 0.245 | 0.075 | 0.135 | 0.162 | OK |
| | C OR 21 50ppb DER TFA | 24.477 | 24.637 | 0.077 | 0.064 | 0.145 | 0.129 | 1.168 | 0.245 | 0.100 | 0.245 | 0.077 | 0.139 | 0.160 | OK |

| DAY 2 | BASE_SCHIFF derivative | 2-FA | 3-FA | $t_R$ (min) | | FWHM (min) | | W (min) | | Resolution | 1%$t_R$ | 0,1 min | Higher value | IDCR criterion | 1,8*IDCR criterion | $\Delta t_R$ | ¿Specific method? |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2-FA | 3-FA | 2-FA | 3-FA | 2-FA | 3-FA | 2-FA | 3-FA | | | | | | | | |
| | D OR 23 50ppb DER TFA | 24.372 | 24.549 | 0.092 | 0.073 | 0.173 | 0.143 | 1.120 | 0.244 | 0.100 | 0.244 | 0.092 | 0.166 | 0.177 | OK |
| | E OR 38 50ppb DER TFA | 24.375 | 24.548 | 0.093 | 0.075 | 0.178 | 0.148 | 1.061 | 0.244 | 1.100 | 1.100 | 0.093 | 0.167 | 0.173 | OK |
| | F OR 46 50ppb DER TFA | 24.365 | 24.551 | 0.093 | 0.074 | 0.175 | 0.142 | 1.174 | 0.244 | 2.100 | 2.100 | 0.093 | 0.167 | 0.186 | OK |

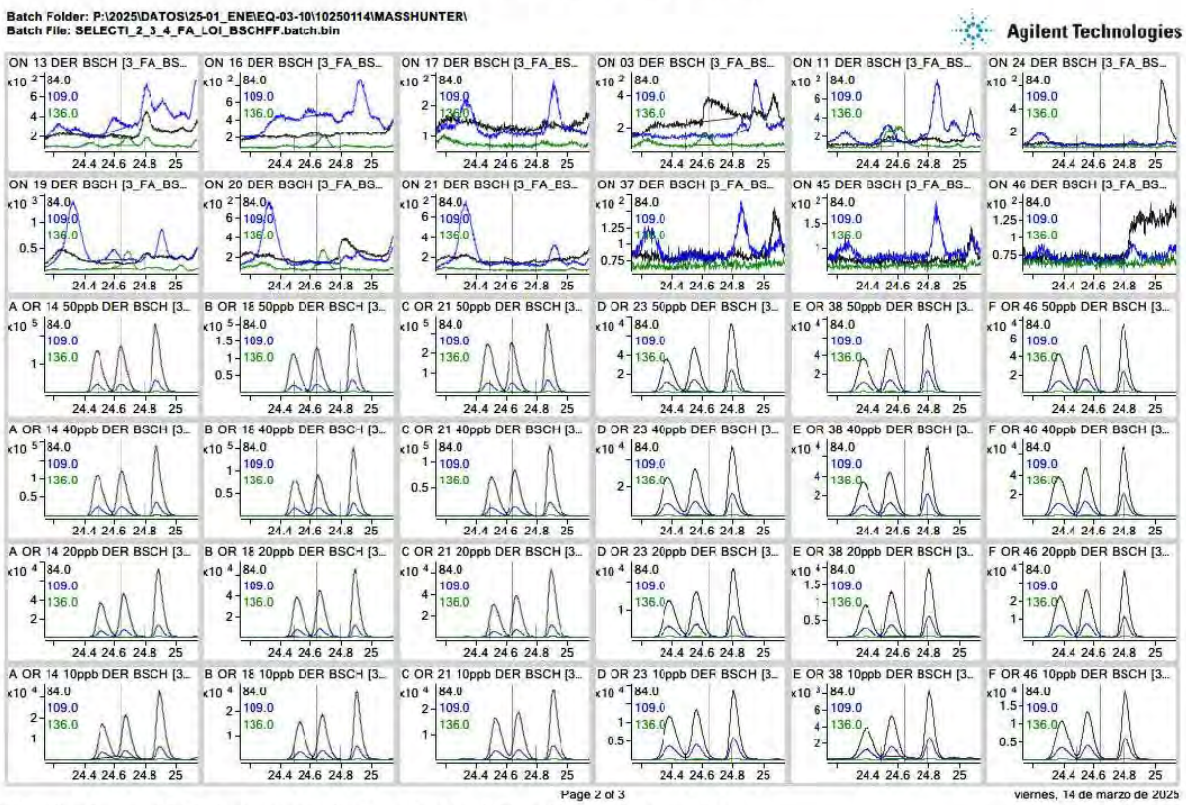
**Figure 2.** Tables of the discrimination robustness

The result showed that the most appropriate ions for confirmation of 2,3,4-FA acetylated derivatives are *m/z* 140, 136 and 109. Moreover, for 2,3,4-FA Schiff base derivatives, the *m/z* values are 84, 109, and 136. The elution sequence in both cases is 2,3,4-FA (Figure 3).

In 100% of the blank samples analyzed, no signals were detected that could interfere with the ions monitored for 2,3,4-FA acetylated derivatives and for 2,3,4-FA Schiff base derivatives. In addition, in 100% of the samples, 2, 3 and 4-FA were discriminated satisfactorily. No interference or signals that could affect the confirmation of FAs were observed in any of the urine samples spiked with other substances.



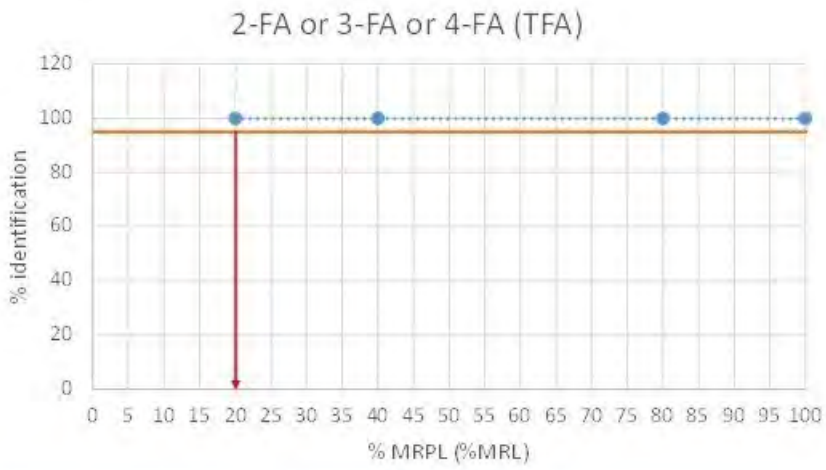

Selectivity and LOI experiment, day 1, day 2 acetylated derivatives.



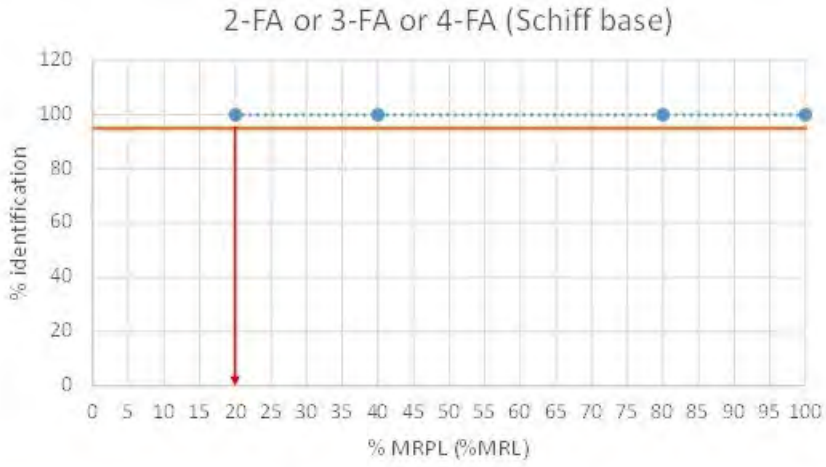

Selectivity and LOI experiment, day 1, day 2 Schiff base derivatives.

**Figure 3.** Selectivity and LOI experiment, day 1, day 2 acetylated derivatives and Schiff base derivatives

The LOI is the lowest concentration which meets the TDIDCR criteria in 95% of samples. According to WADA regulations, the concentration versus percentage identification of the substance at different concentrations could be plotted. In this way, the LOI established for 2,3,4-FA was 10 ng/mL for both derivatives (Figure 4).




| 2-FA or 3-FA or 4-FA (TFA) | | |
|---|---|---|
| Concentration (ng/mL) | % MRPL (% MRL) | % Identification |
| 50 | 100 | 100 |
| 40 | 80 | 100 |
| 20 | 40 | 100 |
| 10 | 20 | 100 |




| 2-FA or 3-FA or 4-FA (Schiff base) | | |
|---|---|---|
| Concentration (ng/mL) | % MRPL (% MRL) | % Identification |
| 50 | 100 | 100 |
| 40 | 80 | 100 |
| 20 | 40 | 100 |
| 10 | 20 | 100 |

**Figure 4.** LOI for 2,3,4-FA where the concentration level is 10 ng/mL

No analytes were detected in the blank samples during the carryover study. Good stability was observed in the extracts, as 2,3,4-FA were detected in 100% of cases.

The recovery achieved for both derivatization procedures was good, 80-90%, although a higher coefficient of variation was observed for the Schiff base derivatives.

Validation experiments performed on different days by different analysts using different batches of reagents and various instrumental equipment yielded equivalent results. It is therefore concluded that the method is robust and fit for purpose.

## Conclusions

The results obtained show that the two derivatization agents allow separation of the isomers, presenting good robustness in specificity, selectivity, the same LOI (5 times below MRL of WADA) and similar and robust resolution (around 1.2) between the pairs of isomers that appear closer. There is no carryover, and the extract is stable in both forms of derivatization.

It is concluded that trifluoroacetyl derivatives are more suitable for confirming the presence of Fluoroamphetamines on a routine basis than Schiff base derivatives, which present a relative abundance of ion 136/ion 84 close to 1%.

However, the combination of both derivatization methods can enable the complete separation and unequivocal identification of the three isomers in cases of complex or tricky samples.

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# A workflow for the identification of proteases in antidoping tests

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## Abstract

The aim of this work is to evaluate an easier, time saving and less expensive analytical strategy to screen for and identify the potential presence of proteases in urine samples with inconclusive erythropoietin (EPO) results. First, the activity of several proteases (Papain, Bromelain, Subtilisin A, Trypsin and Pepsin) and the enzyme Amylase in degrading EPO was assessed by SAR-PAGE analysis of negative urine samples, showing a wide efficacy range among the tested protease, spanning from 2.5 µg/mL for Subtilisin A to 30 µg/mL for Bromelain. In contrast, some enzymes appeared to exhibit no proteolytic activity on EPO, such as pepsin, which was ineffective even at a concentration of 1 mg/mL. Then, the degradation patterns of the whole protein urinary profile following treatment with individual proteases were evaluated by SDS-PAGE followed by Coomassie Blue staining. The results showed that most of the tested proteases induced a distinct and specific degradation pattern and in some cases the molecular mass band corresponding to the protease used could be identified. Finally, a method for the identification of individual proteases in high-resolution mass spectrometry (LC-HRMS) was successfully developed with the aim of providing a confirmation procedure.

## Introduction

Urine manipulation using proteases as masking agent is an illicit practice banned by the World Anti-doping Agency. Their effect is to accelerate the degradation of proteins of doping interest, primarily erythropoietins. Indeed, very low levels of endogenous EPO might be due either to high haematocrit and/or to the adulteration of urines by the addition of proteases during the collection process, leading to the degradation of both endogenous and recombinant EPO. The possible presence of proteases can be assessed using the Protease Fluorescent Detection Kit (Sigma-Aldrich), but there are some limitations: low sensitivity in biological matrices and inability to discriminate, within a single analytical session, which protease is present for a confirmatory test. Therefore, the aim of this work is to identify an easier, time saving and cost-effective analytical strategy to detect  a wide range of proteases in EPO inconclusive urine samples.

## Experimental

To trace the possible origins of undetectable EPO profiles in athletes' urine, data obtained from antidoping urine tests for the detection of recombinant EPO were analysed. Accordingly, the total EPO content was measured in all samples to establish the relationship between EPO concentration and EPO detected by SAR-PAGE analysis.
In the following step, negative urine samples previously verified for the presence of endogenous

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erythropoietin and the absence of exogenous proteases, were spiked with recombinant EPO (rEPO) at a concentration of 2 mIU/mL, with and without different amounts of specific proteases.

The proteolytic enzymes selected for this study were bromelain, papain, pepsin, subtilisin A, trypsin and the digestive enzyme amylase. Negative and spiked urine samples were incubated for 24 h at room temperature and then analysed by SAR-PAGE analysis after an ELISA-based immunopurification, in accordance with the routine analytical procedure covered by ISO17025 accreditation. Then, to investigate changes in the urinary protein profile induced by the addition of the selected enzymes, 4 mL of urine samples were concentrated to 50 µL using a 10 kDa cut-off filter and loaded on a 12% Bis-Tris gel for SDS-PAGE analysis. The gels were then stained with Coomassie Blue solution [1,2]. Confirmation analysis by liquid chromatography coupled to high-resolution mass spectrometry was performed both after band extraction from SDS-PAGE gel (data not shown) and directly on the urine sample. The experimental strategy involved an initial *in silico* analysis for the selection of peptides specific to and uniquely attributable to the digestive enzymes of interest, followed by an *in vitro* analysis for the optimization of the digestion using different enzymes, and finally, an LC-HRMS (qTOF) analysis that allowed characteristic peptides to be detected and their identity confirmed by studying the fragmentation profile.

## Results and Discussion

Our statistical studies showed that all undetectable EPO profiles are related to lower EPO concentrations (< 1 mIU/mL), even if some samples with very low EPO concentrations were still detectable (data not shown); some of the undetectable samples had also high urine specific gravity. Therefore, the impact of proteases on erythropoietins was investigated, showing that their sensitivity to proteases is enzyme specific and dose dependent (only trypsin related results are shown Figure 1, as trypsin was used as a representative protease).

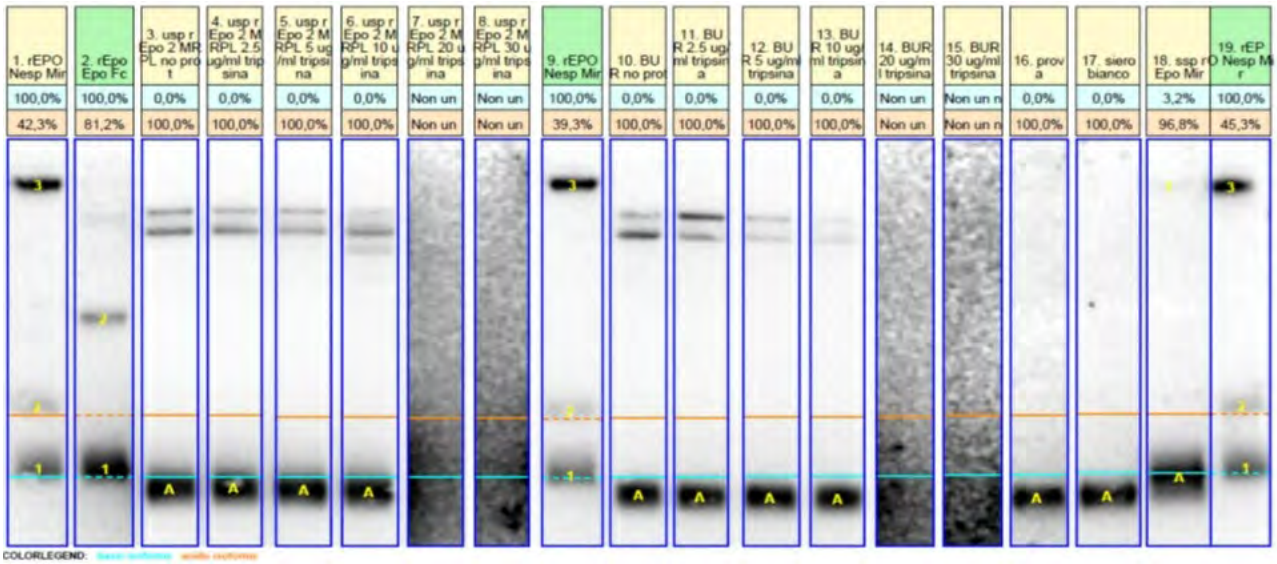


**Figure 1.** SAR-PAGE analysis of negative urine sample spiked with rEPO without (lane 3) and with the addition of 2.5 µg/mL (lane 4), 5 µg/mL (lane 5), 10 µg/mL (lane 6), 20 µg/mL (lane 7) and 30 µg/mL (lane 8) of trypsin; negative urine sample without (lane 10) and with the addition of 2.5 µg/mL (lane 11), 5 µg/mL (lane 12), 10 µg/mL (lane 13), 20 µg/mL (lane 14), 30 µg/mL (lane 15) of trypsin

To further distinguish these enzymes, their overall effect on urine samples was studied by evaluating changes occurring on the protein profiles of negative urines treated with single proteases (Figure 2). The results showed that urine samples without proteases displayed bands in the mass range 17- 150 kDa but after adding subtilisin A, papain or bromelain, most of the high molecular mass proteins disappeared. After treatment with trypsin, several high molecular weight proteins were partially or fully degraded. In addition, a band corresponding to trypsin's molecular weight was detectable even at low concentrations, together with four additional distinct bands (Figure 3). Subtilisin A underwent autolysis, so its band cannot be detected. Pepsin and amylase had no observable effect on the protein profile, and their bands could not be easily detected.

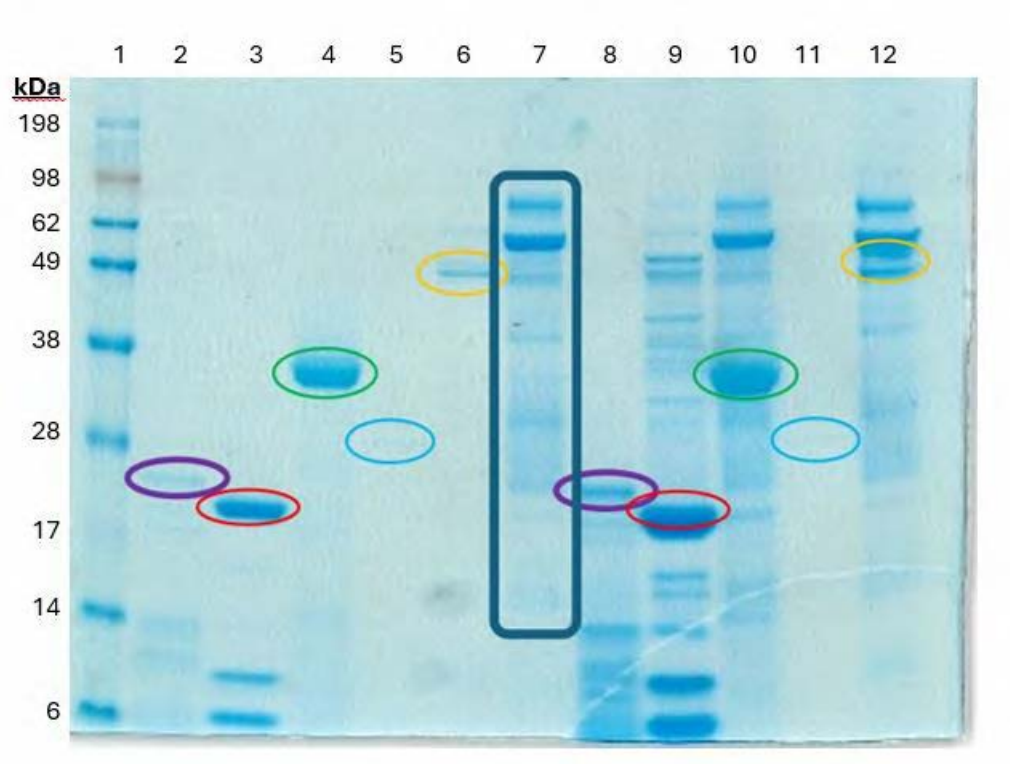


**Figure 2.** Comassie Blue Staining: 1) Protein pre-stained marker; 2)**bromelain** standard (purple); 3)**trypsin** standard (red); 4) **pepsin** standard (green); 5)**subtilisin A** standard (light blue); 6)**amylase** standard (yellow); 7) negative urine sample (blue); 8) negative urine sample + bromelain 50 µg/mL; 9) negative urine sample + trypsin 50 µg/mL; 10) negative urine sample + pepsin 50 µg/mL; 11) negative urine sample + subtilisin A 50 µg/mL; 12) negative urine sample + amylase 50 µg/mL

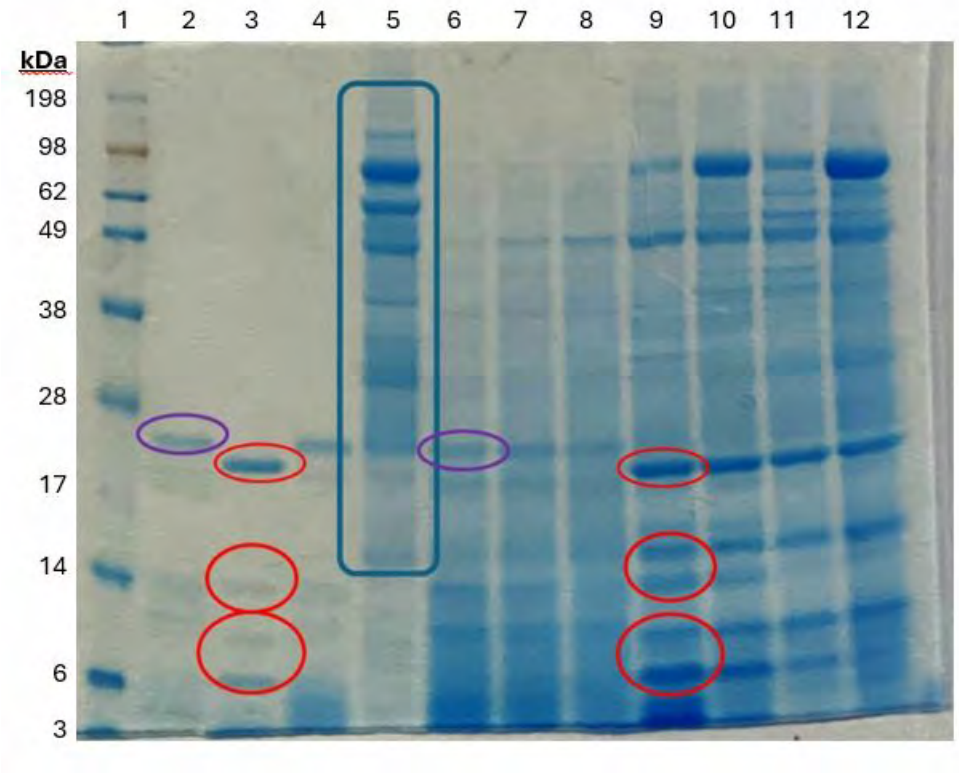


**Figure 3.** Coomassie Blue staining: 1) Protein pre-stained marker; 2)**bromelain** standard (purple); 3)**trypsin** standard (red); 4) bromelain + trypsin standard; 5) **negative urine sample** (blue); 6) negative urine sample + bromelain 50 µg/mL; 7) negative urine sample + bromelain 30 µg/mL; 8) negative urine sample + bromelain 15 µg/mL; 9) negative urine sample + trypsin 50 µg/mL; 10) negative urine sample + trypsin 20 µg/mL; 11) negative urine sample + trypsin 10 µg/mL; 12) negative urine sample + trypsin 5 µg/mL

The results presented here show that not all selected enzymes induce a specific degradation pattern and that identification of their molecular mass band is not always possible.

This method was partially validated. The specificity and robustness of the proposed workflow were evaluated for the screening procedure by assessing the ability to discriminate individual proteases based on their effects on urinary protein profiles. A conventional limit of detection (LOD) was not established, as this study does not aim at quantitative protease determination. Instead, a dose–response approach was adopted, whereby protease concentrations were tested down to levels lower than the minimum concentration shown to effectively degrade EPO, to assess the sensitivity of the workflow in detecting protease-mediated EPO degradation.

In cases where the proteomic profile appeared altered, it was possible to identify the specific protease responsible for protein degradation. An *in-silico* study was performed using the Expasy platform to simulate cleavage patterns and the selection of protease-specific peptides was carried out through interrogation of the RefSeq database. These predictions were then validated *in vitro* by enzymatic digestion, followed by liquid chromatography–high-resolution mass spectrometry (LC-HRMS). Through this integrated workflow, characteristic peptides uniquely associated with individual proteases were identified (Figure 4).

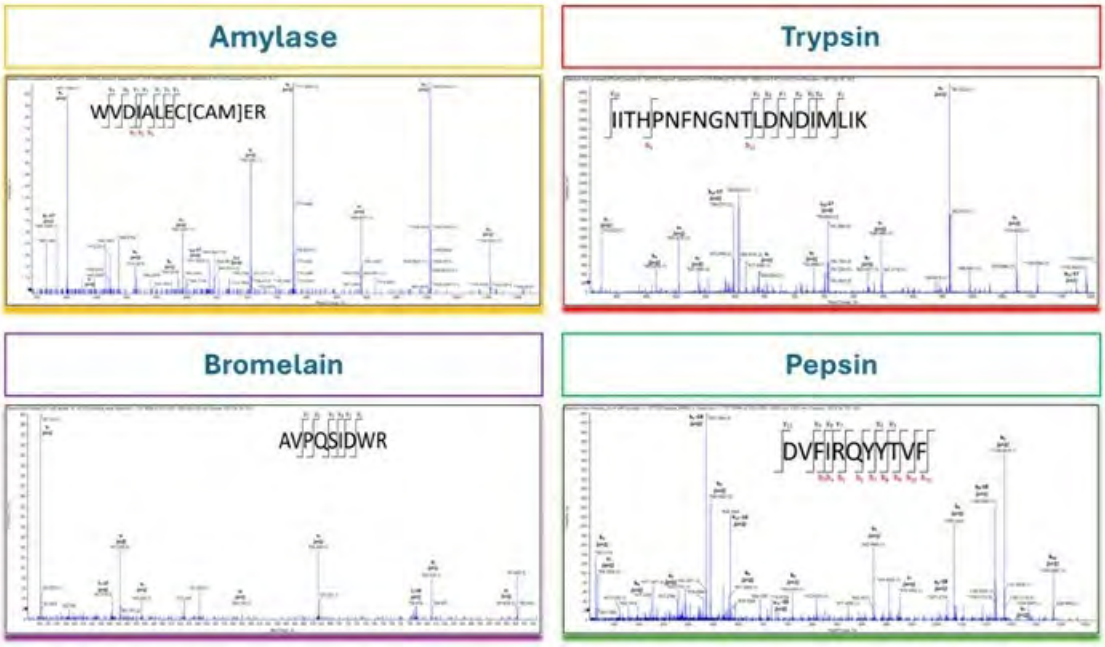


**Figure 4.** Mass spectra of specific peptides of the selected proteolytic enzymes, obtained after enzymatic digestion (with trypsin, for amylase, trypsin, and bromelain; with endoproteinase Asp-N for pepsin)

## Conclusions

Through the proposed procedure, it was possible to verify sample adulteration by proteases by evaluating the urinary protein profile using SDS-PAGE and Coomassie Blue staining in cases of undetectable ERA during routine antidoping tests. Furthermore, in the presence of suspect degradation patterns, confirmatory analysis for the identification of individual proteases can be performed using the newly developed procedure based on liquid chromatography coupled with high-resolution mass spectrometry. This procedure can be applied directly to urine sample and/or following protein extraction from SDS-PAGE gels. Overall, the proposed workflow extends the current analysis of protease for their identification in routine doping analyses and consists of an initial SDS-PAGE screening of urinary protein profiles, followed by confirmatory LC-HRMS analysis directly performed on urine samples, allowing EPO degradation due to protease-mediated sample tampering to be ruled out.

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# Comparison of the detection of epoetin alfa Eprex and the biosimilar Hemax following administration of micro- and therapeutic doses in healthy subjects

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## Abstract

Due to ended patents for the production of recombinant erythropoietin (rEPO), various pharmaceutical companies have started to produce rEPO biosimilars and some of them offer now authorized drugs. This is the case for Hemax®, a copy of Eprex®. Nevertheless, the biosimilars can present small structural differences compared to the original drug. An initial work evaluating the EPO profiles after IEF-PAGE and SDS-PAGE concluded that Hemax may be difficult to identify [1]. An administration study of Hemax and Eprex was performed on six healthy persons. Three subjects received one microdose (10 IU/kg) of Eprex followed by a similar microdose of Hemax and finally three consecutive therapeutic doses (50 IU/kg) of Hemax. Three others subjects followed the same protocol with Eprex only.

Analysis of EPO was performed by SDS-PAGE and IEF-PAGE techniques for urine and plasma samples and SAR-PAGE was used for DBS analysis. While Eprex microdose could be identified until 72h, Hemax microdose was not detected even 24 hours following administration in plasma or urine. Similarly, detection after the therapeutic doses was clear until 9 days for Eprex but limited to 6 days for Hemax in plasma. In DBS, we were able to identify only Eprex microdose following 24h and both drugs up to 3 days after the therapeutic doses.

These results confirmed the possibility to detect the presence of biosimilar Hemax but the elimination/degradation of this drug appears to be faster than the original Eprex shortening the window of detection.

## Introduction

Due to ended patents for the production of recombinant erythropoietin (rEPO) various pharmaceutical companies have started to produce rEPO biosimilars and some of them offer now authorized drugs used in some countries for the same therapeutic applications. This is the case for Hemax® (BioSidus, Argentina), an epoetin alfa drug biosimilar of Eprex® (Janssen-Cilag), which is distributed and used in Algeria to treat anemia linked to chronic kidney disease. Nevertheless the biosimilars can present small structural differences compared to the original drug. An initial work evaluating the EPO profiles of this drug after IEF-PAGE and SDS-PAGE concluded that Hemax may be difficult to identify [1]. An administration study of Hemax and Eprex was performed on six healthy persons.

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## Experimental

### Administration study

The administration study was performed on six healthy volunteers recruited after authorization of the study by the ethics committee of Tizi-Ouzou hospital (Algeria). Three subjects received one microdose (10 IU/kg) of Eprex followed three days later by a similar microdose of Hemax and after a 5-days washout period three consecutive therapeutic doses (50 IU/kg) of Hemax. Three others subjects followed the same protocol with Eprex only. Urine and whole blood (also used to spike DBS) were collected prior to and during administration, up to 11 and 9 days respectively after last administration.

### EPO immuno-purification

All urinary (25 mL), plasma samples (500 µL) and DBS (Mitra, Neoteryx, 2 pebbles) were immuno-purified.

- For urine: Immuno-purification was performed on final retentates resulting from ultrafiltration urine, using a human EPO ELISA immunoplate (STEMCELL Technologies). Retentates were separated in two: one part used for the IEF-PAGE and one part for the SDS-PAGE analysis.
- For plasma: immuno-purification was performed directly on 1 mL of plasma using magnetic beads coated with anti-EPO antibody clone 9C21D11 (R&D systems). Eluates were equally separated in two: one part for IEF-PAGE analysis and one part for SDS-PAGE analysis.
- For DBS: immuno-purification was conducted by EPO Purification Gel Kit 3F6 - for Blood (MAIIA AB), following the manufacturer directions for use. All the eluates was analyzed by SAR-PAGE.

### IEF-PAGE and SDS-PAGE analysis

IEF-PAGE and SDS-PAGE were conducted following the techniques previously described [2].

### SAR-PAGE analysis

SAR-PAGE analysis was performed following the procedure previously described [3].

## Results and Discussion

### EPREX administration

Examples of results of the EPREX administration (subject 1) is presented in Figure 1. By SDS-PAGE analysis, the EPREX pattern shows a broad and intense signal above dynepo line, enabling an extended window of detection up to 72 h in plasma and urine for microdosis and 9 days for therapeutic dosage. By IEF-PAGE, the results showed a quite similar window of detection.

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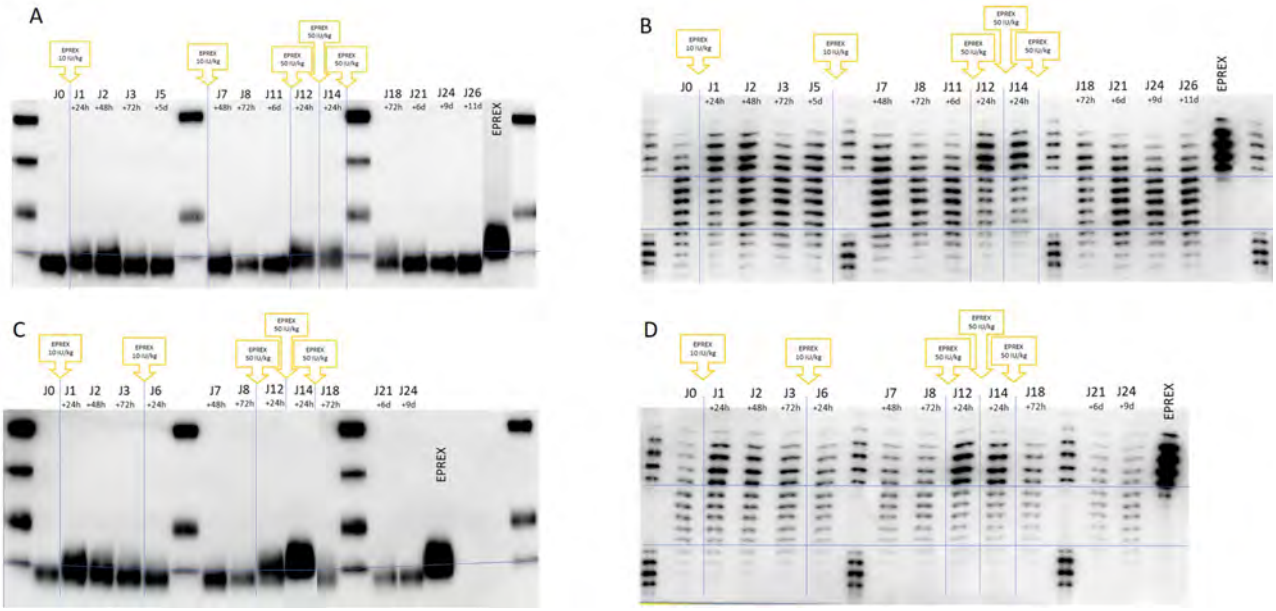


**Figure 1.** Results of the analysis of all the urine samples collected from subject 1, using SDS-PAGE (Panel A) and IEF-PAGE (Panel B), and all plasma samples using SDS-PAGE (Panel C) and IEF-PAGE (Panel D). The subject received only EPREX.

### HEMAX administration

Examples of results of the HEMAX administration (subject 5) is presented below (Figure 2). By SDS-PAGE analysis, the HEMAX pattern shows a narrow signal above dynepo line compared to EPREX, reducing the window of detection after microdosis administration. The window is below 24 h. By IEF-PAGE, the results were quite similar to SDS-PAGE except for a reduced window of detection in urine after therapeutic dose.

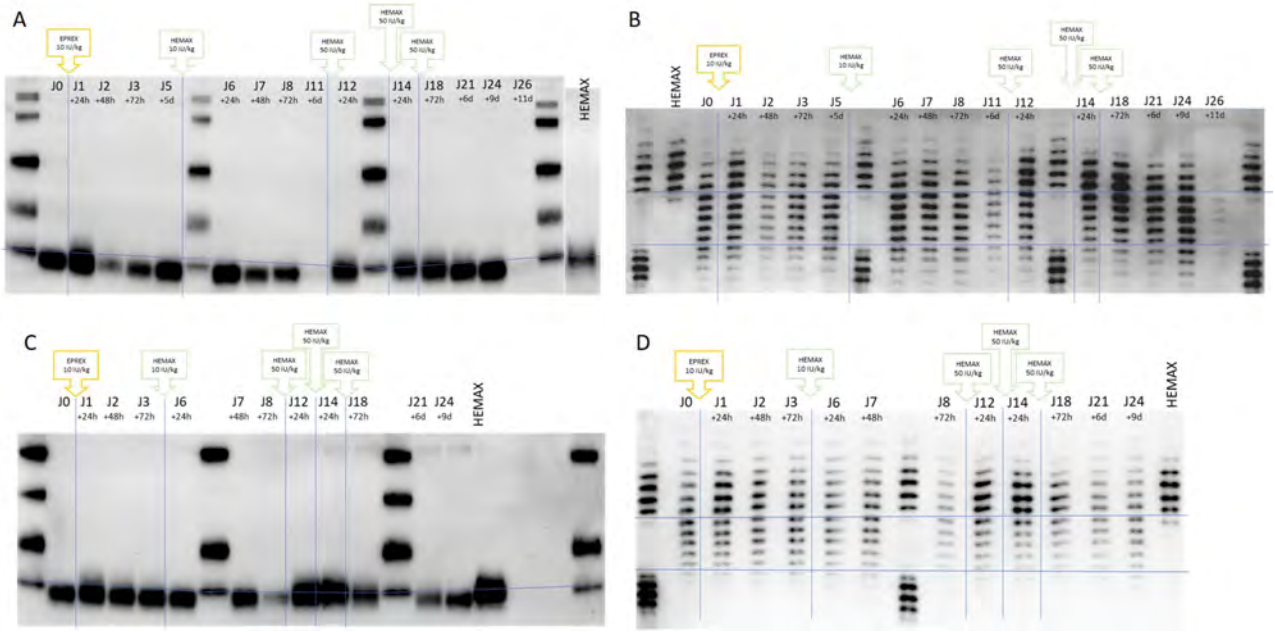


**Figure 2.** Results of the analysis of all urine samples collected from subject 5, using SDS-PAGE (Panel A) and IEF-PAGE (Panel B), and all plasma samples, using SDS-PAGE (Panel C) and IEF-PAGE (Panel D). The subject received EPREX, then HEMAX.

**OVERALL results**

The windows of detection of EPREX/HEMAX administration are presented in Table 1.

By SDS/SAR-PAGE, the rEPO is detected if a signal is present above the endogenous band extended beyond the band apex of dynepo. By IEF-PAGE, the presence of rEPO is proved when the two most intense bands are located in the basic area for urine, as defined by TD2024EPO, and when the ratio of the second most intense band in the basic area to the most intense band in the endogenous area is greater than 1.3 for the blood samples (French anti-doping laboratory's own criteria). We decided to apply a more conservative criteria for blood sample than those cited in the TD2024EPO, as the IEF-PAGE profile is more basic than urine [4], and based on our internal statistic study on 114 negative subjects [5]. By applying the criteria from the TD2024EPO to the plasma, Hemax microdose could be detected up to 48 hours later and therapeutic dose up to 9 days. On the other hand, two of the subjects of this study would fulfil the identification criteria for rEPO before administration. As a reminder, the IEF-PAGE is a complementary analytical method for the presence of rEPO in a sample, the SDS/SAR-PAGE remaining the decisive analysis.

The maximal window of detection was considered when at least one subject fulfilled the criteria descibed above (red color).

MICRODOSE Maximal Window of detection

| PLASMA | Eprex | | | Hemax | | |
|---|---|---|---|---|---|---|
| | Subject 1 | Subject 2 | Subject 3 | Subject 4 | Subject 5 | Subject 6 |
| SDS-PAGE | 48h | 72h | 72h | < 24h | <24h | <24h |
| IEF-PAGE | 24h | 72h | 72h | < 24h | <24h | <24h |

| URINE | Eprex | | | Hemax | | |
|---|---|---|---|---|---|---|
| | Subject 1 | Subject 2 | Subject 3 | Subject 4 | Subject 5 | Subject 6 |
| SDS-PAGE | 72h | 48h | 48h | < 24h | <24h | <24h |
| IEF-PAGE | 24h | 72h | 48h | < 24h | <24h | <24h |

| DBS | Eprex | | | Hemax | |
|---|---|---|---|---|---|
| | Subject 1 | | Subject 3 | Subject 4 | Subject 5 |
| SAR-PAGE | 24h | | 24h | < 24h | <24h |

THERAPEUTIC DOSE Maximal Window of detection

| PLASMA | Eprex | | | Hemax | | |
|---|---|---|---|---|---|---|
| | Subject 1 | Subject 2 | Subject 3 | Subject 4 | Subject 5 | Subject 6 |
| SDS-PAGE | 6 days | 9 days | 9 days | 6 days | 6 days | 6 days |
| IEF-PAGE | 72h | 9 days | 9 days | 72h | 72h | 6 days |

| URINE | Eprex | | | Hemax | | |
|---|---|---|---|---|---|---|
| | Subject 1 | Subject 2 | Subject 3 | Subject 4 | Subject 5 | Subject 6 |
| SDS-PAGE | 6 days | 9 days | <72h | 9days | 9 days | 9 days |
| IEF-PAGE | 6 days | 9days | 72h | 72h | 72h | 72h |

| DBS | Eprex | | | Hemax | |
|---|---|---|---|---|---|
| | Subject 1 | | Subject 3 | Subject 4 | Subject 5 |
| SAR-PAGE | 72h | | 24h | 72h | 72h |

**Table 1.** Maximal window of detection by subject after Eprex and Hemax microdosis and therapeutic administration. The red colour shows the maximum window for all subjects combined

## Conclusions

While Eprex microdose could be identified up to 72h, Hemax microdose was not detected even 24 hours following administration in plasma or urine. Similarly, detection after the therapeutic doses was clear up to 9 days for Eprex but limited to 6 days for Hemax in plasma. No significant differences in sensitivity of detection were identified when using IEF-PAGE or SDS-PAGE except a reduced window of detection for Hemax in urine by IEF-PAGE. In DBS, we were able to identify only Eprex microdose after 24h and both drugs up to 3 days after the therapeutic doses.

Altogether these results confirmed the possibility to detect the presence of biosimilar Hemax in case of doping but the elimination/degradation of this drug appears to be faster than the original Eprex shortening the window of detection.

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# Detection of agents preventing activin receptor IIB activation by capillary electrophoresis immunoassay

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## Abstract

Some members of the TGF-beta superfamily, such as myostatin, signal via activin receptors type II (ActRII) to negatively regulate skeletal muscle growth. Inhibition of this signalling pathway, either by specific binding to myostatin or to the activating receptor type IIB, is a promising therapeutic approach for the treatment of muscle diseases. Due to their potential effects in promoting muscle growth, drugs that prevent ActRIIB activation are banned in sport and are listed in section S4.3 of the WADA Prohibited List. In this study, we present the development of several methods for the detection of humanised monoclonal antibodies that bind to myostatin, namely domagrozumab, landogrozumab, trevogrumab and stamulumab. We also present the detection of bimagrumab, a humanised monoclonal antibody that binds to ActRIIB, and the detection of representative follistatin-based inhibitors FST315-Fc, FST300-His and FST288. The detection methods are based on immunomagnetic purifications followed by automated capillary electrophoresis immunoassay using the Wes Simple Western. Results show that humanised monoclonal antibodies can be detected at levels as low as 50 ng/mL and the follistatin-based inhibitors at levels as low as 50 pg/mL in 100 µL serum samples. Our methods are fast, sensitive, versatile and can all be combined in the same analytical assay.

## Introduction

Due to their potential to promote muscle growth, drugs that prevent ActRIIB activation are banned in sport and listed in Section S4.3 of the World Anti-Doping Agency (WADA) Prohibited List [1]. These agents include the anti-ActRIIB antibody bimagrumab, the myostatin-neutralising antibodies stamulumab, domagzumab and landogzumab, and myostatin-binding proteins such as follistatin. Our laboratory has previously developed analytical strategies for detecting erythropoiesis-stimulating agents (ESAs) and activin ligand traps (such as sotatercept and luspatercept) using automated capillary electrophoresis immunoassay [2, 3]. This study aimed to expand the range of targets that can be detected using this analytical platform to include humanized monoclonal antibodies that bind to myostatin or ActRIIB, as well as follistatin-based inhibitors.

## Experimental

### Reference standards

Recombinant human follistatin FST288 was obtained from Creative Biomart (FST-661H), FST315-Fc (10685-H02H) and FST300-His (10585-H08H) were from Sino Biologicals and FST315 was obtained from RnD Systems (4889-FN). Humanized monoclonal antibodies Domagrozumab (TAB-020ML), Landogro-

zumab (TAB-434CQ), Bimagrumab (AFC-TAB-726) and Stamulumab (TAB-888) were obtained from Creative Biolabs and Trevogrumab (PX-TA1388) was obtained from Proteogenix.

**Sample preparation**

The serum samples (collected in BD Vacutainer® SST™ tubes, US ref. 367986) were obtained from athletes who consented to have their samples used for research purposes and anonymized. When necessary, a pool of 15 mL of serum samples was centrifuged at 4000 x g for 10 min, then filtered on 0.22 µm Steriflip units. For individual samples analysis, 100 µL of serum was centrifuged at 13000 x g for 5 minutes, and the supernatant filtered on Costar Spin-X 0.22 µm filters.

**Immunomagnetic beads preparation**

To isolate humanized monoclonal antibodies, 100 µg of either human recombinant myostatin/GDF-8 (120-00, Peprotech) or recombinant Human Activin RIIB-Fc (R&D Systems, 339-RB) was covalently linked to 165 µL (5 mg) of Dynabeads M-280 Tosylactivated beads solution according to manufacturer's instructions. The bead solution was adjusted to a final volume of 1 mL with PBS 1X/BSA 0.4%. To isolate follistatin-based inhibitors, 500 µL of pre-washed Dynabeads M280 anti-Mouse IgG were incubated with 10 µg of mouse monoclonal anti-human FST MAB6692 (R&D Systems) for 1h at RT with end-over-end rotation in IP Buffer. The beads were then washed twice and resuspended in a final volume of 500 µL of PBS 1X/BSA 0.05% prior to use.

**Immunoaffinity isolation and detection of Follistatin-based inhibitors**

100 µL of serum samples were diluted with 400 µL of IP Buffer in a LoBind Eppendorf tube. Then, 10 µL of the antibody-bead solution was added to the mixture, and the samples were incubated for 16h at 4°C. The beads were washed twice with 750 µL of IP Buffer and once with 500 µLof PBS 1X before elution. Elution was performed with 5 µL of Glycine 0.1M pH2.8 for 10 minutes at RT. Then, 1.25 µL of Fluorescent Master Mix 5X solution was added, and samples were heated at 95°C for 3 minutes before Simple Western analysis. Detection was performed using the anti-FST Rabbit PAb (PAA391Hu01, Cloud-Clone Corp.) as primary antibody and the anti-Rabbit IgG-HRP (Protein Simple) as secondary antibody.

**Immunoaffinity isolation and detection of anti-myostatin monoclonal antibodies**

100 µL of serum samples were diluted with 400 µL of PBS 1X/BSA 0.4% in a LoBind Eppendorf tube. Then, 7.5 µL of the antibody-bead solution was added to the mixture, and the samples were incubated for 30 minutes at 4°C. The beads were then washed twice with 750 µL of PBS 1X/NaCl 0.5M/0.02% Tween-20 and once with 500 µL of PBS 1X. Elution was performed as described above for the follistatin-based inhibitors analysis. Detection was performed using two different protocols.

Protocol 1: Goat Anti-Human IgG (H+L)-HRP (Jackson, 109-036-003) and MAb Anti-Human IgG kappa-LC (R&D Systems, MAB10050) were used as primary antibodies, and anti-Mouse IgG-HRP (Protein Simple) was used as the secondary antibody.

Protocol 2: MAb anti-Human IgG lambda-LC (R&D Systems, MAB10049) was used as primary antibody and anti-Mouse IgG-HRP (Protein Simple) was used as the secondary antibody.

**Wes procedure**

Automated capillary immunoassay (Simple Western) was performed using the Wes system (Protein Simple, San Jose, CA, USA). Analyses were performed using the 12-230 kDa Separation Module (Protein

Simple, SM-W004) according to the manufacturer's instructions with some minor modifications. Briefly, 5 µL of the sample, 10 µL of the blocking solution, 8 µL of the primary antibody solution, 8 µL of the secondary antibody solution and 15 µL of chemiluminescent reagent (West Femto) were loaded onto pre-filled microplates. The Separation Matrix Load time was set to 195 sec., the Stacking Matrix load time to 20 sec., the Sample load time to 12 sec., the Primary Antibody incubation time to 40 minutes and the detection mode was set to HDR (high dynamic range). All other running parameters were set to the default parameters. Wes operation and all calculations were performed using the Compass software (version 4.1.0).

## Results and Discussion

To our knowledge, the analysis of follistatin-based inhibitors and anti-myostatin humanized monoclonal antibodies by means of Simple Western capillary electrophoresis has not been reported in scientific literature. The operating conditions were then adapted from our previous work [2,3] and further optimized them for the detection of these new targets. To detect follistatin, we tested 16 different commercial antibodies before selecting the anti-FST Rabbit PAb (PAA391Hu01) from Cloud Clone Corp. as the optimal primary antibody on the Wes system due to its sensitivity towards follistatin and its optimal signal-to-noise ratio (S/N). As shown in Figure 1, FST288 is detected at approximately 48 kDa, FST315-Fc at approximately 98 kDa, FST315 at 57 kDa (main peak) and FST300-His at 65 kDa (main peak).
While the molecular weights (MW) at which the molecules are detected differ from those observed on traditional SDS or SAR-PAGE analyses [4], their relative discrimination according to electrophoretic mobility aligns with previous work by Reichel *et al.* [4]. We also evaluated the same antibodies for their ability to isolate follistatin molecules from serum samples. Several antibodies demonstrated strong binding properties, with the mouse monoclonal MAB6692 (RnD Systems) yielding the best recovery. Figure 1, panel F shows that samples spiked with 2 ng/mL of representative follistatin-based inhibitors can be clearly detected.

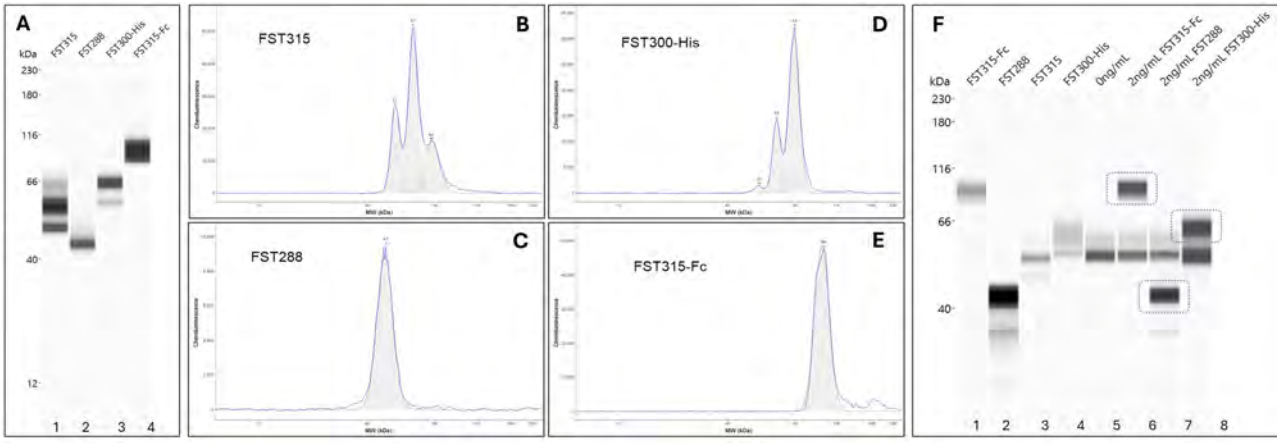


**Figure 1. Representative densitometric profiles of follistatin-based inhibitors by Simple Western.** The different reference preparations of follistatin were analyzed untreated (100 pg, Panel A, Panel F lanes 1-4) or after isolation from 100 µL serum samples spiked at 2 ng/mL (Panel F, lanes 6-8). Lane view analyses, showing the individual chromatograms from the different preparations, are presented in panels B-E. Dotted squares highlight the diagnostic bands (Panel F).

While the presence of endogenous FST isoforms could slightly hinder the detection of FST300-His, FST315-Fc and FST288 can be detected (S/N >10) at concentrations as low as 50 pg/mL as their detection windows are outside that of endogenous FST (see Figure 2).

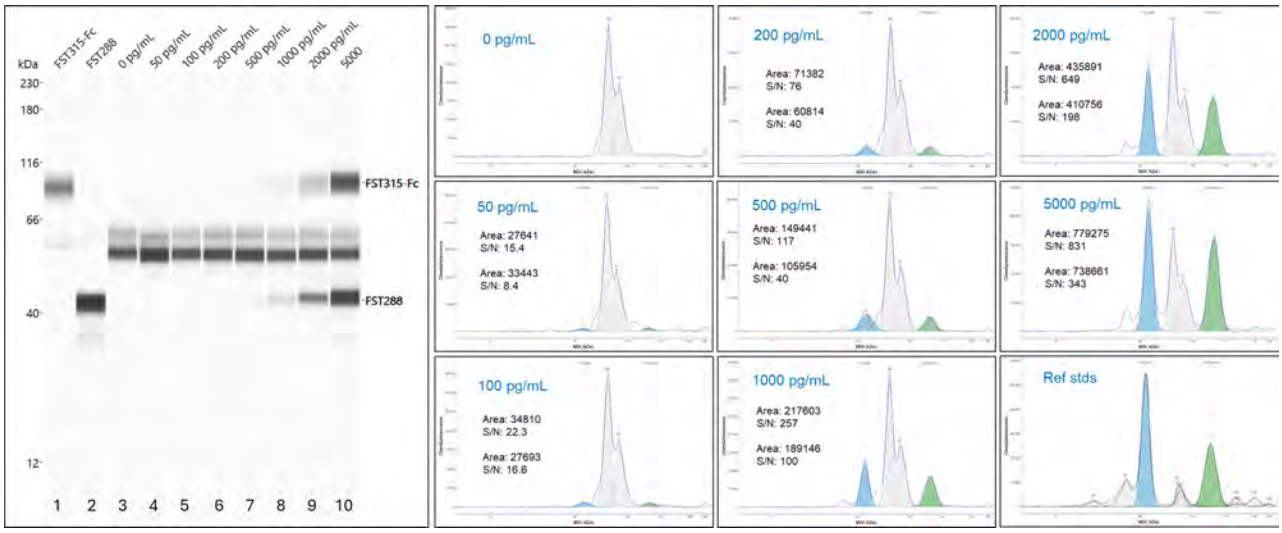


**Figure 2. Representative LOD determination for the detection of FST288 and FST315-Fc in serum samples.** A pool of serum was spiked with increasing concentrations of FST315-Fc and FST288. 100 µL of samples were immunopurified and analysed by capillary electrophoresis for the detection of follistatin. Graph view is presented (left panel). Individual lane views with the relative densitometric analyses of the detected bands are presented in the right panels. On each panel, the area and the S/N of the band detected for FST288 are presented at the top and those for the band detected for FST315-Fc are presented at the bottom.

Humanized monoclonal antibodies were detected by isolating the molecules through their binding to either recombinant human myostatin/GDF-8 (Domagrozumab, Stamulumab, Landogrozumab and Trevogrumab) or ActRIIB-Fc (Bimagrumab). The characteristic immunoglobulin chains were then detected using antibodies that either target the heavy (HC) or light (LC-k or LC-λ) chains. Figure 3 shows that, when analyzed by Simple Western, Landogrozumab (IgG4-k), Domagrozumab (IgG4-k) and Stamulumab (IgG1- k) are detected at 31-32 kDa (LC) and 59 kDa (HC), respectively. Bimagrumab (IgG1-λ) analysis was developed to specifically target the LC and was detected at 35 kDa. Figure 4 (left panel) demonstrates the ability to isolate humanized monoclonal antibodies spiked at 500 ng/mL is serum samples. Additionally, Figure 4 (right panel) shows a representative LOD determination assessment for Stamulumab detection in three different serum samples. Individual chromatograms for each concentration were stacked in the same panel (50, 100 or 250 pg/mL). The results showed that an LOD of 50 ng/mL (based on peak detection at S/N > 10) could be achieved for most monoclonal antibodies. However, since this method can't distinguish between anti-myostatin monoclonal antibodies with similar MW, it is best suited as an initial testing procedure (ITP). Other approaches, such as LC-HRMS/MS will be required for proper identification of the monoclonal antibodies.

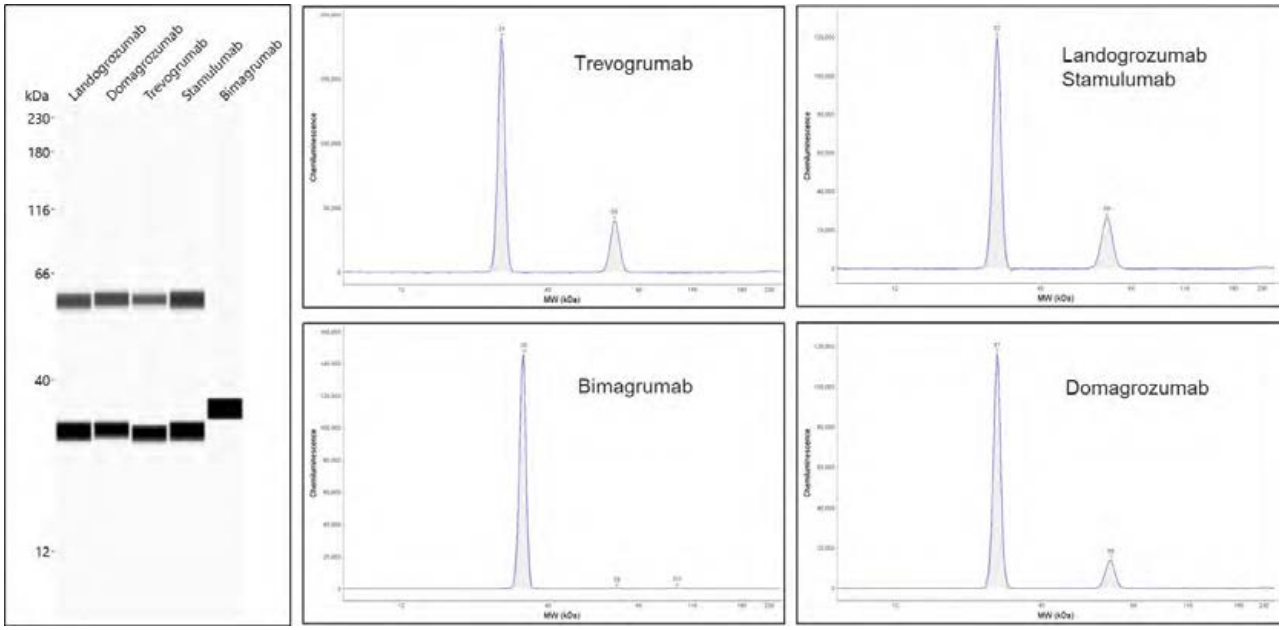


**Figure 3. Representative densitometric profiles of the anti-myostatin humanized monoclonal antibodies by Simple Western.** The humanized monoclonal antibodies are detected by targeting heavy chains (HC) and light chains (LC) of these immunoglobulins. Landogrozumab (IgG4-k), Domagrozumab (IgG4-k), Trevogrumab (IgG4-k) and Stamulumab (IgG1- k) are detected at 31-32 kDa (LC) and 59kDa (HC). Bimagrumab (IgG1-l) analysis was developed for specific detection at 35 kDa (LC). Left panel: Graph view analysis. Right panels: lane view analyses of the individual reference preparations.

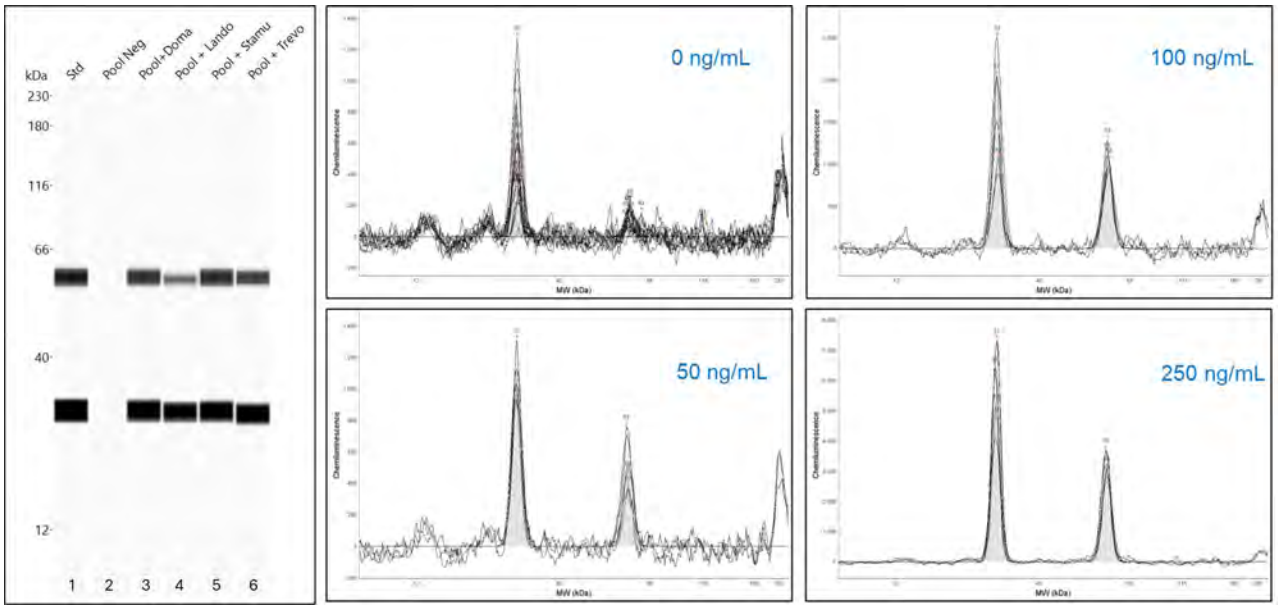


**Figure 4. Isolation of anti-myostatin humanized monoclonal antibodies from serum samples, representative LOD determination for Stamulumab and selectivity assessment.** Left panel: A pool of serum samples was left untreated (lane 2) or spiked at 500 ng/mL with humanized monoclonal antibodies (lanes 3-6), and isolated by immunoaffinity against GDF-8. Right panels: Ten different serums samples were untreated (0 ng/mL) or spiked with concentrations of 50 pg/mL (n=3), 100 pg/mL (n=3), 250 pg/ml (n=3). Stacked chromatograms are presented for each condition. Analysis was performed using Protocol 1.

## Conclusions

This study presents robust and sensitive methods for detecting follistatin-based inhibitors and humanized monoclonal antibodies that prevent activin receptor type IIB (ActRIIB) activation in serum. In summary, the limit of detection was found to be 50 pg/mL for follistatin-based inhibitors and 50 ng/mL for humanized monoclonal antibodies. The successful identification of these molecules at such low concentrations highlights their potential to complement existing analytical techniques [4-6]. Further work is needed to include more follistatin-based inhibitors in the assay and to fully validate the ITP and CP assays. Due to the assay's low sensitivity, future work will also extend to other matrices, such as dried blood spots (DBS). Capillary electrophoresis by Simple Western is a fast method (less than three hours), and one of its main advantages is that the assays can be customised to different lanes. This enables multiple different targets to be detected in the same analytical run. Future work will also aim to develop multiplexed assays.

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# A combined detection of TGF-β signaling inhibitors and ERAs in doping analysis by a multi-step immunopurification and western blotting method

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## Abstract

Sotatercept (activin type IIA receptor linked to the Fc portion of human immunoglobulin IgG1, ACVRIIA-Fc) and luspatercept (activin type IIB receptor linked to the Fc portion of human immunoglobulin IgG1, ACVRIIB-Fc), which could trap the related ligands in transforming growth factor beta (TGF-β) signaling pathway, can enhance the late stage of erythropoiesis, thus were included in the World Anti-Doping Agency (WADA) Prohibited List for several years, under the category of transforming growth factor beta (TGF-β) signaling inhibitors. As these two substances have been approved for clinical therapy, the availability of them increases the risk of misuse by athletes for doping. Thus, it is necessary to develop a sensitive and robust method for doping analysis, especially coupled with erythropoietin receptor agonists (ERAs) analysis.

In this study, we developed a multi-step immunopurification approach with different antibodies, following SAR-PAGE and single-blotting to detect sotatercept, luspatercept and ERAs in one aliquot by one integrated workflow of initial testing procedure (ITP).

With our developed method, both sotatercept and luspatercept can be detected down to 1 pg/mL in urine samples and 30 pg/mL in serum samples while the sensitivity of ERAs are not compromised. Furthermore, the time needed for such ITP can be shortened from 48 hours to 38 hours, along with the low volume needed and increased sensitivity, which are considered essential to Fast-Turnaround request for major events.

**Keywords:** TGF-β signaling inhibitors; doping analysis; immunopurification; western blotting

## Introduction

Since transforming growth factor beta (TGF-β) signaling inhibitors (e.g., luspatercept, sotatercept) can enhance the late stage of erythropoiesis and oxygen-carrying capacity in humans, it has been included in the World Anti-Doping Agency (WADA) Prohibited List [1]. Among the existing method for TGF-β signaling inhibitors in doping analysis, western blotting exhibits relatively high sensitivity [2-5]. Since erythropoietin receptor agonists (ERAs) and TGF-β signaling inhibitors have the similar properties on inducing erythropoiesis, it is viable to develop a method to detect these substances by one integrated workflow.In this study, we developed a multi-step immunopurification approach coupled with western blotting, aiming to detect sotatercept, luspatercept and ERAs simultaneously in one aliquot.

## Experimental

The ultrafiltrated urine and serum samples were added to the prepared magnetic beads coupled with anti-ACVRIIA antibodies to conduct the first immunopurification. Then, the supernatant was pipetted to conduct the second immunopurification with magnetic beads coupled with anti-ACVRIIB antibodies. Next, the supernatant from the second immunopurification was pipetted, for urine samples, STEMCELL ELISA plate was used to conduct the third immunopurification after further ultrafiltration, for serum samples, MAIIA EPO purification kit was used to conduct the third immunopurification. All the beads collected from the previous procedure were washed and eluted, respectively. Each eluate from different step of purification were loaded in different gels. SAR-PAGE was performed at a constant 200 V for 75 min, single-blotting was executed at 1.0 mA/cm$^2$ for 60 min and 1.54 mA/cm$^2$ for 20 min followed by incubation overnight with different biotinylated antibodies in 4 °C (Figure 1).

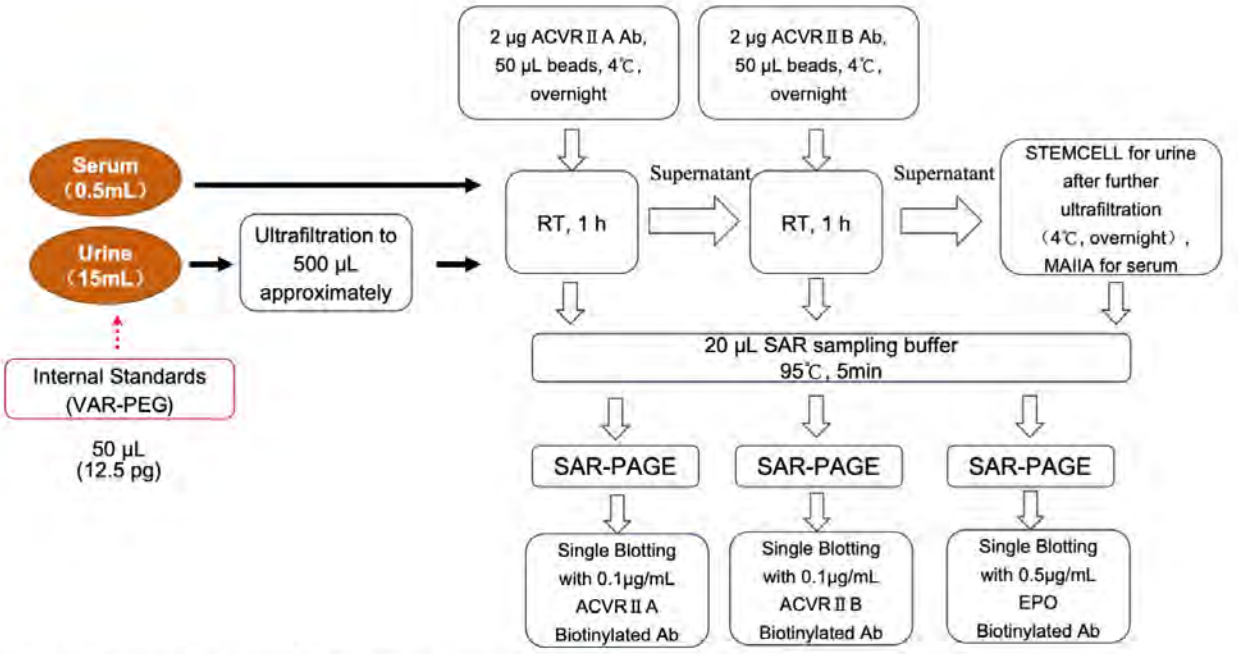



**Figure 1.** Protocol for detection of TGF-β signaling inhibitors and ERAs

## Results and Discussion

### Optimization for immunopurification

Different batches or types of capture antibody were compared (Figure 2A). As shown in the results, for ACVRIIA-Fc, the differences between different batches of antibody was negligible, each batch of antibody from Sino Biological could fulfill the requirement for immunopurification. For ACVRIIB-Fc, the difference between different types of antibody was obvious, #10229-T16 from Sino Biological showed the best results among the three. Next, the amounts of capture antibodies #10257-T16 and #10229-T16 were explored (Figure 2B). Results showed that higher amount of antibody led to an increase in

extraction efficiency. The results obtained with 4 µg usage of beads seemed similar to those using 2 µg (Figure not shown). Considering both the recovery rate and cost-effectiveness, we determined that 2 µg of beads represents the optimal amount for this protocol.

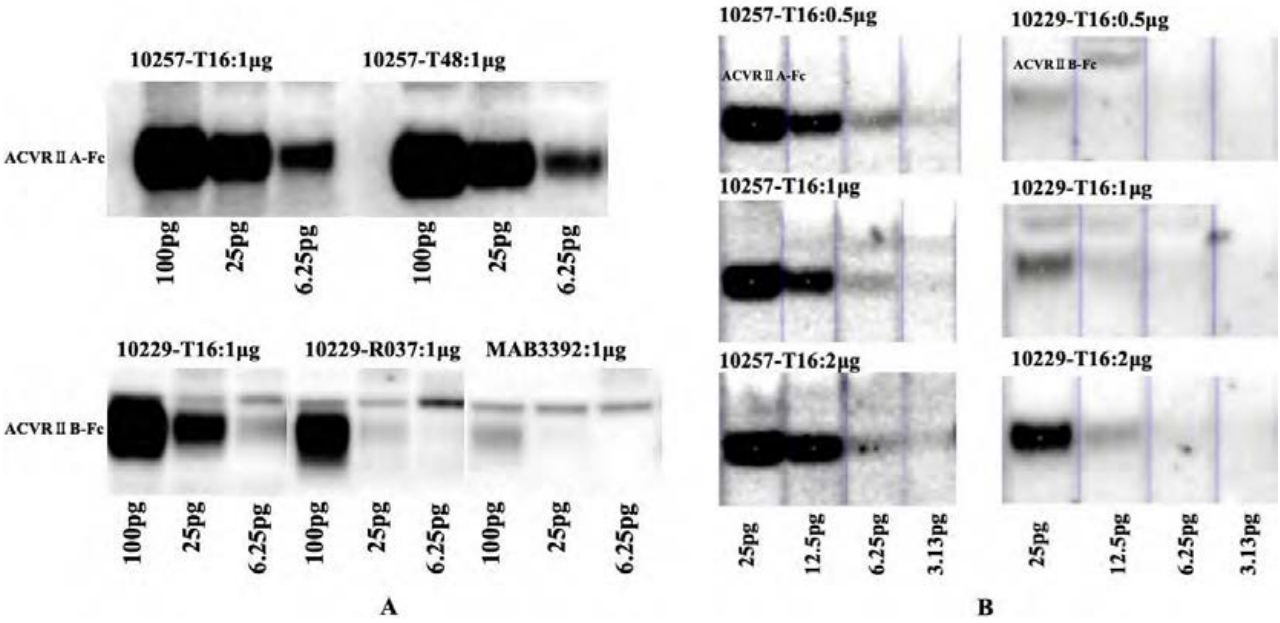
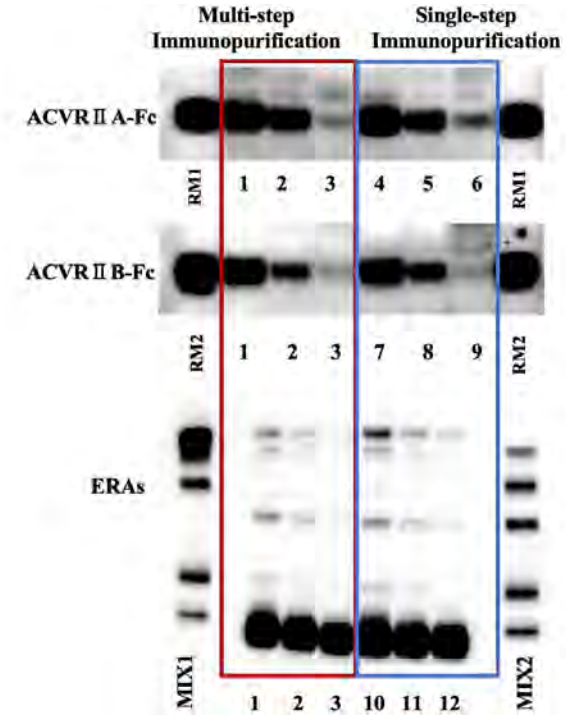



**Figure 2.** Optimization for immunopurification - A: Antibodies selection for immunopurification, B: Exploration of capture antibodies usage

**Sensitivity evaluation of multi-step immunopurification**

The sensitivity of multi-step and single-step immunopurification were compared using one blank urine sample equally divided into twelve aliquots spiked with different target analytes (Figure 3).As shown in the figure, the detection results following multi-step immunopurification showed no significant differences compared to those obtained with single-step immunopurification. Thus, multi-step immunopurification did not compromised the sensitivity of ERAs detection as well as TGF-β signaling inhibitors detection. The detection method we established allow sotatercept and luspatercept to be detected down to 1 pg/mL in urine sample and 30 pg/mL in serum sample.

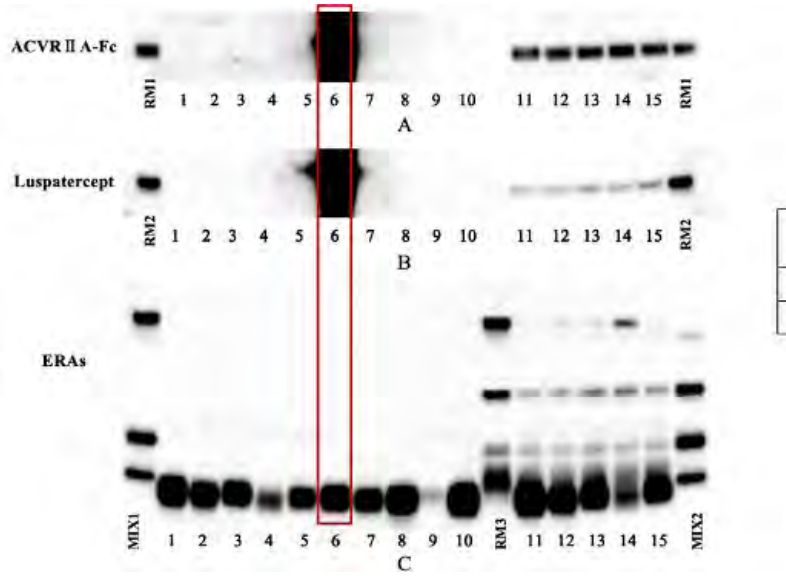



| Sample preparation | ACVR II A-Fc | ACVR II B-Fc | ERAs |
|---|---|---|---|
| 1 | 25 pg | 25 pg | MRPL |
| 2 | 12.5 pg | 12.5 pg | 50% MRPL |
| 3 | 6.25 pg | 6.25 pg | 25% MRPL |
| 4 | 25 pg | / | / |
| 5 | 12.5 pg | / | / |
| 6 | 6.25 pg | / | / |
| 7 | / | 25 pg | / |
| 8 | / | 12.5 pg | / |
| 9 | / | 6.25 pg | / |
| 10 | / | / | MRPL |
| 11 | / | / | 50% MRPL |
| 12 | / | / | 25% MRPL |

**Figure 3.** Comparison of multi-step and single-step immunopurification in urine samples; Reference material 1 (RM1): 25 pg ACVRIIA-Fc; Reference material 2 (RM2): 25 pg ACVRIIB-Fc; MIX-1: DYNEPO+NESP+CERA; MIX-2: DYNEPO+NESP+EPO-Fc

**Unspecific bands detected in urine samples**

During the experiment, we noticed that unspecific band with strong intensity was detected in eluate of step 1 and step 2 from particular sample, which cover the target band and may affect the detection of sotatercept and luspatercept(Figure 4), however, it did not affect the detection of ERAs.




| Sample (Urine) | ACVR II A-Fc | Luspatercept | ERAs |
|---|---|---|---|
| 1-10 | / | / | / |
| 11-15 | 25 pg | 50 pg | MRPL |

**Figure 4.** Unspecific bands in urine samples
A: Results of ACVR?A-Fc detection from multi-step immunopurification (step 1)
B: Results of luspatercept detection from multi-step immunopurification (step 2)
C: Results of ERAs detection from multi-step immunopurification (step 3)
Reference material 1 (RM1): 25pg ACVRIIA-Fc; Reference material 2 (RM2): 50 pg luspatercept; Reference material 3 (RM3): BRP+NESP+EPO-Fc+CERA (MRPL); MIX-1:DYNEPO+NESP+CERA; MIX-2:DYNEPO+NESP+EPO-Fc

## Conclusions

1. Multi-step immunopurification coupled with western blotting can be applicable for detecting ERAs, sotatercept and luspatercept with one aliquot by a single integrated workflow.
2. The method we developed allow sotatercept and luspatercept to be detected down to 1 pg/mL in urine sample and 30 pg/mL in serum sample while the sensitivity of ERAs are not compromised.
3. With this method, the analytical time of ITP for both ERAs, sotatercept and luspatercept was only two days, which can be even shortened to 38 hours in the fast-turnaround for major events.

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Ion RN, Stan C, Toboc A

# Validation of an immunoaffinity purification method using Abcam detector antibody ab253419 and Dynabeads M280 for erythropoietin analysis in blood

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## Abstract

Erythropoietin (EPO) – receptor agonists (ERAs) use is prohibited in sports by World Anti-Doping Agency due to their performance enhancing properties. Several polyacrylamide gel electrophoretic analytical methods have been developed over the years for ERAs detection. According to TD2024EPO, sample immunopurification is a mandatory step before electrophoretic separation. Moreover, confirmation procedure must differ from initial testing procedure and this difference may be achieved by application of a different sample immunopurification procedure among others. In this study, Abcam anti-EPO detector antibody ab253419 coupled to sheep anti-rabbit IgG Dynabeads M280 was used to immunopurify blood samples (serum and plasma) spiked with four ERAs (BRP, NESP, EPO-Fc, CERA) at MRPL, 0.5 MRPL, 0.25 MRPL and 0.1 MRPL. Immunopurified samples were further subjected to SAR-PAGE and western blot using biotinylated anti-EPO antibody BAM2871. Selectivity, reliability of detection at MRPL, limit of detection and recovery were evaluated. Results have shown that this immunopurification method can be used for ITP or CP of plasma samples due to its high selectivity and sensitivity.

## Introduction

Sample purification is a mandatory step in erythropoietin (EPO)-receptor agonists (ERAs) analysis [1]. Recently, Reichel *et al.* [2] proposed an effective immunoaffinity purification step-by-step protocol using immunoprecipitation of EPO and it's analogs from human urine and blood doping control samples based on magnetic beads with non-covalent immobilization of the capture antibody. As the Sino Biological #51099-T42 polyclonal rabbit antibody used in the study has limited availability, Reichel *et al.* [2] suggested the use of recombinant rabbit monoclonal antibodies Abcam #ab253419 as an alternative. The objective of this study was to validate the immunoaffinity purification method that uses Abcam detector antibody ab253419 coupled to sheep anti-rabbit IgG Dynabeads M280 for erythropoietin analysis in blood.

## Experimental

Blood sample immunopurification, both serum and plasma, was performed using Abcam anti-EPO detector antibody ab253419 coupled to single use sheep anti-rabbit IgG Dynabeads M280 according to the protocol proposed by Reichel *et al.* [2]. Immunopurified samples were further subjected to SAR-PAGE and semi-dry western blotting using biotinylated anti-EPO antibody BAM2871. SAR-PAGE analysis was performed at 200V for 70 min. After SAR-PAGE, proteins were transferred on Immobilon-P membrane

using a transfer buffer with 48 mM Tris base, 39 mM glycine, 0.00375% SAR, and 5% methanol. Single blotting was performed at 1.56 mA/cm$^2$ for 45 min. using biotinylated Mab anti-EPO, clone AE7A5 (0.5 µg/mL in 1% NFM/PBS) as a primary antibody. West Femto substrate was used for chemiluminescence detection. Method validation was performed according to the WADA Laboratory Technical Note- Validation of polyacrylamide gel electrophoretic (PAGE) analytical methods for the analysis of EPO-receptor agonists (ERA) in doping control, version 1.0, April 2021.

## Results and Discussion

### Selectivity

Figure 1 shows that the method is highly selective for plasma samples, where only endogenous EPO was observed. However, in serum samples interfering signals were detected above EPO-Fc, reaching CERA region.

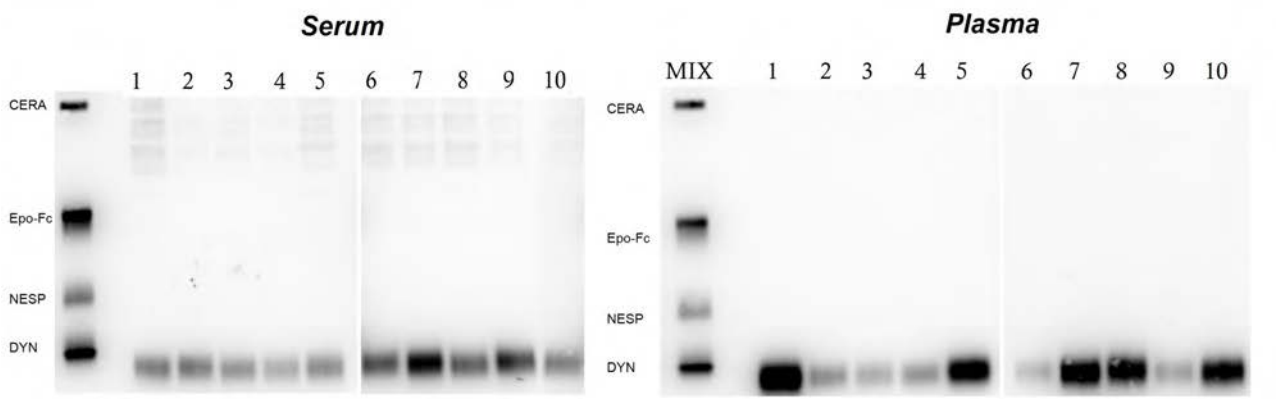


**Figure 1.** SAR-PAGE analysis of 10 different serum and plasma samples (1-10)

### Reliability of detection at MRPL

All ERAs spiked into the blank samples at their MRPLs could be detected in all experimental conditions as it can be seen in Figure 2.

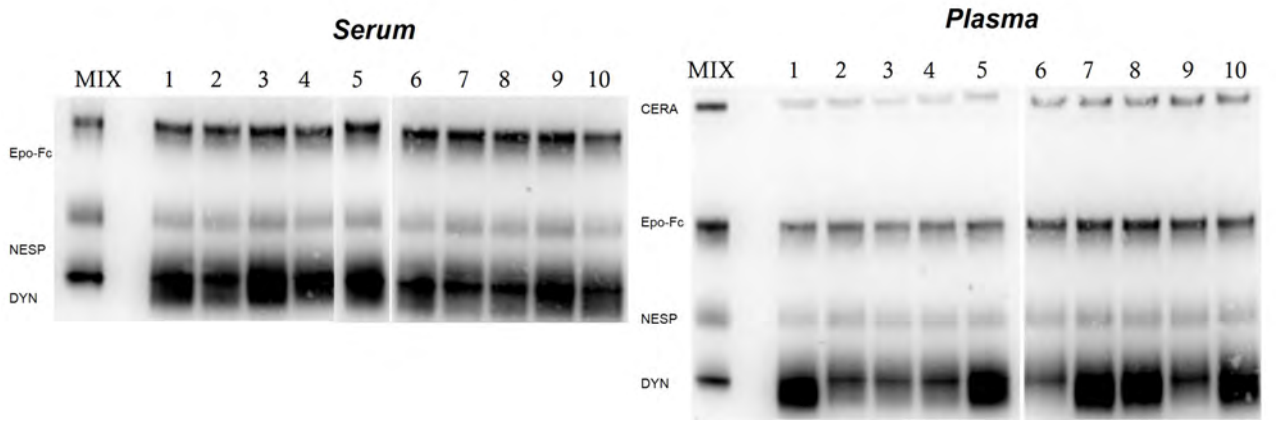


**Figure 2.** SAR-PAGE analysis of 10 different serum and plasma samples (1-10) spiked with four ERAs at MRPL

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**Limit of detection**

Figure 3 shows the results of LOD determination. These were identical for the two matrices and estimated at 3 mIU/mL for BRP, 3 pg/mL for NESP and 15 pg/mL for EPO-Fc.LOD for CERA was estimated only for plasma samples at 15 pg/mL.

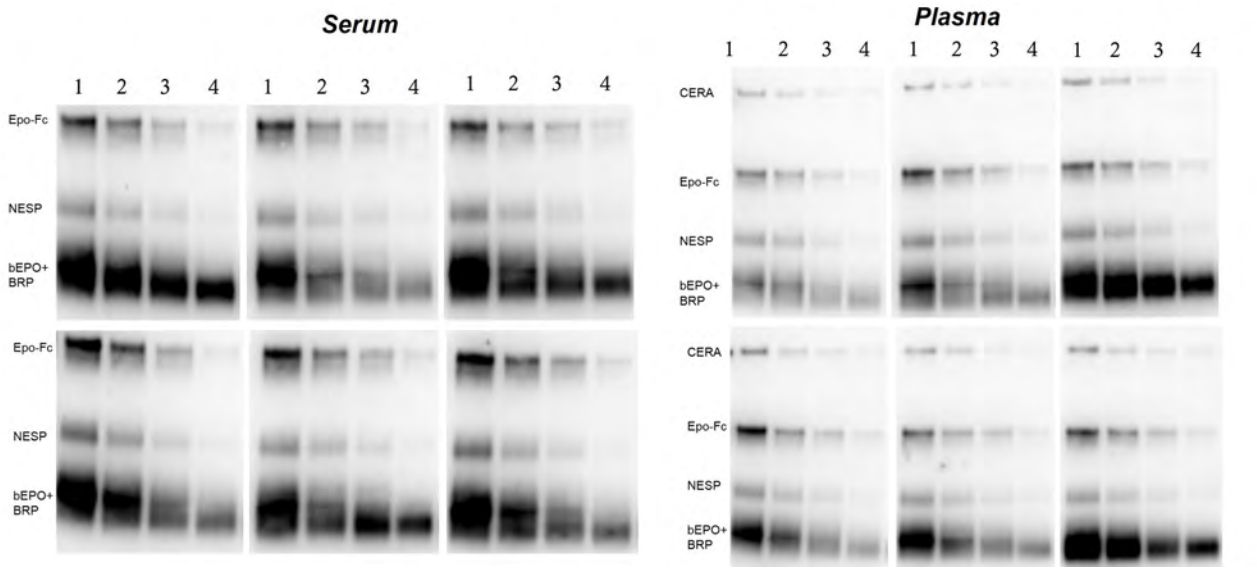


**Figure 3.** LOD determination after spiking six serum and six plasma samples with an ERA-Mix at 1.0/0.5/0.25/0.1 MRPL (1 to 4)

**Recovery**

Recoveries varied between 39% and 68%. For BRP, NESP and EPO-Fc these were similar in different samples of each matrix. The recovery of CERA in different samples of plasma were also nearly the same. Slightly better recovery percentages were found for plasma samples versus serum samples.

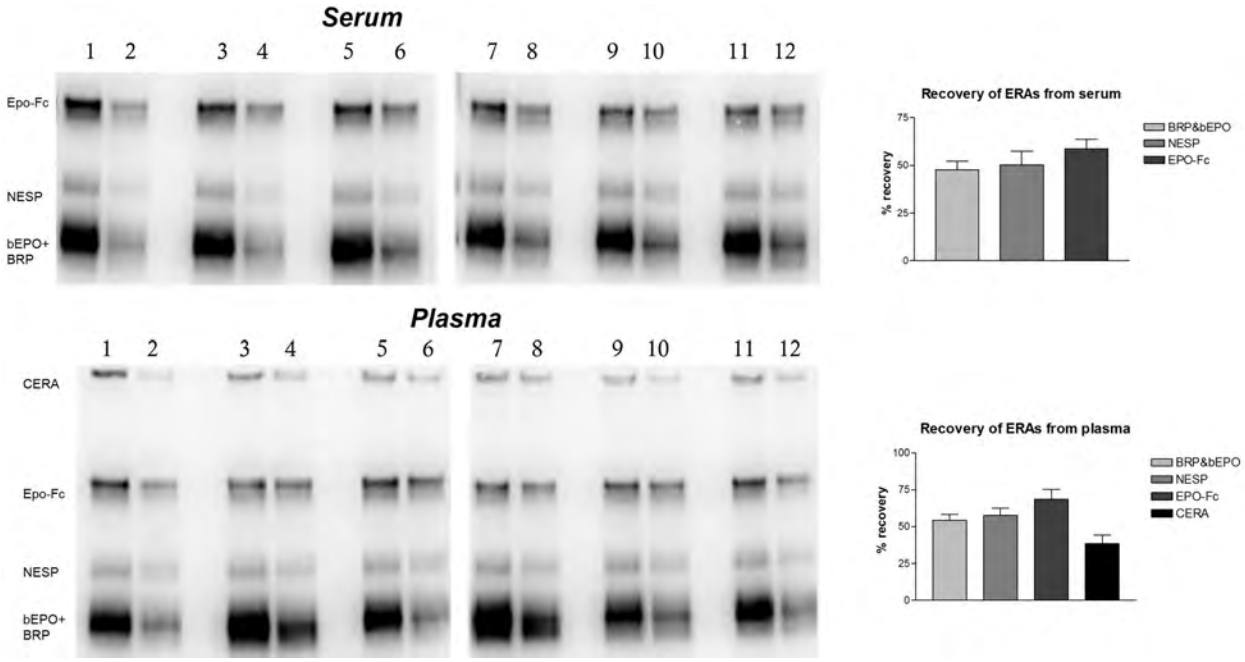


**Figure 4.** Recoveries by SAR-PAGE using magnetic beads immunopurification method of blood samples

## Conclusions

The method is capable of detecting analytes in serum and plasma samples at low concentrations (0.1 MRPL). The magnetic beads immunopurification method is recommended for ITP or CP of plasma samples due to its high selectivity and sensitivity.

For serum samples, this method can be applied only for the confirmation procedure of BRP, NESP and EPO-Fc due to the interfering signals present in the CERA region. In a previous study, evaluating detection of ERAs from dried blood spots extracted and immunopurified with EPO Purification Gel Kit for Blood (MAIIA Diaagnostics), non-specific bands above EPO-Fc have also been reported, but it was concluded that they did not interfere with ERA identification [3]. Moreover, Reichel *et al.* used the same magnetic beads with non-covalent immobilization of the capture antibody, namely Sino Biological 51099-T42, to immunopurify serum samples spiked with ERAs and found that these were detectable at concentrations lower than 0.1 MRPL, without reporting cross-reactive bands [2]. Recently, Salamin *et al.* brought arguments to support the stability and consistent detectability of CERA in serum and DBS [4]. The clean results in plasma samples in this study suggest that the cross-reactive bands in serum samples are artefacts of the coagulation process and related protein-interactions. Understanding the impact of hemolysis and long term storage on serum stability could help reveal the cause of our contradictory results.

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Kaliszewski P[1], Zamlynski M[2], Zalewska Z[1], Grucza K[1], Woznica M[1], Zamlynski J[2], Olejek A[2], Kwiatkowska D[1,3]

# Pregnancy and status of being a professional athlete can contribute to elevated levels of PIIINP, IGF-I and $GH_{2000}$

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Biological and Chemical Research Centre, University of Warsaw, Warsaw, Poland[3]

## Abstract

The use of growth hormone (GH) is prohibited in sport by the World Anti-Doping Agency (WADA) [1]. The detection of GH misuse relies on measuring GH-dependent biomarkers, namely insulin-like growth factor-1 (IGF-I) and the N-terminal propeptide of type III procollagen (PIIINP). However, limited data are available on physiological conditions that may influence these biomarkers and potentially lead to false-negative or false-positive results. Literature suggests that pregnancy [2,3] and high-intensity physical activity associated with elite athleticism may elevate IGF-I and PIIINP levels.

To investigate this, biomarker levels were analyzed in three groups of women: 101 in early pregnancy (gestational weeks 10.6–12.4), 101 non-pregnant professional athletes, and 101 non-pregnant non-athletes. PIIINP was measured using the Siemens ADVIA Centaur kit, and IGF-I using IDS-iSYS. Results show elevated levels of IGF-I, PIIINP, and GH2000_F scores in both professional athletes and pregnant women compared to controls. These findings highlight a significant risk of false-positive GH biomarker results in early-pregnant athletes, whose GH2000_F scores may exceed population thresholds.

## Introduction

Human growth hormone (hGH) misuse detection relies on the measurement of hGH-dependent biomarkers, primarily IGF-I and PIIINP. This method offers a detection window of up to seven days following GH administration. However, current population-based thresholds do not account for inter-individual variability, reducing detection sensitivity. To address this, individualized thresholds are being incorporated into the Athlete Biological Passport (ABP), which requires that biomarkers demonstrate intra-individual stability.

In male subjects, IGF-I and PIIINP have shown low coefficients of variation (<37%). However, corresponding data in females are limited. Hormonal fluctuations, particularly those associated with the menstrual cycle, suggest higher variability in women, as evidenced in the ABP's steroid module. Furthermore, the influence of pregnancy on IGF-I and PIIINP levels remains underexplored, though literature indicates increases in these markers during late pregnancy [2,3]. Understanding the effects of pregnancy and professional athletic activity on these biomarkers is essential for accurate result interpretation.

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## Experimental

- PIIINP was measured using the Siemens ADVIA Centaur PIIINP assay (Siemens Healthcare Diagnostics, Camberley, UK), and IGF-I using the IDS-iSYS IGF-I assay (Immunodiagnostic Systems, Boldon, UK). Assays were performed according to manufacturers' protocols.
- Method validation followed WADA's hGH Biomarkers Test Guidelines Version 3.0 [4].
- The GH2000_F score was calculated using the formula:
  GH2000_F = –8.459 + 2.454·ln(PIIINP) + 2.195·ln(IGF-I) – (73.666 / age) [4]
- Statistical analysis was performed using one-way ANOVA, with a significance threshold of $p < 0.05$.
- Samples were collected from three groups:
  - 101 women in early pregnancy (10.6–12.4 weeks),
  - 101 non-pregnant professional athletes (various disciplines),
  - 101 non-pregnant non-athlete controls.
- Participants' mean age was 30 ± 10 years (standard deviation).

Anova: Single Factor

SUMMARY

| *Groups* | *Count* | *Sum* | *Average* | *Variance* |
|---|---|---|---|---|
| IGF-1 spotswomen group | 101 | 24091.0 | 238.5 | 4392.9 |
| IGF-1 non-sportswomen group | 104 | 21277.8 | 204.6 | 5567.1 |
| IGF-1 pregnant non-sportswomen group | 101 | 19227.2 | 190.4 | 3045.8 |

ANOVA

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Between Groups | 123774.9 | 2.0 | 61887.4 | 14.2 | 1.2E-06 | 3.03 |
| Within Groups | 1317283.4 | 303.0 | 4347.5 | | | |
| Total | 1441058.2 | 305.0 | | | | |

Anova: Single Factor

SUMMARY

| *Groups* | *Count* | *Sum* | *Average* | *Variance* |
|---|---|---|---|---|
| P-III-NP spotswomen group | 101 | 938.3 | 9.3 | 12.2 |
| P-III-NP non-sportswomen group | 104 | 646.9 | 6.2 | 3.0 |
| P-III-NP pregnant non-sportswomen group | 101 | 1101.1 | 10.9 | 14.2 |

ANOVA

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Between Groups | 1162.4 | 2 | 581.2 | 59.8 | 1.30E-22 | 3.03 |
| Within Groups | 2945.4 | 303 | 9.7 | | | |
| Total | 4107.8 | 305 | | | | |

Anova: Single Factor

SUMMARY

| *Groups* | *Count* | *Sum* | *Average* | *'ariance* |
|---|---|---|---|---|
| GH 2000 score spotswomen group | 101.0 | 582.6 | 5.8 | 0.9 |
| GH 2000 score non-sportswomen group | 104.0 | 475.4 | 4.6 | 1.3 |
| GH 2000 score pregnant non-sportswomen group | 101.0 | 615.3 | 6.1 | 1.7 |

ANOVA

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Between Groups | 132.2 | 2.0 | 66.1 | 50.4 | 1.3E-19 | 3.03 |
| Within Groups | 397.4 | 303.0 | 1.3 | | | |
| Total | 529.5 | 305.0 | | | | |

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

## Results and Discussion

The highest average IGF-I concentration was observed in the athlete group (239 ng/mL), compared to 205 ng/mL in pregnant women and 190 ng/mL in controls. This suggests that status of being professional athlete has a more pronounced effect on IGF-I levels than early pregnancy.

PIIINP levels were elevated in both the pregnant group (10.9 ng/mL) and athletes (9.3 ng/mL), compared to the control group (6.2 ng/mL). Consequently, GH2000_F scores were also elevated in athletes (mean 5.8) and pregnant women (mean 6.1) compared to controls (mean 4.6).

The additive effects of early pregnancy and elite athletic activity could result in GH2000_F scores exceeding the population-based threshold of 9.35 established for the IDS-iSYS/Siemens assay pair [4], increasing the likelihood of false positives.

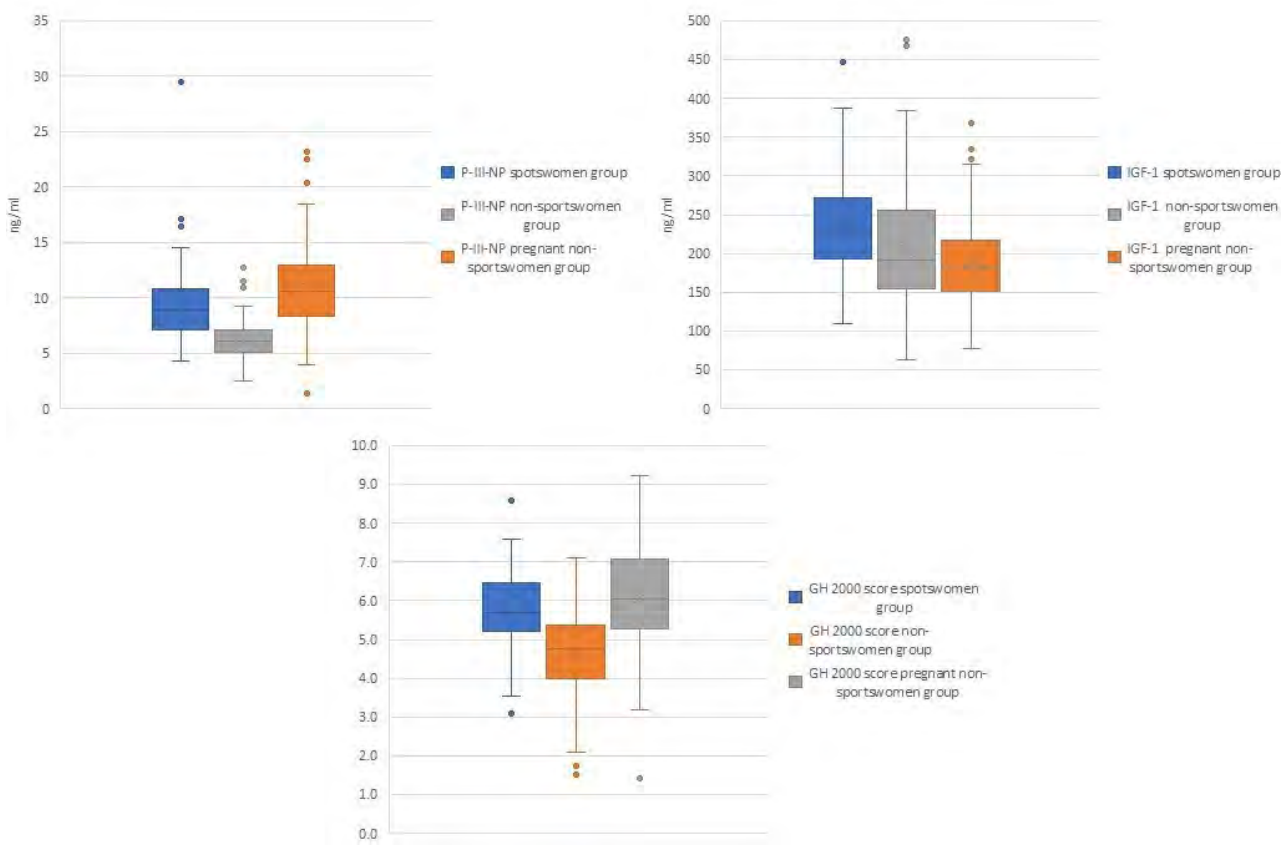


**Figure 1.** Results of IGF-1, P-III-NP. and GH2000 score

## Conclusions

Both early pregnancy and elite athletic status significantly affect levels of IGF-I, PIIINP, and GH2000_F scores. These elevations may result in false-positive findings when population-based thresholds are applied. Additionally, such variability poses challenges for implementing these markers within the Athlete Biological Passport.

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Yepez Gavilanes E, Kozhakhmetova Z, Bartlett C, Ahi S, Wolff K

# Validation of an initial testing procedure to detect haemoglobin-based oxygen carriers (HBOCs) in serum and plasma using the Sysmex XN-1000

Drug Control Centre, King's Forensics, Drug Control Centre King's College, London, Great Britain

## Abstract

Haemoglobin-based oxygen carriers (HBOCs), including Hemopure™ and Oxyglobin™, are oxygen-therapeutic agents developed as substitutes for red blood cells (RBCs) in surgical and emergency settings. Due to their ability to enhance oxygen delivery and potentially improve endurance, HBOCs pose a risk for misuse in elite sport. Under normal physiological conditions, haemoglobin (HGB) is confined to RBCs, with minimal free HGB present in serum or plasma. Therefore, elevated concentrations of free HGB in serum and/or plasma may serve as an indicator of illicit HBOC administration [1].
Currently, no Technical Document or Guideline has been issued by the World Anti-Doping Agency (WADA) for the detection of HBOCs, nor is a screening threshold established.
This study aimed to develop and validate an Initial Testing Procedure (ITP) for the quantification of free HGB in human serum and plasma using the Sysmex XN-1000 haematology analyser. The method employed Sodium Lauryl Sulphate (SLS) for HGB detection, offering a cyanide-free, high-throughput analytical approach. Controlled haemolysis experiments were conducted at room temperature and 4°C using serum and plasma from healthy volunteers. Additionally, HGB concentrations were measured in athlete samples collected over a two-month period to assess inter-individual variability and baseline haemolysis. Based on these investigations, a threshold of ≥ 0.3 g/dL was established as a presumptive adverse analytical finding (PAAF), distinguishing samples spiked with HBOCs from those exhibiting mild haemolysis. Spiking experiments using bovine haemoglobin (0.1–1.0 g/dL) demonstrated assay linearity with mean recoveries of 97% (serum) and 98% (plasma). Samples with HGB concentrations ≤ 0.2 g/dL were classified as negative, while those ≥ 0.3 g/dL met criteria for PAAF. These results support the application of this validated ITP for HBOC detection in anti-doping settings and provide a scientifically justified threshold for further confirmatory analysis.

## Introduction

Blood doping is defined by the World Anti-Doping Agency (WADA) as the use of substances or methods that enhance the uptake, transport, or delivery of oxygen by increasing the number of red blood cells in the bloodsteam to enhance athletic performance. Historically, this has included blood transfusions and the administration of recombinant erythropoietins (EPOs). More recently, attention has shifted to the use of haemoglobin-based oxygen carriers (HBOCs) and other synthetic oxygen transport molecules. HBOCs are designed to increase the oxygen-carrying capacity of blood and serve as universal substitutes for red blood cells (RBCs)[1,2], making them attractive for performance enhancement in endurance sports.

These products are derived from either Bovine, Lugworm and/or human haemoglobin and are chemically modified – via intra- or inter-molecular cross-linking, polymerization, or conjugation – to maintain stability and function in circulation [4-6].

Under physiological conditions, haemoglobin (HGB) is confined to RBCs, and only trace amounts are present in plasma or serum. In contrast, HBOCs circulate as free haemoglobin in plasma or serum, providing a potential analytical marker for detection of abuse. Thus, elevated concentrations of free HGB in serum and/or plasma may indicate the administration of a HBOC. Due to their deep red coloration, HBOCs may be visually detectable in plasma or serum samples. However, visual inspection is subjective and cannot reliably differentiate between exogenous HBOCs and haemoglobin released through haemolysis. Therefore, an objective, semi-quantitative/qualitative method is required to distinguish these sources of free haemoglobin as an Initial Testing Procedure, which can be used to prompt a more HBOC specific confirmation procedure.

HBOCs are currently included under Section M1 of the WADA 2024 Prohibited List, which covers manipulation of blood and blood components. However, no specific WADA Technical Document or Guideline exists for the screening or confirmation of HBOC use. Nevertheless, HBOCs are included in the External Quality Assessment Scheme (EQAS) for 2024, highlighting the need for validated testing methodologies. Since 2019, WADA has harmonized the use of flow cytometry-based instruments, including the Sysmex XN-1000, for measurement of haematological parameters within the Athlete Biological Passport (ABP). This transition presents an opportunity to validate procedures for the detection of HBOCs using this platform. The Sysmex XN-1000 haematology analyser offers a reproducible and standardized alternative to visual inspection eliminatng the objectivity of visual inspections.

This study outlines the validation of an Initial Testing Procedure (ITP) for the detection of HBOCs in human serum and plasma using the Sysmex XN-1000. The analyser employs a cyanide-free method using Sodium Lauryl Sulphate (SLS) for haemoglobin detection. Specifically, ferrous iron ($Fe^{2+}$) in haemoglobin is oxidized to ferric iron ($Fe^{3+}$) by the hydrophobic region of SLS, after which $Fe^{3+}$ binds to the hydrophilic region, producing turbidity. Any free HGB present in serum and/or pleasma undergoes conformational changes that enable spectrophotometric measurement. The absorbance at 555 nm is then measured, with the recorded absorbance directly proportional to the HGB concentration (expressed in g/dL). The objective of this work is to establish a robust and reproducible ITP of HBOCs in human serum and plasma using the Sysmex XN-1000. This procedure aims to support anti-doping laboratories in identifying presumptive use of HBOCs in the absence of formal WADA guidelines.

## Experimental

The objective of this study was to establish a threshold concentration of free haemoglobin (HGB) in human serum and plasma that could trigger confirmatory analysis for suspected administration of haemoglobin-based oxygen carriers (HBOCs).

The study comprised six main components:

(i) selectivity assessment,

(ii) evaluation of haemolysis impact,

(iii) supplementary haemolysis analysis,

(iv) linearity assessment using bovine haemoglobin, and
(v) haemolysis monitoring in athlete samples (vi) Inter-laboratory study to verify the detection / absence of different HBOCs in blind samples provided by the FMSI Rome Anti--doping Laboratory.

All whole blood/ blood samples in EDTA tubes and SST tubes, were centrifuged at 1300 rcf for 15 minutes, and the plasma or serum layer removed for analysis. QCs were analysed using the Sysmex XN-1000 with standard settings, these settings were then altered to allow for the analysis of serum and/or plasma samples due to the difference in viscosity.

The following parameters were changed using the Manual Analysis Menu to:
READ ID - this was switched OFF
CAP OPEN - this was turned ON
ASPIRATION SENSOR - this was turned OFF
RET - was turned OFF
DIFF - was turned OFF

**i. Selectivity Assessment**

To determine baseline concentrations of free haemoglobin in athlete samples, 20 serum and 20 plasma specimens were analysed using the Sysmex XN-1000 haematology analyser. All samples were obtained with prior informed consent for research use.
Free haemoglobin concentrations were measured using the sodium lauryl sulphate (SLS) method, which permits cyanide-free quantification of haemoglobin via spectrophotometric absorbance at 555 nm. This data were used to define the expected range of HGB concentrations in non-haemolysed samples and to inform the development of a screening threshold for presumptive adverse findings.

**ii. Effect of Haemolysis on Free Haemoglobin Concentration**

Venous blood samples were collected from six healthy adult volunteers (three male, three female). Each participant provided three samples in EDTA tubes and three in Serum Separator Tubes (SSTs) to simulate different haemolysis conditions.

Baseline (Non-haemolysed) Condition

One EDTA and one SST sample per participant were processed within 48 hours of collection. Whole blood haemoglobin concentration was first measured using the Sysmex XN-1000 to establish individual baselines. Following centrifugation at 1300 × *g* for 15 minutes, the resulting serum and plasma fractions were analysed in duplicate for free haemoglobin.

Room Temperature Haemolysis

A second set of EDTA and SST samples were stored at room temperature (20–22°C) for seven days. On day 7, samples were centrifuged at 1300 × *g* for 15 minutes, and free haemoglobin concentrations in plasma and serum were measured in duplicate. Statistical analyses included calculation of mean values, standard deviations (SD), and coefficients of variation (CV).

Refrigerated Haemolysis

A third set of EDTA and SST samples was stored at 4°C for seven days under the same conditions. After

centrifugation, free haemoglobin levels were determined as above and subjected to the same statistical evaluations.

**iii. Supplementary Haemolysis Study**

To expand the haemolysis dataset, an additional six serum and six EDTA samples were subjected to identical haemolysis conditions – storage at room temperature and 4°C for seven days. Following centrifugation at 1300 × *g*, free haemoglobin concentrations were measured in duplicate using the Sysmex XN-1000, and results were evaluated for mean, CV, and SD.

**iv. Linearity Assessment: Bovine Haemoglobin Spiking**

To assess the linear response of the Sysmex XN-1000 to increasing haemoglobin concentrations, pooled human serum and plasma samples (obtained from the NHS Blood Bank) were spiked with bovine haemoglobin at defined levels.

A stock solution was prepared by dissolving 1 g of bovine haemoglobin in 25 mL of 0.1% BSA in 0.1% insulin in 0.01 M phosphate-buffered saline (PBS). The concentration of haemoglobin in the stock solution was confirmed prior to use. Aliquots of pooled serum and plasma were then spiked to obtain a range of haemoglobin concentrations and analysed using the Sysmex XN-1000 to assess linearity across the target range.

**v. Haemolysis Monitoring in Routine Athlete Samples**

Over a two-week period, whole blood samples received in EDTA tubes at the Drug Control Centre were visually inspected on day three post-receipt for evidence of haemolysis in the plasma fraction. Where research consent was available, samples were photographed to document haemolysis severity, centrifuged at 1300 × *g*, and analysed for free haemoglobin concentrations using the Sysmex XN-1000.

**vi. Inter-Laboratory Study with FMSI Rome Anti-doping Laboratory**

The FMSI Rome Anti-doping Laboratory prepared 2 human serum and 2 human plasma samples that were spiked with different amount of HBOCs (Oxyglobin at 0.5 g/dL and Hemopure at 0.3 g/dL). A third serum and plasma sample was used as blank reference. Analysis was performed by both laboratories using the Sysmex- XN 1000 haematological analyzer. The concentration of 0.2 g/dL was used as the decision limit. Samples with HGB concentrations ≤ 0.2 g/dL were classified as negative, while those ≥ 0.3 g/dL met criteria for Presumptive Adverse Analytical Finding (PAAF).

## Results and Discussion

**i. Selectivity**

Selectivity analysis was conducted to determine baseline free haemoglobin (HGB) concentrations in serum and plasma samples from athletes. Using the Sysmex XN-1000 haematology analyser, 20 serum and 20 plasma samples were assessed. The mean free haemoglobin concentration in serum was 0.03 g/dL, with a coefficient of variation (CV) of 1.46%, while plasma samples exhibited a slightly higher mean of 0.08 g/dL, with a CV of 0.98%.

**ii. Effect of Haemolysis on Free Haemoglobin Concentration**

To investigate the effect of delayed processing and storage conditions on haemolysis, samples were

analysed after being stored either at room temperature (20–22°C) or refrigerated (4°C) for seven days. Analysed on day 0 and Day 7 after collection.

Day 0 (fresh samples)

To simulate physiological haemolysis and assess its impact on free HGB measurements, fresh venous blood samples were analysed within 48 hours of collection.

- Plasma samples showed a mean free haemoglobin concentration of 0.03 g/dL (CV = 1.41%)
- Serum samples showed a mean of 0.02 g/dL (CV = 2.24%)

These values were in close alignment with the selectivity data, and the low variability suggests minimal spontaneous haemolysis. The comparability between serum and plasma matrices at this early timepoint further supports the stability of non-haemolysed samples under proper handling conditions.

Room Temperature Storage – Day 7

- Plasma samples exhibited a mean free HGB concentration of 0.36 g/dL (CV = 0.60%)
- Serum samples showed a mean of 0.16 g/dL (CV = 0.24%)

These results indicate a substantial increase in free haemoglobin concentrations compared to day 0, confirming that prolonged room temperature storage promotes red blood cell lysis. Both matrices demonstrated elevated means, with plasma showing a higher susceptibility to haemolysis than serum.

Refrigerated Storage – Day 7

- Plasma samples showed a mean free HGB concentration of 0.06 g/dL (CV = 0.65%)
- Serum samples had a mean of 0.03 g/dL (CV = 1.67%)

Under refrigerated conditions, the extent of haemolysis was considerably lower compared to room temperature storage. While the mean free HGB concentrations remained slightly higher than fresh samples, they were still well below the proposed threshold for a presumptive adverse finding (0.3 g/dL). Notably, plasma again demonstrated a higher CV than serum, suggesting greater sensitivity to environmental stress and storage conditions.

**iii. Additional Haemolysis Study**

In response to a request from the Deputy Director, a supplementary haemolysis investigation was undertaken to expand the dataset and further validate the impact of storage conditions on free haemoglobin (HGB) concentrations in serum and plasma. An additional set of six serum and six plasma samples were stored at room temperature (20–22 °C) for seven days to simulate delayed sample processing. Following centrifugation, free HGB concentrations were measured using the Sysmex XN-1000. The results were as follows:

- Mean free haemoglobin concentration in plasma samples was 0.10 g/dL, with a coefficient of variation (CV) of 0.63%
- Mean free haemoglobin concentration in serum samples was 0.03 g/dL, with a CV of 1.22%

Importantly, the free HGB levels observed in this extended study remained below the proposed PAAF threshold of **0.3 g/dL**, suggesting that even under suboptimal storage conditions, endogenous haemolysis rarely reaches levels that would be mistaken for HBOC use. These data support the robustness of the proposed threshold and underscore the importance of matrix selection and sample handling protocols in haemoglobin-based doping detection.

**iv. Linearity Assessment: Bovine Haemoglobin Spiking**

The aim was to assess the system's ability to quantify exogenous haemoglobin accurately and reproducibly across a relevant concentration range.

The instrument successfully detected bovine haemoglobin in both serum and plasma across all tested concentrations, demonstrating a linear response with minimal variability. The recovery rates were consistent, and no significant variation was observed between replicates, indicating high analytical precision. Importantly, carryover was not observed during sequential analysis of high- and low-concentration samples, further confirming the instrument's suitability for routine screening of HBOCs.

**v Haemolysis Monitoring in Routine Athlete Samples**

As part of routine quality monitoring, a two-week observational study was conducted to assess the prevalence and extent of haemolysis in whole blood samples received at the Drug Control Centre. Samples were visually inspected three days post-receipt for signs of haemolysis in the plasma fraction.

Approximately 29.6% of the samples exhibited visible signs of haemolysis within three days of arrival. However, subsequent analysis using the Sysmex XN-1000 revealed that the mean free haemoglobin concentration among these samples was 0.07 g/dL, a value well below the proposed threshold of 0.3 g/dL for a presumptive adverse analytical finding (PAAF).


**Figure 1.** Haemolysis monitoring

**vi. Inter-Laboratory Study with FMSI Rome Anti-doping Laboratory**

Both laboratories identified the positive samples correctly. The results obtained by the two laboratories were perfectly comparable both in qualitative and quantitative terms.

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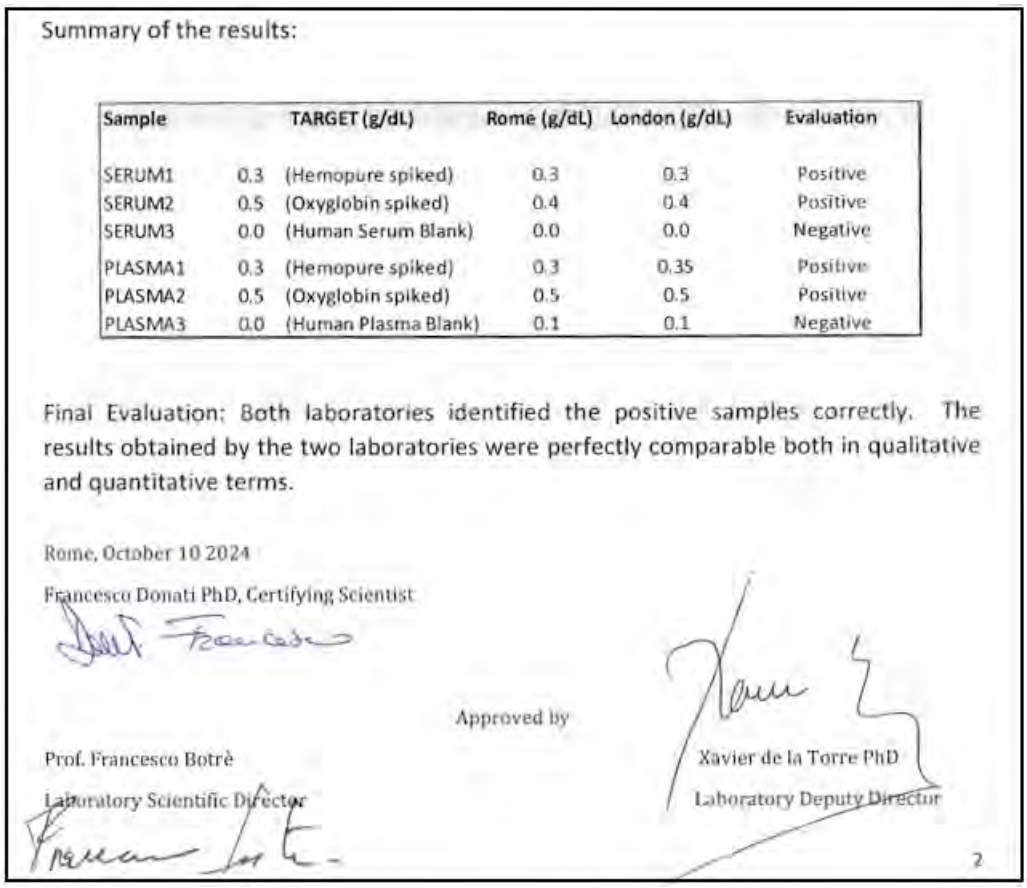

Summary of the results:

| Sample | | TARGET (g/dL) | Rome (g/dL) | London (g/dL) | Evaluation |
|---|---|---|---|---|---|
| SERUM1 | 0.3 | (Hemopure spiked) | 0.3 | 0.3 | Positive |
| SERUM2 | 0.5 | (Oxyglobin spiked) | 0.4 | 0.4 | Positive |
| SERUM3 | 0.0 | (Human Serum Blank) | 0.0 | 0.0 | Negative |
| PLASMA1 | 0.3 | (Hemopure spiked) | 0.3 | 0.35 | Positive |
| PLASMA2 | 0.5 | (Oxyglobin spiked) | 0.5 | 0.5 | Positive |
| PLASMA3 | 0.0 | (Human Plasma Blank) | 0.1 | 0.1 | Negative |

Final Evaluation: Both laboratories identified the positive samples correctly. The results obtained by the two laboratories were perfectly comparable both in qualitative and quantitative terms.

Rome, October 10 2024

Francesco Donati PhD, Certifying Scientist

Approved by

Prof. Francesco Botrè
Laboratory Scientific Director

Xavier de la Torre PhD
Laboratory Deputy Director

2

**Figure 2.** Inter-laboratory study summary of results

## Conclusions

The findings of this study collectively demonstrate the analytical validity and operational robustness of using the Sysmex XN-1000 for the detection of free haemoglobin (HGB) in serum and plasma, with direct application in the initial screening for haemoglobin-based oxygen carrier (HBOC) misuse.

- **Selectivity assessments** confirmed that under normal physiological conditions, free haemoglobin concentrations in serum and plasma remain low, with minimal variability. These results reflect the expected confinement of haemoglobin within erythrocytes and provide a reliable reference range for identifying abnormal elevations potentially associated with HBOC administration.
- **Baseline measurements from freshly processed samples** demonstrated consistently low free HGB concentrations, reaffirming that minimal haemolysis occurs when samples are handled and analysed promptly.
- **Room temperature storage** for seven days significantly increased haemolysis, particularly in plasma samples, whereas **refrigerated storage** mitigated but did not fully prevent this effect. Notably, plasma exhibited greater susceptibility to haemolysis-induced artefacts compared to serum, emphasising the importance of matrix selection and storage conditions in pre-analytical workflows.

- The haemolysis data support the adoption of a free HGB threshold of ≥ 0.3 g/dL as a Presumptive Adverse Analytical Finding (PAAF). This cut-off effectively distinguishes between natural haemolysis and potential HBOC use, providing a scientifically defensible trigger for confirmatory testing.
- The **l**inearity study confirmed the ability of the Sysmex XN-1000 to detect a wide range of bovine haemoglobin concentrations in both matrices with high reproducibility and no observable carryover. These results validate the system's suitability for quantitative screening in anti-doping applications.
- Routine haemolysis monitoring of athlete samples revealed that while visual haemolysis was observed in approximately 29.6% of samples within three days of receipt, corresponding free HGB concentrations remained well below the PAAF threshold. This demonstrates that incidental haemolysis during transport or handling does not typically interfere with the interpretation of results in the context of HBOC detection.
- The inter-laboratory study showed that the results obtained by the two laboratories were perfectly comparable both in qualitative and quantitative terms.

In summary, the data support the implementation of a Sysmex XN-1000-based Initial Testing Procedure (ITP) for HBOC screening. The proposed threshold of ≥ 0.3 g/dL offers high specificity, with limited risk of false-positive findings due to endogenous haemolysis, thereby contributing to a more reliable anti-doping testing framework for the detection of HBOCs in human Serum and Plasma.

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# Application of miRNA analysis in the detection of hemolysis of blood samples subjected to doping control

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## Abstract

We studied the variation in the expression of specific human miRNAs following red blood cells hemolysis with the aim of developing a strategy to distinguish a physiological hemolysis from the interfering plasma discoloration resulting from the illicit use of hemoglobin-based blood substitutes (HBOCs). Two subsets of plasma samples corresponding to different degrees of hemolysis were analyzed. Total RNA was extracted from plasma, quantified, and converted to complementary DNA (cDNA) using specific primers for the following red blood cells specific miRNAs (mi27a, mi210, mi451, mi16). The change in miRNA expression was quantified using two different approaches: i) real-time qPCR and Endpoint digital PCR. We observed that the expression of mi451, mi16 and mi210 increases progressively with the level of hemolysis and that these markers seem to be sensitive enough to detect samples even with mild hemolysis (approximately 0.2 g /dL of free hemoglobin). We concluded that the application of miRNA analysis can be a complementary test to effectively distinguish between natural hemolysis and the use of prohibited substances, to support decision for proceed to confirmatory analysis of suspected or false positive samples for HBOCs abuse.

## Introduction

Blood hemolysis, described as the breakdown or damage of erythrocytes is accompanied by a discoloration of the plasma which progressively turns from yellow to red in relation to the quantity of Hemoglobin released. Hemolysis is a particularly troublesome interfering factor during the antidoping screening analysis for the detection of Hemoglobin-Based Oxygen Carriers abuse. Current anti-doping screening procedures, based on a spectrophotometric approach, can suffer from this interference. An interesting and innovative approach to detect a real hemolysis can be based on the analysis of microRNA content within human serum or plasma. We studied the variation in plasma of some specific miRNAs of red blood cells following hemolysis to verify the possibility of developing an appropriate strategy to identify an in-vitro hemolysis in a blood sample.

## Experimental

HBOCs screening method involves the spectrophotometric analysis of a small quantity of serum or plasma (2-3 µL). The reading is performed at 415 nm corresponding to the maximum absorption wave-length. An appropriate threshold was selected as a decision limit to establish the need to perform a confirmatory analysis for a suspect sample. This screening strategy is very sensitive, nevertheless it suffers from possible interference due to in-vitro hemolysis event.

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RBC-derived miRNAs represent most of the miRNAs content in whole blood. miRNAs in RBC are the residue of miRNAs present within red blood cells during their immature phases and may participate in gene expression regulation through translational repression. We selected 4 mature RBC-specific miRNAs (miR16, miR27a, miR210, miR451) known to be among the most abundant and which are therefore more likely to be detected in circulation after RBC hemolysis.

Within a non-hemolyzed physiological blood sample, HGB and RBC's microRNAs are found within the intact Red Blood Cells. The resulting plasma is yellow in color and contains minimal quantities of free HGB and miRNAs. As hemolysis occurs, the integrity of the RBCs breaks down. The plasma is red in color due to the presence of free HGB and, depending on the degree of hemolysis, contains increasing quantities of miRNAs specific to red blood cells.

12 blood samples with absent or minimal hemolysis (below 0.1 g/dL free HGB), 12 blood samples with mild (moderate) hemolysis (approximately 0.3 g/dL free HGB) and as many samples with severe hemolysis (approximately 1 g/dL free HGB) were subjected to analysis. Small Nucleolar RNU48 was used as housekeeping normalizer gene for qPCR based on Delta-Delta Ct relative quantification method.

## Results and Discussion

A progressive decrease in the average amount of total RNA extracted was observed with the increasing degree of hemolysis. This can be indicative of RNA degradation occurred during the period between extraction times, thus reducing its quantity. However, it should also be considered that during hemolysis, cellular contents spill into the plasma fraction or may interfere with the total RNA extraction process. Nevertheless, the purity ratios showed a constant trend over time.

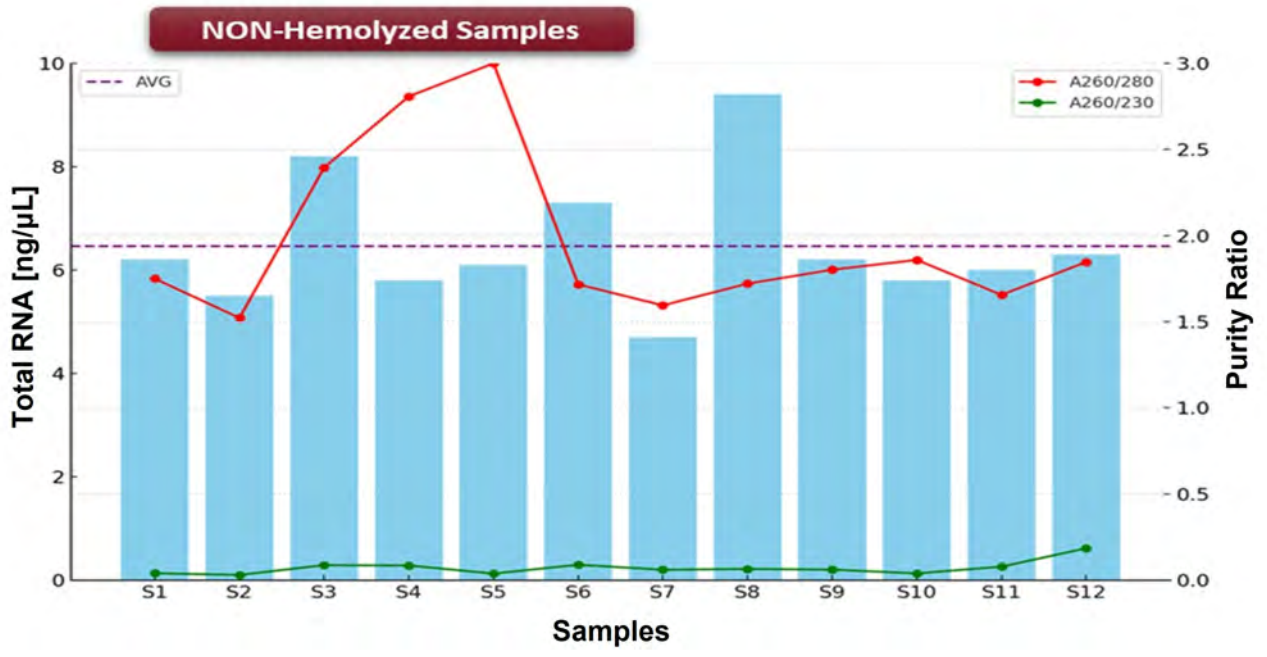


**Figure 1**: Total RNA content extracted from 12 non-hemolyzed plasma samples and their degree of purity

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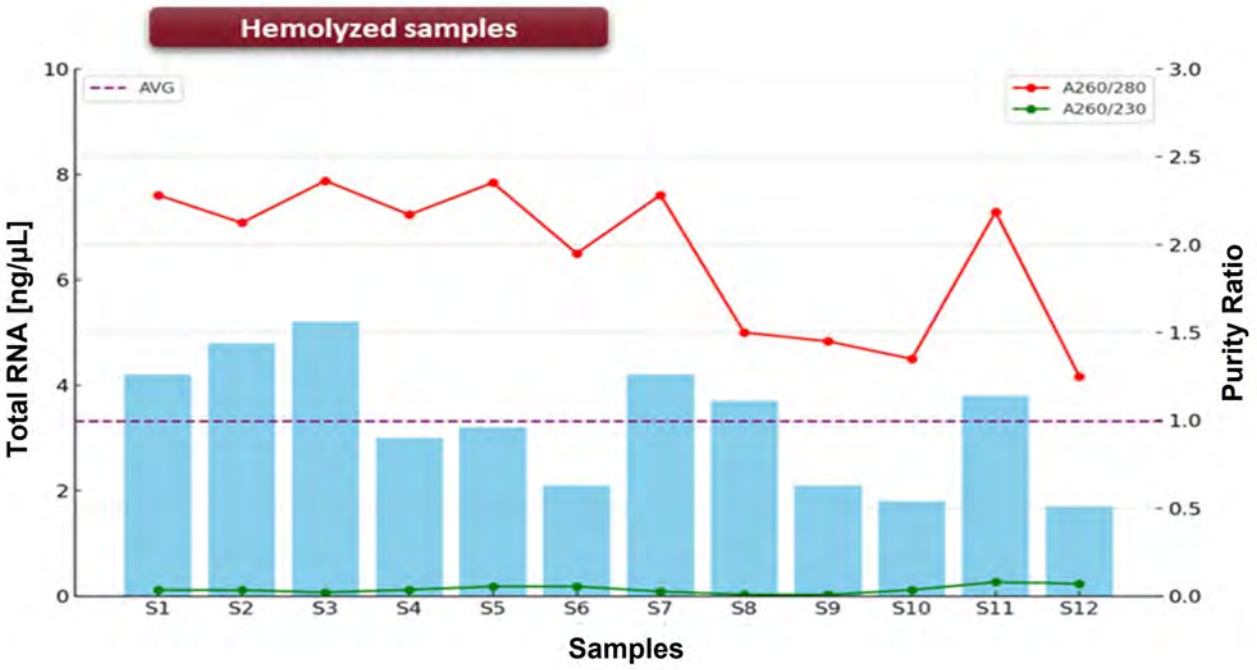


**Figure 2**: Total RNA content extracted from 12 hemolyzed plasma samples and their degree of purity

An increase in the quantity of miRNAs in severe hemolyzed plasma is highlighted compared to non-hemolyzed samples. This is demonstrated by a statistically significant reduction in the Cycle Threshold (CT) of miR210, miR451 and miR16. As expected, RNU48 used as an endogenous control was stable. However, miR27a is not affected by severe hemolysis. No statistically significant differences were detected between the CTs of samples with moderate hemolysis compared to samples without hemolysis (data not shown).

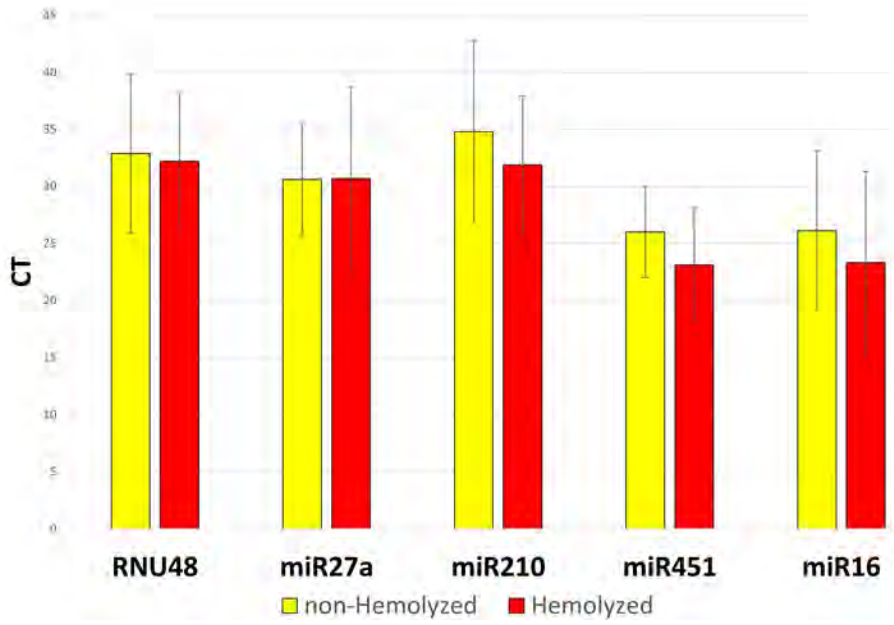


**Figure 3.** Real Time qPCR: hemolyzed vs. non-hemolyzed samples

The analysis performed with the Endpoint Digital PCR confirms the qPCR data but with higher analytical sensitivity. Here, the variations in the quantity of miRNAs are clearly detectable in both samples with severe and moderate hemolysis compared to samples with absence of hemolysis. The differences detected between these 3 degrees of hemolysis are much more evident and marked for miR16 and miR451 which are therefore more ideal markers for the very sensitive identification of even weak degrees of hemolysis.

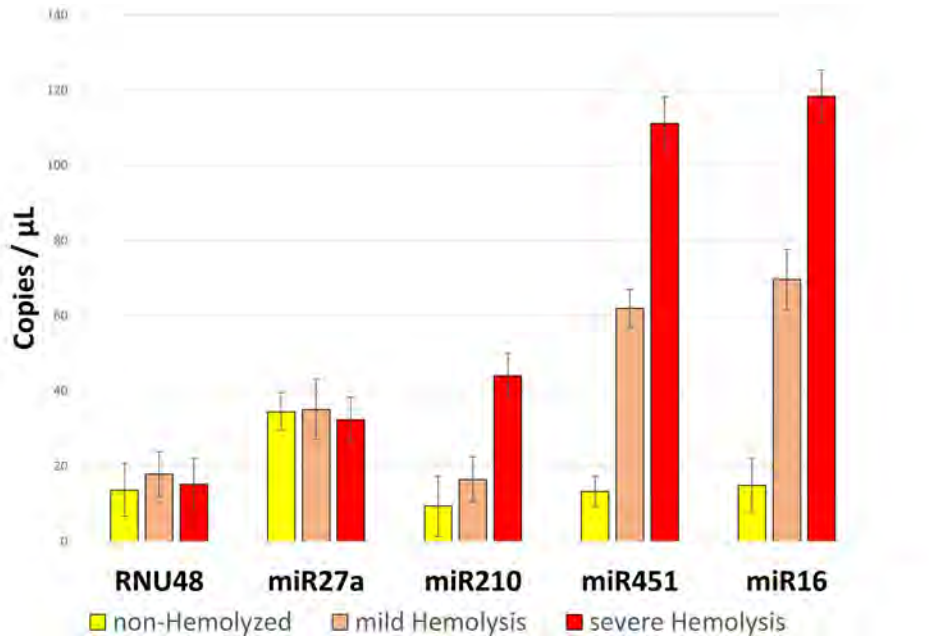


**Figure 4.** Endpoint digital PCR: hemolyzed vs. non-hemolyzed samples

## Conclusions

- miR-451, miR-16, and miR-210 can be used as sensitive markers of an hemolyzed sample.
- Due to its high sensitivity and its ability to perform absolute quantification, the use of digital PCR is ideal both because it allows the detection of even samples with mild hemolysis and because it would allow the application of discrimination thresholds.
- This approach allows the identification of a true hemolysis and therefore can be of help in the attempt to discriminate the samples that should be considered truly suspicious for HBOC abuse. In any case, some interfering phenomena (such as hemolysis due to sample transport or bad storage) must be taken into consideration before giving a final opinion on the sample under examination.
- The inclusion of a greater number of miRNAs in the analysis panel will improve both the specificity and sensitivity of this analytical approach.

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# Detection of phosphatidylserine externalization on red blood cell membrane: A sensitive biomarker to detect blood transfusion abuse in sport doping

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## Abstract

The aim of this work was to analyze the externalization of phosphatidylserine (PS) on the membrane of red blood cells (RBC) and verify whether this strategy can be applied for the development of a direct method for the detection of the abuse of blood transfusions in sports doping. PS is a phospholipid that, under physiological conditions, is expressed on the internal side of the RBC's plasma membrane. PS translocation from the inner to the outer leaflet of the membrane occurs in case of cell damage, senescence and/or apoptosis and constitutes a phagocytosis signal for macrophages which have the task of removing senescent RBC from the bloodstream by phagocytosis through the reticuloendothelial system. We have analyzed by flow cytofluorimetry 10 whole blood samples:
i) 10 blood samples within 24 hours of collection (t=0),
ii) after 40 days of storage simulating blood banking conditions (addition of Saline-Adenine-Glucose-Mannitol (SAGM) solution in a 1:5 ratio and storage between 2-8 °C) and
iii) after a simulation of a blood transfusion using 10% spiked t=0 blood in a new fresh blood sample.

We found that the variation in the relative percentages of different RBC populations (mature RBC, Intermediate RBC and RBC microvesicles) in association with the counting of senescent cellular events showing externalization of phosphatidylserine, allows the identification of blood cells that underwent a preservation process for the purpose of reinfusion both in the stored samples and in the transfused samples. This analytical strategy seems very promising for the development of a method that can detect the abuse of both homologous and autologous transfusions in sports doping.

## Introduction

During blood transfusion, blood is taken from a donor to be stored refrigerated in a blood bag mixed with preservatives. During the storage period, red blood cells (RBC) undergo a series of morphological changes that induce their senescence (Figure 1). Phosphatidylserine (PS) is detectable during the process of cellular senescence. PS is a membrane phospholipid physiologically is found on the internal surface of the RBC membrane. Translocation of PS from the internal surface to the outer leaflet of RBC occurs in the event of senescence. We developed a flow cytometry protocol for the detection of senescent red blood cells by staining the externalized PS to verify the ability to detect senescent RBC in samples in which an erythrocyte transfusion was simulated.

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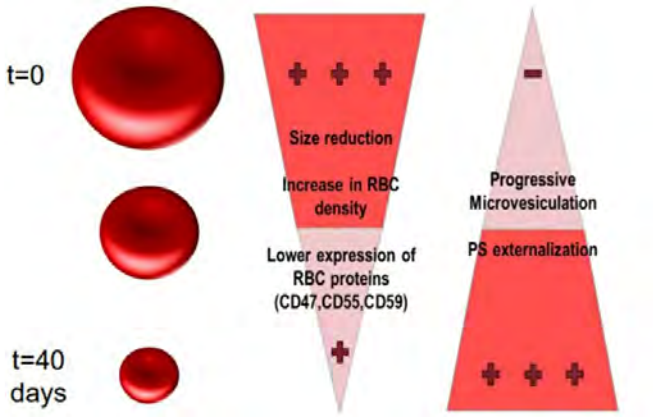


**Figure 1.** Red Blood Cells' morphological and biochemical changes during a 40 days storage period

## Experimental

10 whole blood (WB) samples were added with Saline-Adenine Glucose Mannitol (SAGM) preservative solution in a 1:5 SAGM:Whole Blood ratio and stored refrigerated simulating blood bank condition (40 days, 4°C). At the end of the storage period, ex-vivo transfusions were performed on new fresh blood samples by using the preserved portion of blood as a 10% donor. All sample aliquots were then subjected to antibodies staining and FACS analysis targeting the externalized PS according to the following procedure: 100 µL of blood containing 1 million RBC were added to 10 µL anti human Glycophorin-PE antibody (Beckman Coulter) and to 10 µL of AnnexinV-FITC antibody (Invitrogen). All antibodies have been previously titrated to use them at best performance. After incubation of 15±5 minutes at room temperature in mild agitation, RBC were washed with 100 µL of PBS. RBC were centrifuged at 1000*g* for 10 minutes and, after removal of the supernatant, the RBC pellet was recovered in a final volume of 200 µL of PBS. FACS analysis were conducted at Flow 15 µL/second and a minimum of 15000 events were collected each sample.

## Results and Discussion

Both a morphological and immunological gating strategy were applied to identify and count only CD235a positive events (corresponding to Red Blood Cells) while excluding cellular aggregates and the interfering CD235a negative events. Red Blood Cells subpopulation (mature, intermediate and Red Blood Cells-derived Microvesicles, RBC-MPs) were identified on the Forward Scatter detector after calibration with fluorescent microspheres with known diameter ranging from 0.5 to 9 µm in size (Megamix-Plus Biocytex). These spheres span the size range expected for both mature and RBC-MPs.

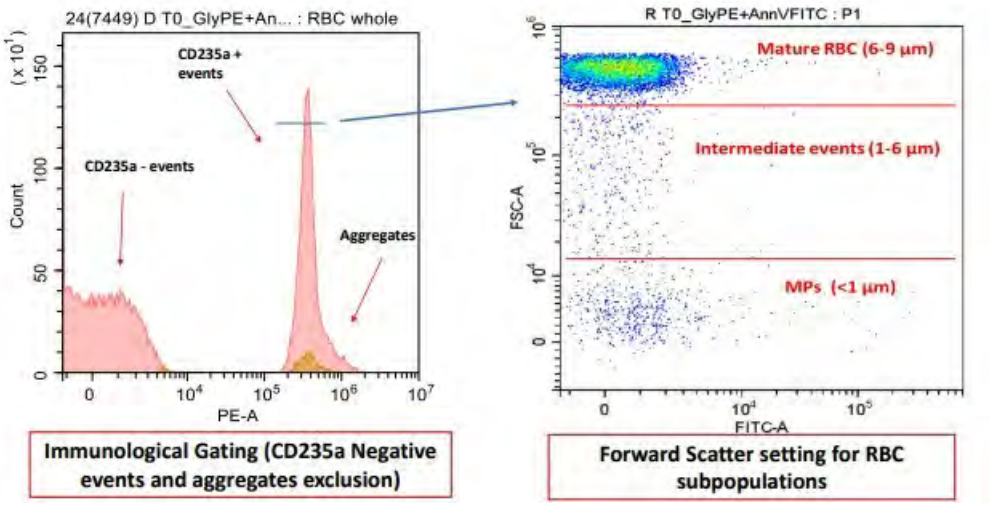


**Figure 2.** Flow Cytometry identification of Red Blood Cells subpopulations. CD235a positive events were separated according to their size on Forward Scatter channel so to distinguish mature RBC, Intermediate Sized events and RBC-MPs

FACS analysis of the size of the RBCs, have highlighted three main changes as a consequence of 40 days storage. Firstly, a decrease of mature RBC relative content (from 93 to 42%) and a contemporary increase of intermediate events (from 2 to 10%). Secondly, a remarkable increase of events related to RBC-MPs subpopulation (from 5 to 48%). Lastly, in the mixed samples, the percentage difference of RBC subpopulations, compared to the samples at t=0, is statistically significant for RBC-MPs events (p value $< 0.05$ calculated by conventional t test).

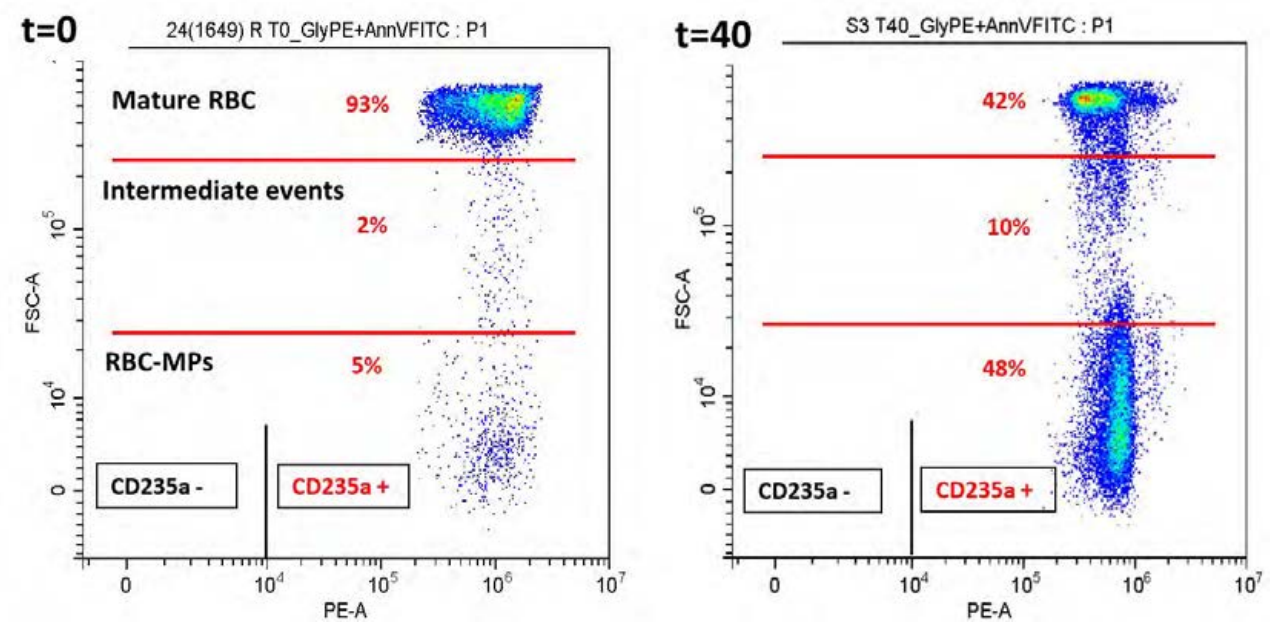


**Figure 3.** Changes of relative percentages of Red Blood Cells subpopulations after 40 days of storage period (t=40) compared to fresh whole blood (t=0)

At the same time, the analysis of externalized phosphatidylserine showed that first of all there is a an increase of externalized PS positive events at t=40 compared to t=0 in each of the RBC subpopulations (mature, intermediate and RBC-MPs) as well as in the count of total events taken together. Furthermore, a significant statistical difference (p value < 0.5) were detected for all RBC subpopulations in the number of externalized PS events in the 10% mixed samples after 40 days of storage.

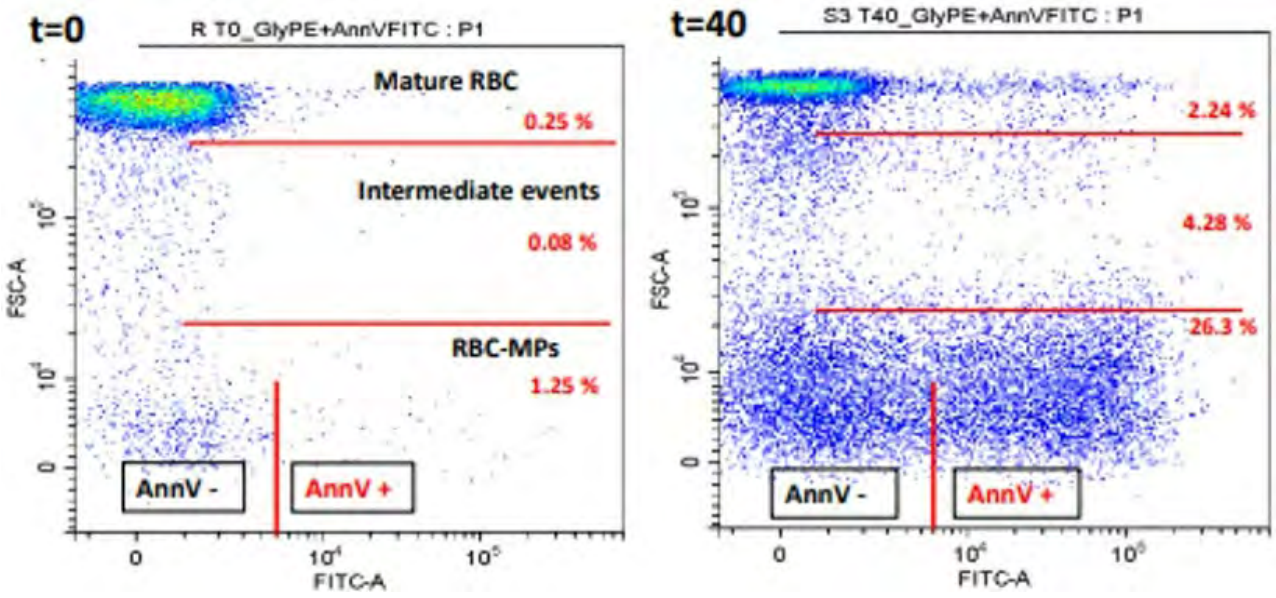


**Figure 4.** Flow Cytometry Phosphatidylserine detection in mature Red Blood Cells and Microparticles-derived Red Blood Cells. As explained in the text, the relative percentage of Annexin V-positive events increases significantly after a 40-day storage period. The most significant increase is observed in the RBC-MPs population

## Conclusions

1. The variation in the relative percentages of RBC subpopulations, together with the counting of senescent cellular events showing externalization of phosphatidylserine, allows the identification of RBC that have undergone a preservation process for the purpose of reinfusion even in the mixed sample.
2. PS is a sensitive and irreversible marker that could be used to implement a detection method for both ABT and HBT. PS exposure is a key signal for cellular and red blood cell senescence. Although apoptosis (or rather, erythroptosis) is the primary driver, the externalization can occur in various physiological and/or pathological conditions (such as senescence or physiological aging, or in the case of pathological hemolytic anemia). Moreover, it cannot be excluded that cellular damage during blood storage and/or transport may have been a source of interference, having caused PS staining also internally. In any case, the externalization serves as a cellular activation signal that triggers the removal of senescent RBC from the cellular circulation by macrophages. We focused on developing a staining procedure for the accurate identification of PS externalization using FACS. Determining the difference between physiological and pathological externalization or externalization caused by improper specimen storage or transport was not our goal at this stage.

3. This experimental approach could be applicable to any type of mixed samples which involves the use of erythrocytes preserved in the typical blood bank conditions (2-8 °C with preservative solutions). Flow cytometry allows to standardize sample preparation and, most of all, the analytical conditions so to guarantee high inter-laboratory reproducibility.
4. Realistic “Thresholds of Suspicion” and “Windows of Detection” as well as other confounding factors need to be determined using real transfused samples.

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Yan K, Liu L, Dong T, Wang Z, Zhang L

# Co-eluting interference tentatively identified during blood steroid profile analysis

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## Abstract

The implementation of the endogenous blood steroidal profile is an important update of World Anti-Doping Agency (WADA)'s Athlete Biological Passport modules. It was reported much sensitive to individuals carrying dis-functioning UGT2B17 genotype, especially in females. Two of the parameters, namely testosterone (T) and androstenedione (A4), are quantified using a liquid chromatograph mass spectrometry technique, with their concentration ratio (T/A4) calculated.

Beijing Anti-Doping Laboratory developed and validated a protein precipitation followed by UPLC-HRMS method for this purpose. During the very first analytical batches of application, a co-elution interference to T at the same retention time with molecular weight difference of only 0.0365 were observed. It causes overestimated concentration of T which resulted in elevated T/A4 for some of the athletes' profile.

This poster described our investigation where the source of this co-elution was tentatively identified in the separating gel of the blood collection tube (BD Vacutainer® SST™-II), the re-validation of the method with optimized chromatographic condition, and a comparison of different sample pre-treatments. It was also deduced that this interference might be less detected after freeze-thaw circles.

In conclusion, this incident reminded us that method validation should be designed with conditions simulating actual routine practice as much as possible, specifically in this case, fresh blood should be taken into account beyond stored samples.

## Introduction

The implementation of the endogenous blood steroidal profile is an important update of WADA's Athlete Biological Passport modules. Two of the parameters, namely testosterone (T) and androstenedione (A4), are quantified using a liquid chromatography mass spectrometry technique, with their concentration ratio (T/A4) calculated [1-3]. Beijing Anti-Doping Laboratory developed and validated a protein precipitation followed by UPLC-HRMS method for this purpose. During the very first analytical batches of application, an unexpected co-eluting interference to quantification Ion of testosterone was observed with m/z difference of 0.0365 Da. It causes overestimated concentration of T which resulted in elevated T/A4 for some of the athletes' profile. This paper describes our investigation and corrective action to solve this situation.

## Experimental

### Optimization of chromatographic condition and mass spectrometry data

It was observed that the ratio between the quantitative ion (*m/z* 109.0645) and qualitative ion

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(*m/z* 97.0645) does not match the QC during the ITP. LC condition of the method was firstly modified to separate the interference as shown in Table 1.

| UPLC | | | | MSD | |
|---|---|---|---|---|---|
| **Type** | UltiMate 3000 | | | Thermo Q-Exactive Plus | |
| **Mobile phase** | A: 10 mM aqueous ammonium formate<br>B: Methanol | | | **Acquisition mode** | Full Scan<br>PRM |
| **Column** | Thermo Hypersil GOLD<br>2.1×100mm, 1.9μm | | | **Resolution** | 17500<br>35000 |
| | | | | **AGC target** | $1e^6$ |
| **Flow-rate** | 0.25 ml/min ⟹ **0.26 ml/min** | | | **NCE** | 35 eV |
| | | | | **Isolation widow** | 1.0 m/z |
| **Injection** | 15 μL | | | | |
| **Gradient elution** | 0 -11 min | 50% - 80%B | 0 -11 min **50% - 65%**B | **Quantification Ion** | T: 289.2160 – 109.0645 |
| | 11-14min | 80% - 100%B ⟹ | 11-14min **65% - 100%**B | | |
| | 14-18min | 50%B | 14-18min 50%B | | |
| **Retention Time OF Testosterone** | 8.2 min ⟹ **10.8 min** | | | | |

**Table 1.** Optimization of LC condition and mass spectrometry parameters

Meanwhile, we also analyzed the mass spectral data of the full-scan and diagnostic ions of the sample for co-elution interference by using a high resolution mass spectrometer (Thermo Q-Exactive Plus). The mass spectrometry parameters are also shown in Table 1.

**Re-validation of the method with freshly collected sample**

By reviewing data, the observation were made:The samples which showed significant interfering responses relative to other normal samples were fresher serum that had been stored for a very short time, and The serum matrix used in method validation were either depleted by Charcoal, or anonymized athletes' samples store for a long time.

Therefore, it was suspected that, this interference might only be observed in freshly collected blood sample. Hence, a partial validation of selectivity with freshly collected blood (n=13) was proceeded. After that all 7 samples in question were then confirmed with both the "old" method and the optimized method.

**Identification of the source of the interference**

Contamination to reagents, consumables and laboratory equipment were excluded. As mentioned above, it was presumed that this interference is suspected to be degraded/removed by storage time and number of freeze/thaw circles. Hence, A suspicion was made that it came from the blood collection tube (BD Vacutainer® SSTTM-II). Therefore, a small portion of separation gel was taken out of a new BD serum tube, tested in combination with 50 μL of Quality Control sample.

In addition, we choose an alternative pretreatment method of solid phase extraction (SPE) described in literature [1] to verify if the interference can be removed.

## Results and Discussion

**The result of re-validation with optimization method**

Compared to “old” method, the new method that optimizes liquid phase conditions successfully separates target compound from interfering substance. IDCR criteria were met for testosterone, and an interference peak is well separated in all samples (n=13) as shown in Figure 1A. Figures 1B and 1C illustrated the impact on the results of aliquot before and after optimization.

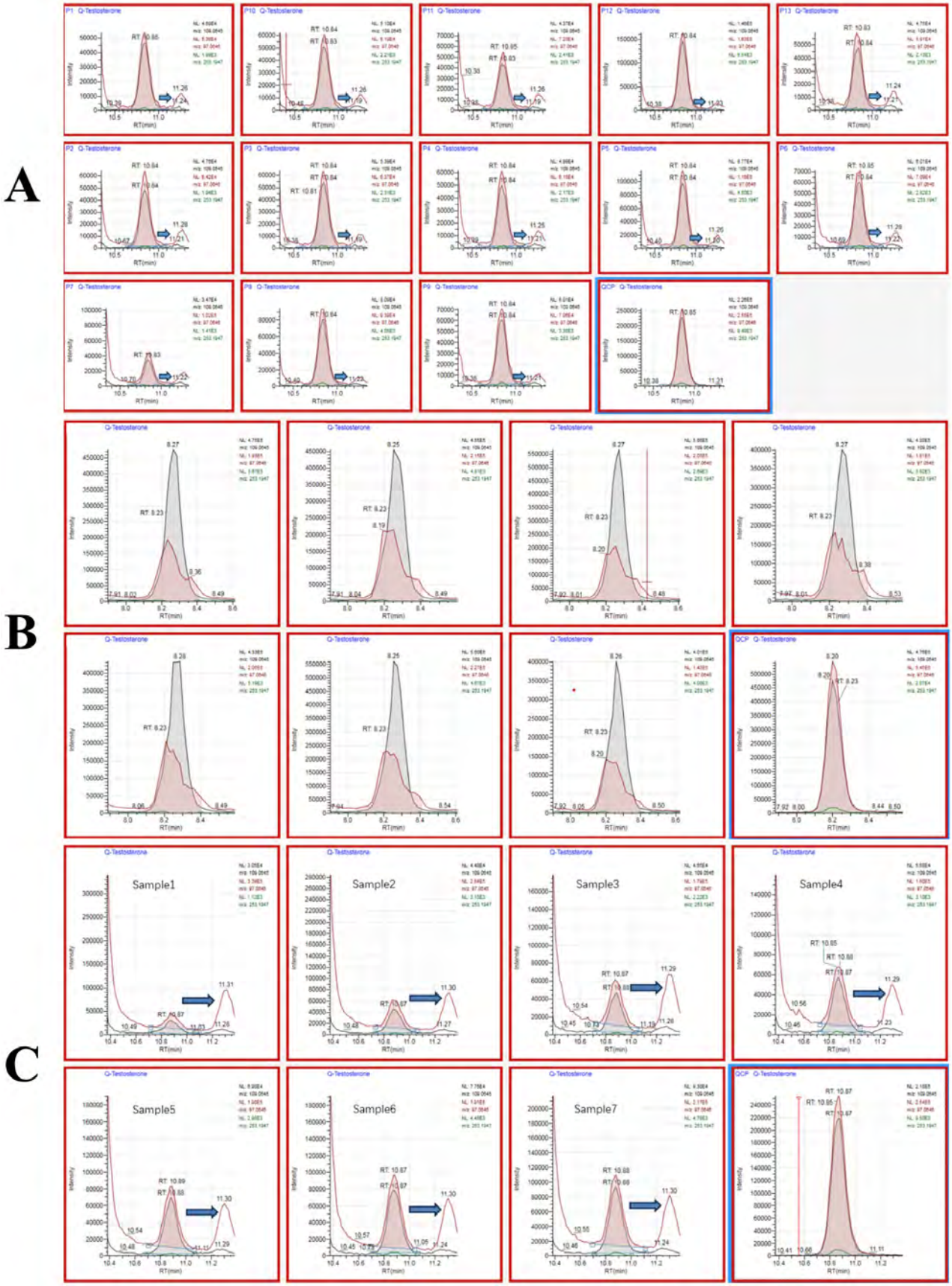


**Figure 1.** Chromatographic data of freshly serum for selectivity (**A**), the results of aliquot by original method (**B**) and optimization method (**C**)

**Mass spectrometry data**

The mass spectrometry data for testosterone and the interfering substance were further analyzed. The results showed that the precursor ion of testosterone *m/z* 289.2160 (retention time: 10.85 min), while that of the interference was *m/z* 289.2005 (retention time: 11.30 min). The quantitative ion was *m/z* 109.0645 for testosterone and *m/z* 109.1010 for the interference. The qualitative ion was *m/z* 97.0645 for testosterone and *m/z* 97.1010 for the interference, with a minimal difference of only 0.0365 Da. Based on these observations, the mass spectrometry information of compounds was tentatively identified as shown in Figure 2.

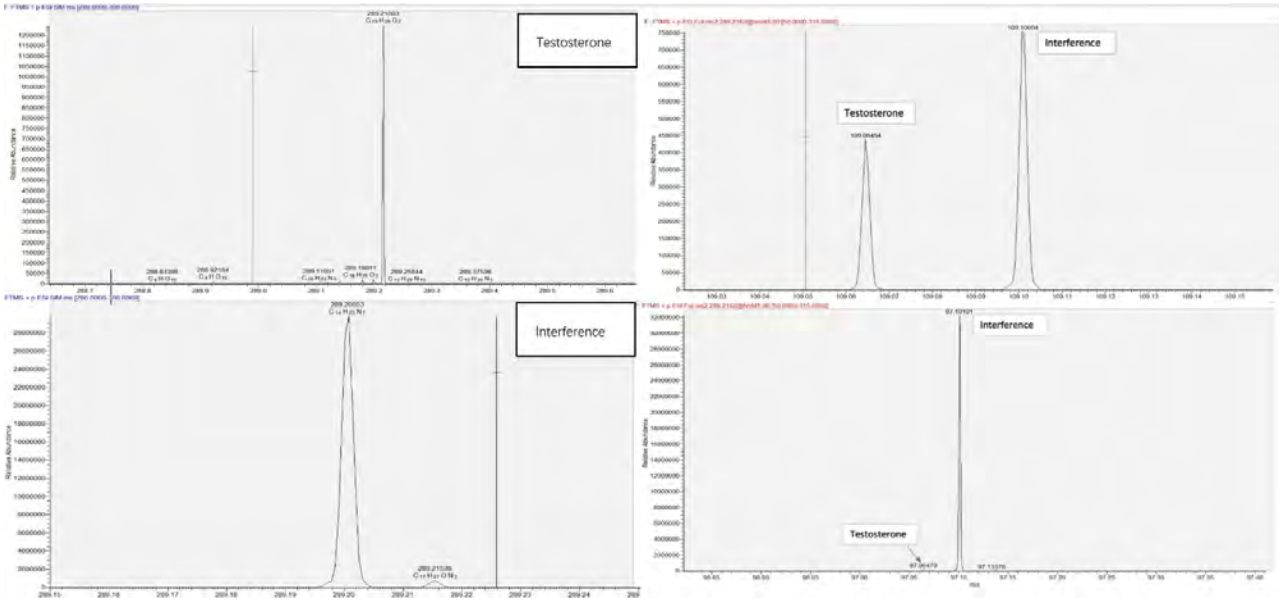


**Figure 2.** The Mass spectrometry information of testosterone and interference

In mass spectrometry analysis, we attempted to separate target compounds from interferences using high-resolution mass spectrometry (maximum resolution 70,000). As shown in Figure 2, while the two could be sufficiently separated at the mass spectrum level, their retention times were extremely close and their precursor ion masses were similar, the instrument minimum isolation window was 0.4 *m/z*, which is significantly greater than the difference between the precursor ions of the two substances (0.015 m/z). Consequently, they were still collected together during the ion acquisition phase, making it impossible to completely distinguish them at the quantification data. Even employing PRM mode and setting an exclusion list to filter out interference parent ions proved ineffective. Their product ions closely resemble those of the target compound, while the target-specific product ion (*m/z* 234) exhibits weak response, rendering it unsuitable for quantification. Ultimately, repeated experiments demonstrated that baseline separation achieved solely through optimized liquid chromatography conditions is the fundamental solution to this interference issue. As shown in Figures 1A and 1C, although only the target quantification ion *m/z* 289.2005-109.0645 was put into the method, and the optimized method already achieves baseline separation of the interferent and target compound by chromatographic, the instrument still displays the acquisition of the interferent ion *m/z* 289.2160-109.1010 due to inability to resolve it. This further corroborates the aforementioned situation.

**The result of the Confirmation Procedure with optimization method**

It is observed that the interference is well separated in CP by the optimized method as shown in Figure 1B. Afterwards, we repeated the experiment on these samples again. It is then observed that, after 20 days of storage and one more freeze/thaw circle, testosterone concentration significantly decreased even with the old method.

**The source of the interference**

The result shows that quantitative ion of *m/z* 109.0645 is enhanced (97.0645 showing shoulder peak and less peak height) in the old method, and the new method mitigating the interference, using *m/z* 109.0645 as quantitative ion is fit-for-purpose.

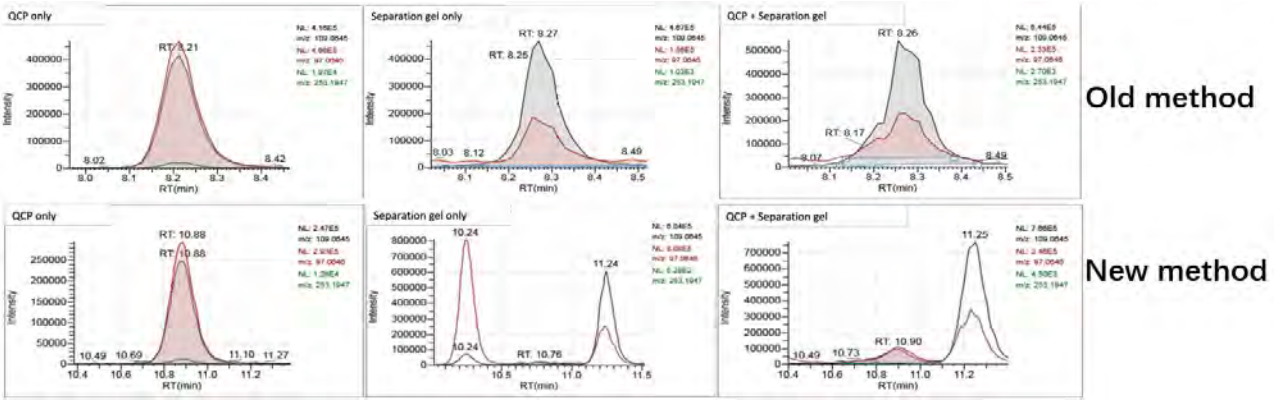


**Figure 3.** Chromatographic data of identification experiment

Hence, the source of this interference is tentatively identified from the separation gel of BD Vacutainer ® SSTTM-II serum collection tube is very possible. Notably, the use of SPE as a pretreatment method does not eliminate this interference either.

## Conclusions

An interfering substance, potentially derived from blood collection tube separation gels, was found in seven samples and was found to degrade with storage or repeated freezing and thawing. An optimized liquid-phase gradient separates testosterone from the baseline of the interfering substance so that it no longer interferes with the quantification of testosterone concentrations.

This incident reminded us that Method Validation should be designed with conditions simulating actual routine practice as much as possible, specifically in this case, fresh blood should be taken into account beyond stored samples.

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Momobayashi A, Akiyama K, Okano M

# Detection of homologous blood transfusion doping by targeting major histocompatibility antigens

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## Abstract

Homologous blood transfusion (HBT) doping is typically determined by analyzing population distributions of red blood cell (RBC) antigens via flow cytometry. The detection of double populations (DPs) of RBC antigens indicate the presence of transfused donor RBCs. In current initial testing a DP triggers a confirmation step that reanalyzes the same antigen using altered antibody concentrations; however, underlying biological marker remains unchanged, limiting orthogonality. To address this, we explored a confirmation strategy that targets histocompatibility antigens expressed on other blood cells. Our rationale is that whole blood transfusion introduces exogenous white blood cells (WBCs) and platelets (PLTs), and even packed RBC transfusions can leave residual donor PLTs present in circulation. We therefore investigated an in vitro MHC (HLA)-based approach using flow cytometry on WBCs and PLTs. Within individuals, HLA reactivity patterns were concordant between WBCs and PLTs , and negative versus positive populations were well separated across multiple specificities. In mixtures designed to mimic matched-RBC transfusion (95:5 combination of two samples with identical RBC antigen profiles), conventional RBC antigen analysis showed no DP, whereas platelet HLA analysis revealed clear DPs for three HLA specificities, supporting its value as a complementary marker when RBC phenotypes are matched. Sensitivity titrations indicated that platelet HLA analysis detects DPs at donor fractions of approximately 1%, with signals approaching the visual detection limit at 0.1%. These findings support PLTs-based HLA analysis as a practical adjunct for HBT detection and confirmation.

## Introduction

Homologous blood transfusion (HBT) analysis in anti-doping laboratories typically targets red blood cell (RBC) blood group antigens. Transfused cells are inferred by population analysis of RBC antigen expression, where the presence of a double population (DP) indicates HBT. Here, we assess an alternative strategy that shifts the analytical target from RBC blood group antigens to Major Histocompatibility Complex (MHC) antigens. Our premise is that whole-blood transfusion introduces exogenous WBCs and PLTs, whereas transfusion of packed RBCs may still introduce a small number of donor-derived PLTs into circulation. We therefore investigate whether exogenous WBCs and/or PLTs can serve as additional of complementary indicators of HBT, detectable via their MHC profiles.

## Experimental

### Materials

Samples: Peripheral whole blood collected in EDTA-2K tubes from in-house volunteers who provided

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informed consent for use in this study.

RBC antigens reagents:

- Primary antibodies: anti-C, c, E, e, Jka, Jkb, Fya, Fyb, and S (BIO-RAD, CA, USA)
- Secondary antibodies: rabbit anti-human IgM FITC (Dako, Yishun, Singapore); goat anti-human IgG FITC, (Thermofisher, MA, USA).

MHC reagents:

- Primary antibodies: anti-HLA-A3, -A24/11/2403+, -A30/31, -B7/27/42+, -B44/75/17+, -B15/57, -B57/58 (One Lambda, CA,USA), anti-HLA-A2 and anti-HLA-B7 (Bio Legend, CA,USA); anti-HLA-A24 (LSBio, CA,USA).
- Secondary/labeling reagents: goat anti-mouse IgG+IgM PE (Jackson ImmunoResearch, PA,USA); PE Streptavidin (BD Biosciences, NJ, US).

**Instrumentation**

Flow cytometer: Navios EX (Beckman Coulter)

**RBC antigens Analysis**

RBCs were suspended in PBS and aliquoted into tubes. Cells were incubated with human IgM antibodies (anti-C, -c, -E, -e, -Jka, -Jkb) or human IgG antisera (anti-Fya, -Fyb, -S) following the optimal dilutions at room temperature for 90 minutes. After centrifugation and wash with PBS, secondary staining was performed with rabbit anti-human IgM FITC or goat anti-human IgG FITC. Cells were washed, resuspended in PBS, and analyzed by flow cytometry (FCM)[1].

**MHC Analysis (White blood cells)**

Whole blood (EDTA-2K) was aliquoted and incubated with anti-HLA antibodies. For directly fluorescent-labeled antibodies, erythrocyte lysis was performed post-staining using lysing reagent. For unlabeled/biotinylated primaries, samples were washed with PBS, incubated with goat anti-mouse IgG+IgM PE or PE-streptavidin as appropriate, then subjected to erythrocyte lysis. All samples were washed with PBS, resuspended, and analyzed by FCM [2]. WBCs were identified by scatter characteristics.

**MHC Analysis (Platelets)**

The Platelet-rich plasma (PRP) fraction was collected as the PLT sample. Antibody staining followed the same procedure as for WBCs, omitting erythrocyte lysis. After washed in PBS, PLT suspensions were analyzed by FCM. PLTs were gated by low forward/side scatter; and were examined the MHC expression by PE signal.

## Results and Discussion

**RBC antigens Analysis**

RBC antigen profiling was performed on four healthy individuals (Table 1). To model HBT In a scenario where donor and recipient RBC antigen profiles are fully matched, a mixed sample by combining Sample 1 and Sample 2 at a 95:5 ratio was prepared. Flow cytometric RBC antigen testing did not reveal a

double population (DP) in the 95:5 mixture (Figure 2a), consistent with the identical RBC antigen phenotypes of the components.

| | ABO | RBC antigen expression patterns (current methods) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | C | c | E | e | Jka | JKb | Fya | Fyb | S |
| Sample 1 | A | + | + | + | + | - | + | + | - | - |
| Sample 2 | A | + | + | + | + | - | + | + | - | - |
| Sample 3 | B | + | - | - | + | + | + | + | - | - |
| Sample 4 | O | + | - | - | + | + | - | + | - | - |

**Table 1.** RBC antigen expression patterns by current methods

**MHC Analysis**

Within each subject, HLA reactivity patterns were concordant between WBCs and PLTs, and negative *vs* positive populations were well separated for multiple specificities, yielding clear bimodality where applicable (Figure 1). These results indicate that several HLA targets provide sufficient dynamic range and resolution for DP detection in the context of HBT.

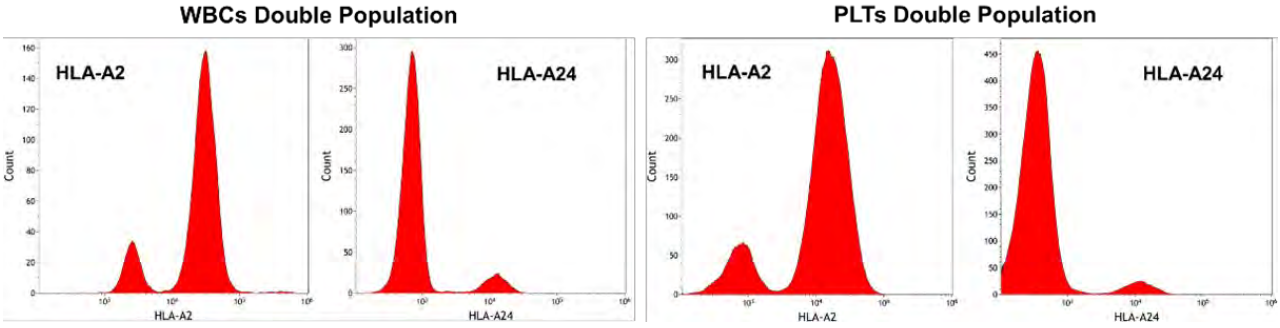


**Figure 1.** Bimodal HLA staining on WBCs and PLTs within individuals; clear separation of negative/positive for specified HLA targets

Despite this, WBC-based analysis can be complicated by subset heterogeneity. By contrast, PLTs lack such immunophenotypic subsets and show narrower scatter and fluorescence distributions, improving the detectability of minor exogenous populations by DP analysis.

**Comparison of RBC antigens Analysis and MHC analysis of PLTs**

We directly compared the RBC antigen method with platelet MHC analysis on the 95:5 mixture. RBC antigen analysis showed no DP (Figure 2a), reflecting the matched RBC phenotypes. In contrast, platelet MHC analysis revealed clear DPs in three HLA specificities (Figure 2b).

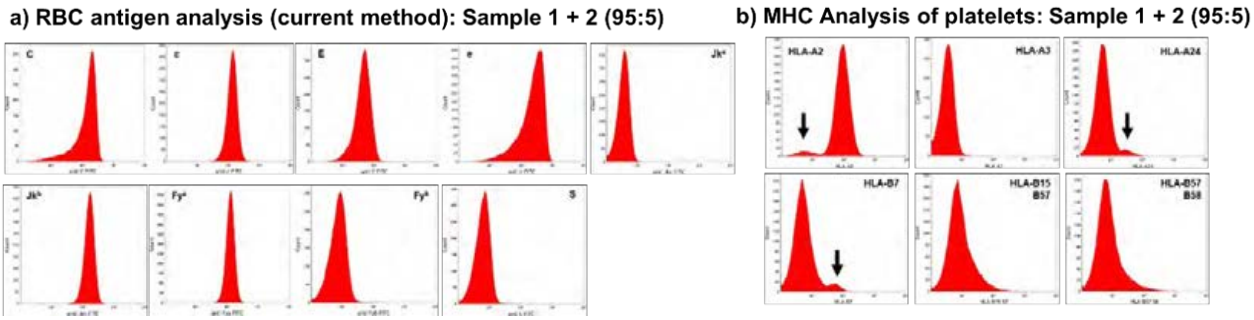


**Figure 2.** 95:5 matched-RBC mixture: (a) RBC antigen analysis shows no DP; (b) PLT HLA analysis shows DPs in three HLA specificities

**Limit of detection of platelet MHC analysis**

To estimate analytical sensitivity, we prepared titrations of HLA-A24-positive blood into HLA-A24-negative blood and assessed PLTs by MHC staining. A distinct DP was detected at 1% donor fraction, whereas DP was near the visual detection limit at 0.1% (Figure 3). These findings suggest a practical limit of detection near 1% for HLA-A24 with the current staining and acquisition settings, with potential variability across HLA specificities due to affinity and antigen density.

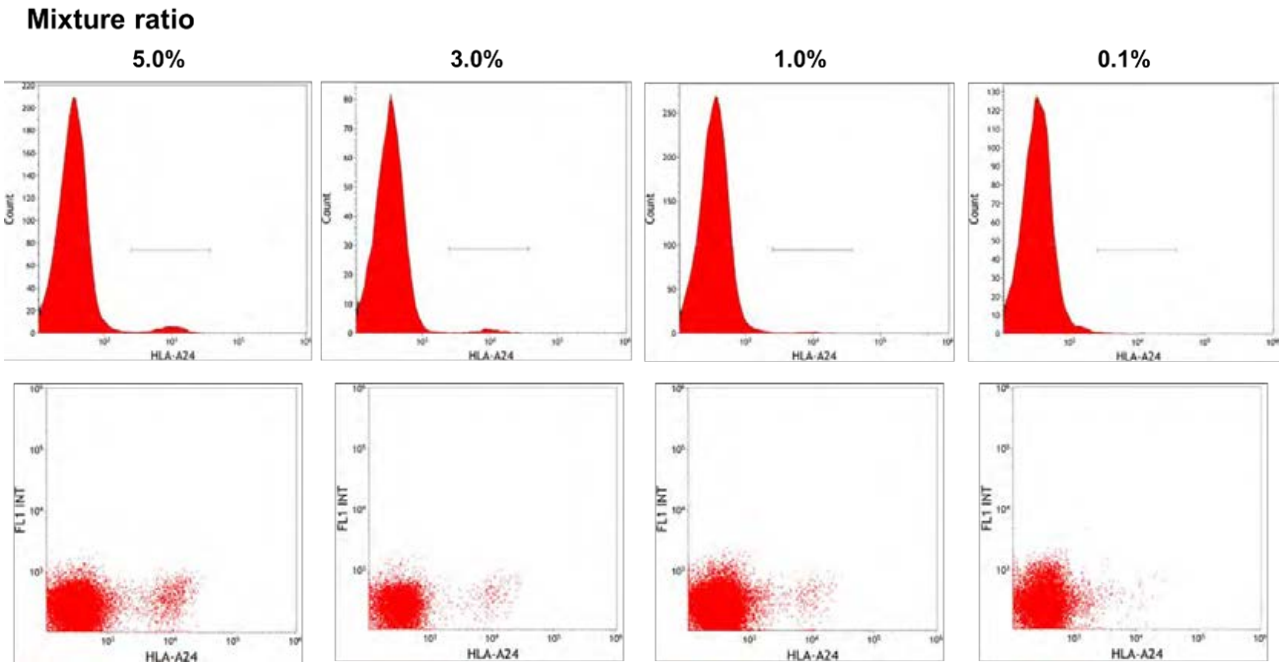


**Figure 3.** PLT HLA (HLA-A24) titration series; DP detectable at 1% donor; near visual limit at 0.1%

## Conclusions

We showed that HBT can be detected not only via RBC blood group antigens but also by profiling HLA antigens on leukocytes and PLTs. Platelet HLA profiling yielded clear DPs and detected donor fractions down to approximately 1% in our titration experiments, and it successfully identified DPs even when RBC antigen analysis showed none. These findings support platelet MHC analysis as a practical and

complementary approach for HBT detection, particularly even RBC phenotypes are matched. Future work will focus on sensitivity enhancement (e.g., increased event acquisition, enrichment or sorting of PLTs, optimized antibody panels and fluorophores), evaluation across additional HLA specificities and donor-recipient combinations, and validation in large cohorts to define specificity, reproducibility, and operational thresholds for routine application.

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Momobayashi A, Kojima T, Okano M

# Modification of antigen panel to improve detection power of homologous blood transfusion doping

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## Abstract

Homologous blood transfusion (HBT) analysis typically targets 8 to 9 antigens on red blood cells; the Tokyo laboratory uses C, c, E, e, Jka, Jkb, Fya, Fyb, and S, inferring transfusion from double population (DP) signals. In 75 Japanese athletes, nine-antigen typing yielded 25 profiles; 13 individuals showed the most common profile, indicating potential false negatives for HBT between such donors and recipients. Therefore, we evaluated panel optimization by incorporating antigens with high per-cell expression, balanced population frequencies, and narrow, well-resolved histograms. Antigens with low abundance, wide expression ranges, or extreme population skew impaired DP discrimination. Incorporating additional markers enabled clear DP detection even in mixtures fully matched on the current panel. This study outlines criteria for population-tailored antigen panels to improve HBT detection sensitivity in Japanese cohorts.

## Introduction

Homologous blood transfusion (HBT) can be detected in anti-doping laboratories by flow- cytometric analysis of red blood cell (RBC) surface antigens [1]; notably, two positive cases were identified at the Tokyo 2020 Olympics [2]. In this approach, multiple RBC antigens are typed, and donor-recipient discordance is inferred from double-population (DP) signals. Although this method relies on interindividual differences in antigen expression, several antigens commonly used in current panels exhibit pronounced racial and geographical frequency biases. To enhance HBT detection efficiency, panels should prioritize antigens with substantial interindividual variation and balanced population frequencies. We evaluated the performance of the current analytical panel used for Japanese athletes and attempted to identify antigens suitable for constructing a more population-appropriate panel.

## Experimental

### Materials

Samples: EDTA 2K-anticoagulated peripheral blood collected from in-house volunteers who provided informed consent for use in this study.

Primary antibodies: anti-C, c, E, e, Jka, Jkb, Fya, Fyb, S, M, N and Leb (BIO-RAD, CA, USA).

Secondary antibodies: rabbit anti-human IgM FITC (Dako, Yishun, Singapore), goat anti-human IgG FITC (Thermofisher, MA, USA), and goat anti-mouse IgG+IgM PE (Jackson ImmunoResearch, PA, USA).

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**Analytical methods**

RBC antigen analysis using the current panel

RBCs were washed by centrifugation with phosphate-buffered saline (PBS) and adjusted to $5 \times 10^7$ RBCs/mL . All centrifugation conditions were 1500 g for 10 minutes. Aliquots of the RBC suspension were dispensed into tubes and centrifuged with PBS. For primary staining, human IgM antibodies (anti-C, c, E, e, Jka, Jkb) or human IgG antiserum (anti-Fya, Fyb, S) were added to the respective tubes. After mixing, samples were incubated at room temperature for 90 minutes. Following the primary reaction, cells were washed with PBS by centrifugation. Fluorescently labeled secondary antibodies matched to the primary isotyperabbit anti-human IgM-FITC or goat anti-human IgG-FITC were then added.

After the secondary incubation, cells were washed, resuspended in PBS, and analyzed by flow cytometry (Navios EX, BECKMAN COULTER)[1].

RBC antigens analysis of the additional panel

RBC suspensions prepared as above were dispensed into tubes. Additional candidate primary antibodies (anti-Leb, -M, and -N) were added to the respective tube, followed by incubation. Cells were then washed with PBS by centrifugation. The PE-conjugated goat anti-mouse IgG+IgM was added as the secondary antibody. After the secondary incubation, cells were washed, resuspended in PBS, and analyzed by flow cytometry.

## Results and Discussion

HBT analysis with the current panel (Expression frequencies)

We screened 75 Japanese athletes; all samples were negative for HBT by the current flow-cytometric panel. As summarized in Table 1, the antigen-negative frequencies observed in this cohort closely matched those reported for the Japanese population by the Japanese Red Cross Society [3].

| Antigen-negative frequency (%) | Current Panel Antigens | | | | | | | | | Additional Antigens | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | c | E | e | $Jk^a$ | $Jk^b$ | $Fy^a$ | $Fy^b$ | S | $Le^b$ | M | N |
| Value based on results at Tokyo Laboratory | 10.7 | 41.3 | 48.0 | 10.7 | 26.7 | 22.7 | 2.7 | 76.0 | 85.3 | - | - | - |
| Values reported by the Japanese Red Cross Society | 12 | 44 | 50 | 9 | 27 | 23 | 1 | 80 | 89 | 32 | 22 | 28 |

**Table 1.** Comparison with the antigen-negative frequency of Japanese published by the Japanese Red Cross Society

Using the nine-antigen panel, 25 distinct antigen-profile patterns were identified (Table 2). Of these, 8 patterns were shared by four or more individuals.

| Antigen Profile Pattern | Current Panel Antigens | | | | | | | | | Number of Japanese athletes |
|---|---|---|---|---|---|---|---|---|---|---|
| | C | c | E | e | $Jk^a$ | $Jk^b$ | $Fy^a$ | $Fy^b$ | S | |
| 1 | + | - | - | + | + | + | + | - | - | 13 |
| 2 | + | + | + | + | + | + | + | - | - | 10 |
| 3 | + | + | + | + | - | + | + | - | - | 5 |
| 4 | + | + | + | + | + | - | + | - | - | 5 |
| 5 | + | - | - | + | + | - | + | - | - | 4 |
| 6 | + | - | - | + | - | + | + | - | - | 4 |
| 7 | + | - | - | + | + | + | + | - | + | 4 |
| 8 | + | + | + | + | - | + | + | + | - | 4 |
| 9 | - | + | + | - | + | - | + | - | - | 3 |
| 10 | - | + | + | - | + | + | + | - | - | 3 |
| 11 | + | - | - | + | + | + | + | + | - | 3 |
| 12 | + | + | + | + | - | + | - | + | - | 2 |
| 13 | + | + | + | + | + | + | + | - | + | 2 |
| 14 | + | + | - | + | - | + | + | + | - | 2 |
| 15 | + | + | - | + | - | + | + | - | - | 1 |
| 16 | + | - | - | + | + | - | + | + | + | 1 |
| 17 | + | + | + | + | + | - | + | - | + | 1 |
| 18 | - | + | + | - | - | + | + | + | - | 1 |
| 19 | + | + | + | + | + | + | + | + | + | 1 |
| 20 | + | - | - | + | + | - | + | - | + | 1 |
| 21 | + | + | - | + | + | + | + | - | - | 1 |
| 22 | + | + | - | + | - | + | + | + | + | 1 |
| 23 | - | + | + | - | + | + | + | + | - | 1 |
| 24 | + | + | + | + | + | - | + | + | - | 1 |
| 25 | + | - | - | + | + | - | + | + | - | 1 |

**Table 2.** HBT survey of Japanese athletes (antigen expression)

HBT analysis with the current and additional antigens

The current panel using EDTA2K-anticoagulated peripheral blood from four healthy Japanese volunteers who consented to participate. Notably, two samples (Samples 1 and 2) exhibited completely identical antigen profiles by the current panel (Table 3).

| | ABO Blood Group | RBC antigen expression patterns by current methods | | | | | | | | | Antigen Profile Pattern |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | C | c | E | e | $Jk^a$ | $Jk^b$ | $Fy^a$ | $Fy^b$ | S | |
| Sample 1 | A | + | + | + | + | − | + | + | − | − | No.3 |
| Sample 2 | A | + | + | + | + | − | + | + | − | − | No.3 |
| Sample 3 | B | + | − | − | + | + | + | + | − | − | No.1 |
| Sample 4 | O | + | − | − | + | + | − | + | − | − | No.5 |

**Table 3.** Antigen expression of four healthy volunteer samples

To model transfusion scenarios, simulation mixtures at 95:5 (vv) were prepared. One pair combined Samples 1 and 2 (identical profiles), and the other combined Samples 3 and 4, which differed only at Jkb. The mixtures were analyzed using the current panel (Figure 1a and 1b). Moreover, we assessed additional antigens—M, N, and Leb—that show minimal bias in antigen-negative frequency among Japanese individuals (Table 1). As shown in Figure 1c and 1d, DP signals were detectable with these added antigens even in the mixture derived from Samples 1 and 2, for which HBT could not be detected using the current panel alone.

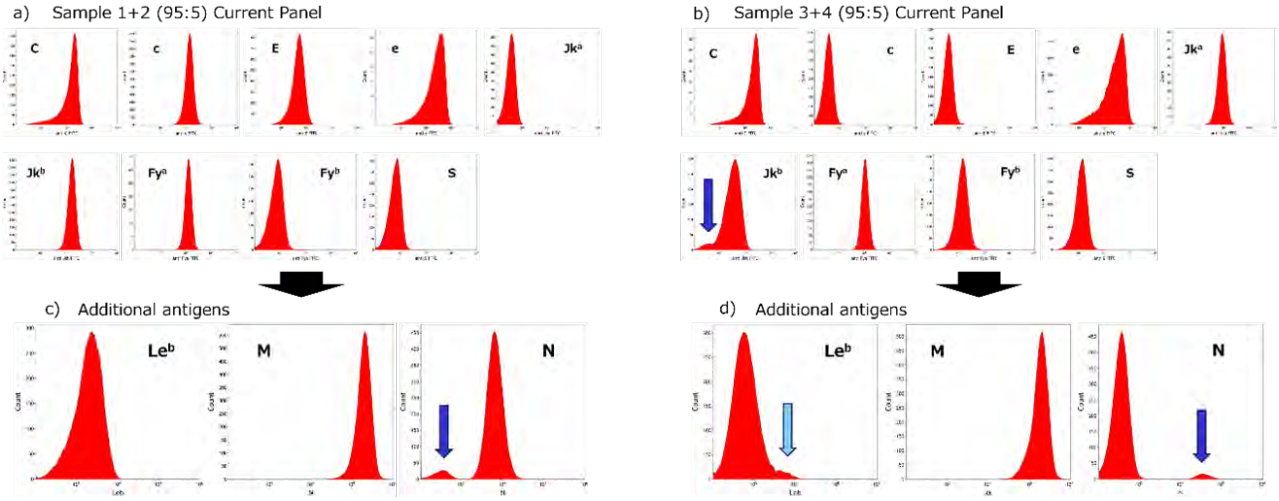


**Figure 1.** HBT analysis of current panel and additional antigens using simulated transfusion samples

These findings highlight two key points. First, the high prevalence of shared antigen profiles within a relatively homogeneous population can limit HBT detectability when relying solely on a constrained set of antigens. Second, adding markers with balanced frequencies and complementary inheritance patterns (e.g., M/N and Lewis) increases the likelihood of donor–recipient discordance and thus DP detection.

## Conclusions

Expanding the antigen panel enabled clear DP detection even in mixtures derived from samples with completely matched profiles by the current panel. In this study, we prioritized markers with balanced population frequencies (M, N, and Leb). While N antigen performed robustly, further optimization for Japanese athletes is warranted, particularly considering cell-level Leb expression intensity. In the future, panel design should integrate both antigen frequency in the target population and per-cell expression levels to maximize donor-recipient discordance and DP signal strength. Overall, these findings underscore the need to tailor HBT panels to population-specific antigen distributions and expression characteristic to improve detection performance.

## References

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# DBS in ESA analytics – an optimized approach for immunopurification

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## Abstract

In order to simplify sample collection and sample transportation and to reduce the overall analysis costs, the world anti doping agency (WADA) has increasingly propagated the analysis of “dried blood spots” (DBS) in recent years [1]. The focus of work with DBS in the field of doping analysis was and is primarily on small molecules, as the mass spectrometers used here have high sensitivity and therefore enable the detection of very small quantities. In contrast, there has been relatively little work to date in the field of EPO/ERA analysis of DBS. The main difficulty in detecting large molecules (proteins such as EPO) in DBS is the limited sample volume. While the methods used in routine testing typically require 500 µL of serum or plasma, only approx. 20-75 µL of dried whole blood is available for DBS. An ELISA-based IP-method was developed for DBS and fully validated according to the requirements of WADA.

## Introduction

WADA has recently implemented dried blood spots as a matrix for ERA analysis in the technical document [2]. According to this TD, it is mandatory that the initial testing procedure (ITP) differs from the confirmation procedure (CP), for example by using different methods for immunopurification. For DBS, this can be achieved by using commercially available kits from MAIIA Diagnostics with different antibodies. Our laboratory has decided to use a MAIIA gel kit for CP. For ITP, it has been decided, for time-flexibility and financial reasons to optimize an extraction protocol [3] followed by concentration and to use StemCell$^{TM}$ ELISA plates for immunopurification. Immunopurified proteins were separated by SAR-PAGE as developed in our laboratory [4].

## Experimental

In the present study, non-volumetric (DBS cards) and volumetric (TASSO) dried blood spotting devices were used. For sample preparation, 20 µL of blank or spiked whole blood from 10 healthy donors (5 male, 5 female) was spotted onto the card and TASSO and were allowed to dry for a minimum of 2h. One 6 mm punch taken from the centre of the spot (corresponding to approx. 12 µL) or one tip from TASSO was used for extraction. An overview of the two different types of extraction and immunopurification used for ITP and CP is presented in Figure 1.

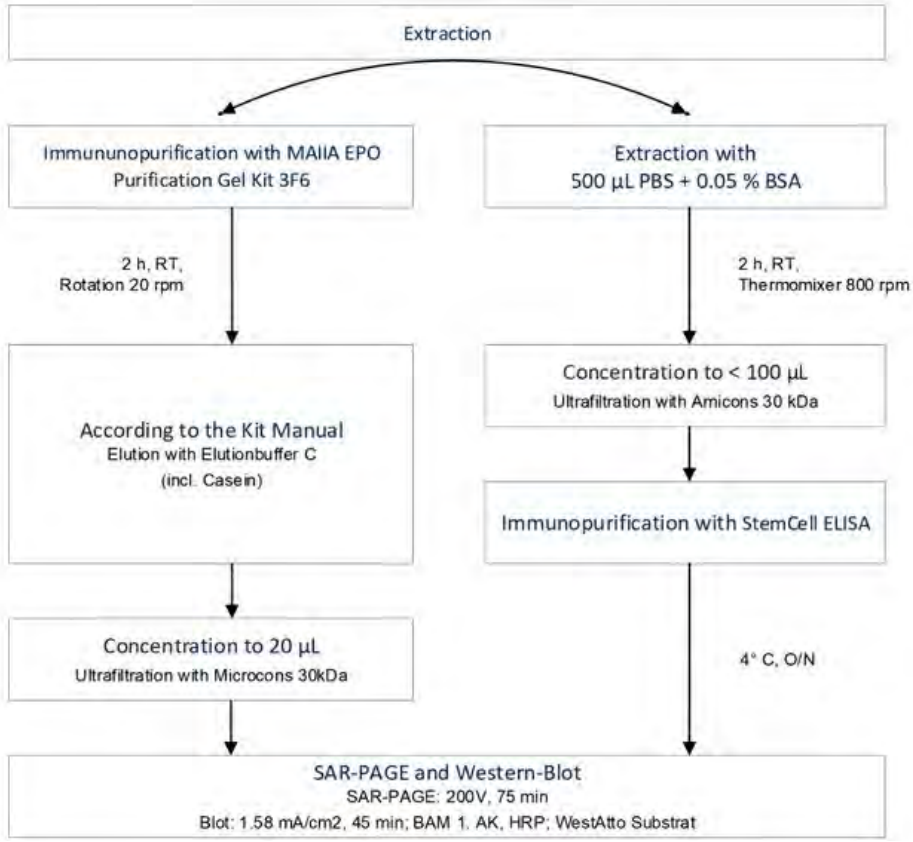


**Figure 1.** Overview of analytical steps of CP on the left and ITP on the right

For CP, the MAIIA EPO Purification Gel Kit 3F6 for blood is used. With this method, extraction and EPO enrichment are carried out in one step (Figure 2A).
For ITP, we wanted to use a method that is more flexible and fits better into our routine work. Therefore, we optimized an extraction protocol previously described [3] followed by immunopurification with ELISA (Figure 2B).

After optimization, the method was validated for its selectivity, reliability at minimum required performance levels (MRPLs), limit of detection (LOD), recovery, and robustness following the recommendations of WADA.

Selectivity was assessed by analyzing ten different blank samples, reliability by analyzing ten samples spiked with ERAs at the MRPL. The LOD was estimated by analyzing six different blood samples spiked with four different ERAs at four different concentration levels (100% MRPL, 50% MRPL, 25% MRPL. 10% MRPL). Recovery was assessed by comparing signal intensities of ERA solutions in buffer without immunopurification to samples spiked at the MRPL. Robustness was assessed by using different analysts and different extraction conditions (2h at RT and o/N at 4°C) on different days.

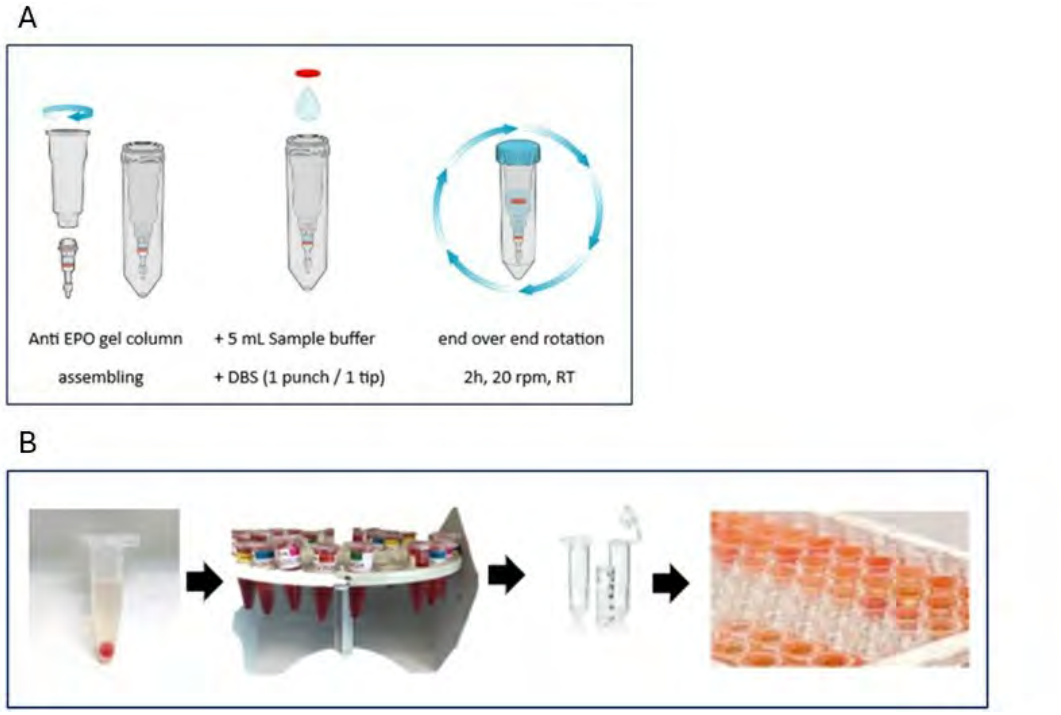


**Figure 2.** **A:** MAIIA EPO Purification gel kit for blood (https://maiiadiagnostics.com/wp-content/uploads/2024/04/DfU-EPGK-B-2024.pdf). **B:** PBS extraction and immunopurification with ELISA

## Results and Discussion

The concentrations of target analytes (rEPO, dEPO, CERA, EPO-Fc) reflecting the different concentration levels (MRPL, 50% MRPL, 25% MRPL. 10% MRPL) according to TD2024EPO are shown in Table 1.

### Selectivity

All samples analyzed (ten DBS-TASSO samples and ten DBS-Cards samples) were evaluated as negative. Therefore, it can be demonstrated that the present method can clearly distinguish between endogenous erythropoietin and other recombinant erythropoietins and their analogues. It can thus be considered *selective.*

### Reliability

All mandatory target analytes were detected at the MRPL in 100% of the samples**.** The method can therefore be considered as *reliable at the MRPL*.

### Limit of Detection (LOD)

According to TD2024EPO, the LODs shall not be higher than 50% of the corresponding Minimum Required Performance Levels (MRPLs). Our results (shown in Figure 3A and 3B) demonstrate that we overfulfil this requirement by reaching 25% of MRPL.

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A

| ERA | MRPL | 1 tip (20 µL) | 50 % MRPL | 1 tip (20 µL) | 25 % MRPL | 1 tip (20 µL) | 10 % MRPL | 1 tip (20 µL) |
|---|---|---|---|---|---|---|---|---|
| rEPO | 30 mIU/mL | 0,6 mIU | 15 mIU/mL | 0,3 mIU | 7.5 mIU/mL | 0,15 mIU | 3.0 mIU/mL | 0,06 mIU |
| dEPO | 30 pg/mL | 0,6 pg | 15 pg/mL | 0,3 pg | 7.5 pg/mL | 0,15 pg | 3.0 pg/mL | 0,06 pg |
| EPO-Fc | 150 pg/mL | 3,00 pg | 75 pg/mL | 1,50 pg | 37.5 pg/mL | 0,75 pg | 15 pg/mL | 0,30 pg |
| CERA | 150 pg/mL | 3,00 pg | 75 pg/mL | 1,50 pg | 37.5 pg/mL | 0,75 pg | 15 pg/mL | 0,30 pg |

B

| ERA | MRPL | 1 spot (12 µL) | 50 % MRPL | 1 spot (12 µL) | 25 % MRPL | 1 spot (12 µL) | 10 % MRPL | 1 spot (12 µL) |
|---|---|---|---|---|---|---|---|---|
| rEPO | 30 mIU/mL | 0,36 mIU | 15 mIU/mL | 0,18 mIU | 7.5 mIU/mL | 0,09 mIU | 3.0 mIU/mL | 0,04 mIU |
| dEPO | 30 pg/mL | 0,36 pg | 15 pg/mL | 0,18 pg | 7.5 pg/mL | 0,09 pg | 3.0 pg/mL | 0,04 pg |
| EPO-Fc | 150 pg/mL | 1,8 pg | 75 pg/mL | 0,90 pg | 37.5 pg/mL | 0,45 pg | 15 pg/mL | 0,18 pg |
| CERA | 150 pg/mL | 1,8 pg | 75 pg/mL | 0,90 pg | 37.5 pg/mL | 0,45 pg | 15 pg/mL | 0,18 pg |

**Table 1.** MRPLs according to TD2024EPO, **A**: absolute values for TASSO calculated based on 20 µL blood/tip. **B**: absolute values for cards calculated based on 12 µL blood/spot

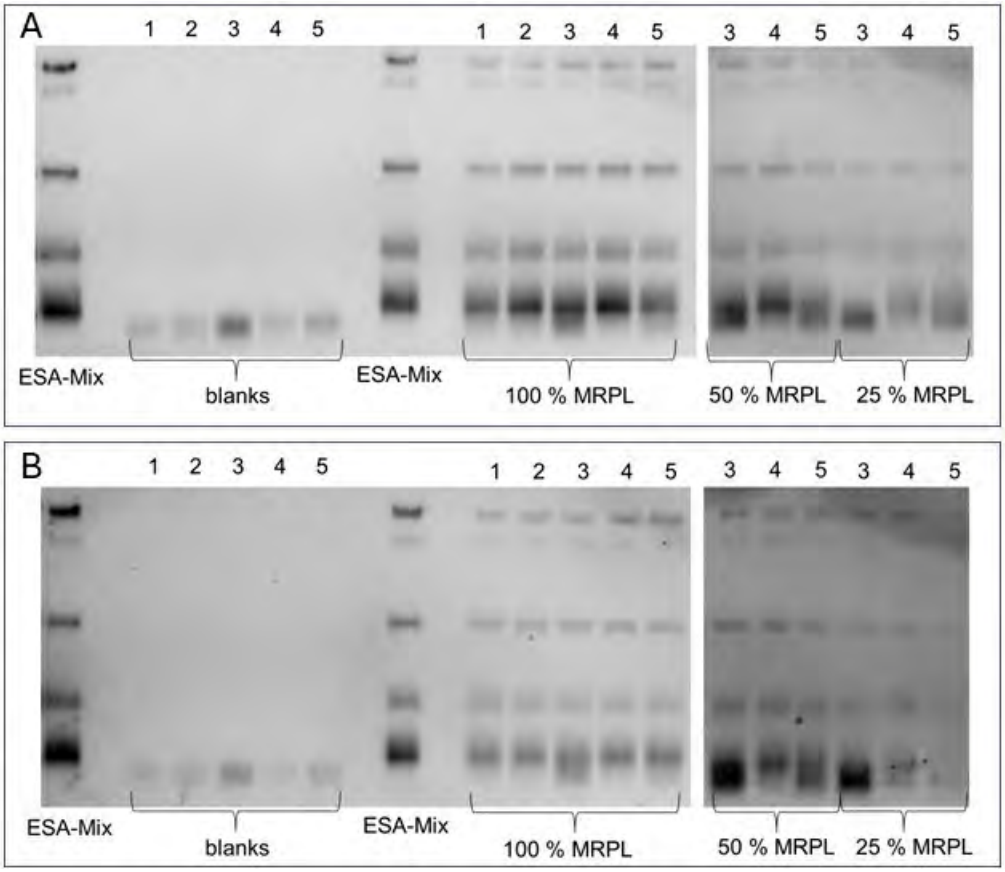


**Figure 3. A:** TASSO; male, blanks and sample 1 - 5 spiked with 100% MRPL of rEPO, dEPO, EPO-Fc and CERA, sample 3 - 5 spiked with 50% and 25%. **B:** Cards; male, blanks and sample 1 - 5 spiked with 100% MRPL of rEPO, dEPO, EPO-Fc and CERA, sample 3 - 5 spiked with 50% and 25%

This results in the following LODs in accordance with TD2024EPO in DBS for TASSO and Cards:

- rEPO: 7,5 mIU/mL
- dEPO: 7,5 pg/mL
- EPO-Fc: 37,5 pg/mL
- CERA: 37,5 pg/mL

**Recovery**

To calculate the recoveries of the method, a total of 6 different blood samples were spiked with the ESA mix at MRPL and then compared with the ESA mix at MRPL in sample buffer. The respective pixel volume of the bands after background correction was used for the calculation. Average recoveries ranged from approximately 20% to 80%. When performing the calculations, it must be considered that this is not a quantitative but a qualitative method.

**Robustness**

All tested variants of the method delivered reliable results compared to the routinely used method. The method can therefore be considered as *robust.*

## Conclusions

The optimized protocol with PBS + 0.05% BSA extraction and immunopurification with StemCell™ ELISA was successfully validated for cards and TASSO and can be applied, after accreditation, for routine analysis.

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# Detection of testosterone esters in DBS by LC-MS/MS

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## Abstract

The detection of testosterone esters in blood represents a crucial advancement in anti-doping efforts, complementing existing analytical techniques such as IRMS analysis in urine. Our study focuses on dried blood spots (DBS) as an alternative matrix for steroid ester detection, aiming to enhance sampling convenience and broaden anti-doping applications. Hence, we present a validated ITP for testosterone esters detection in DBS using LC-MS/MS.

DBS samples were prepared using the Tasso M-20 device as a support for blood spots and analyzed using the Thermo Vanquish-Altis Plus LC-MS/MS system. Sample preparation involved liquid-liquid extraction (LLE) followed by derivatization with a Girard-P mixture to improve sensitivity. The ITP was validated following WADA guidelines, assessing parameters such as selectivity, limit of detection (LOD), carry-over, reliability of detection, extract stability and robustness. The detection reliability was confirmed at 0.5 ng/mL across 10 different DBS matrices for all targets. Selectivity was acceptable for most targets, although the selectivity for testosterone enanthate and testosterone cypionate was worse on the Tasso M-20 support.

To conclude, this study confirms the potential of DBS for detecting testosterone esters, building on previous advancements made in serum analysis. However, it also points to the need for further development of more selective confirmatory procedures for DBS, using an LC-MS-HR system. The successful application of this method enhances anti-doping efforts, offering a practical and efficient approach for detecting exogenous androgenic steroids in athletes.

## Introduction

Steroid esters are prohibited substances in sports, classified under S1.1 (anabolic androgenic steroids) of the WADA Prohibited List. Direct detection of testosterone esters in blood is difficult due to rapid enzymatic hydrolysis [1] of the ester bond. DBS sampling reduces enzymatic activity [2] and increases the stability of these compounds, while also providing a minimally invasive and convenient alternative for blood doping control [3]. The present study aimed to establish an initial testing procedure (ITP) for nine testosterone esters in DBS. The ITP was validated in accordance with the International Standard for Laboratories [4] (ISL) guidelines.

## Experimental

DBS collected with the Tasso-M20 device were transferred into 2 mL polypropylene tubes and extracted with 300 µL of methanol containing 25 µL of an internal standard solution (T-undecanoate-D3). After sonication and centrifugation, the supernatant was transferred to glass tubes. One milliliter of deionized

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water and 4 mL of diethyl ether were added, followed by vortex mixing and centrifugation. The upper ether layer was transferred to glass tubes and evaporated under nitrogen at 50°C. For derivatization, 60 µL of Girard-P [5] solution (1 mg/mL in 10% acetic acid/methanol) was added to the dry extract. The mixture was vortexed, transferred to LC vials, and heated at 70 °C for 30 minutes before centrifugation. The extraction workflow for DBS samples is illustrated in Figure 1.

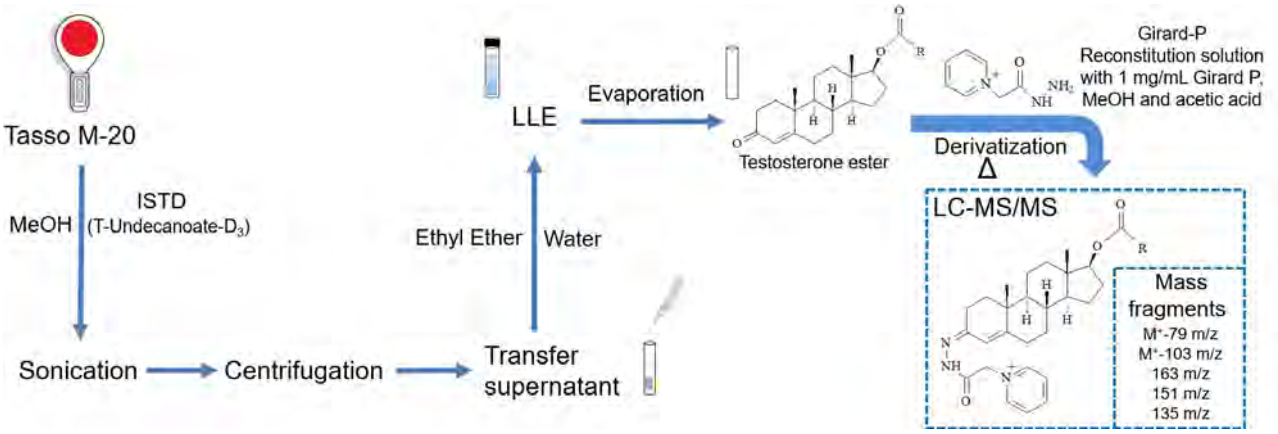


**Figure 1.** Extraction and derivatization procedure for testosterone esters in Tasso-M20 DBS

Chromatographic separation was performed on a Halo Peptide ES-C18 column (2.1 × 100 mm, 2.7 µm) at 50°C, using water with 0.1% formic acid (A) and methanol with 0.1% formic acid (B) at 0.4 mL/min. The gradient was: 0–3.0 min, 50–72.5% B; 3.0–6.0 min, 72.5–100% B; 6.0–8.0 min, 100% B; 8.0–11.0 min, 50% B. Injection volume was 5 µL.

LC-MS/MS measurements were performed on a Vanquish UPLC coupled to a Altis Plus triple quadrupole mass spectrometer (Thermo Fisher Scientific) equipped with a heated electrospray ionization (H-ESI) source operated in positive ionization mode. The spray voltage was set at 3500 V, the ion transfer tube temperature at 325°C, and the vaporizer temperature at 350°C. Sheath, auxiliary, and sweep gases were respectively set at 55, 10, and 1 arbitrary units. Argon was used as the collision gas at a pressure of 2.0 mTorr. The system was operated in selected reaction monitoring (SRM) mode with three transitions monitored for each analyte (see Table 1).

| Compound | Precursor (m/z) | Product ions (m/z) | Retention time (min) | Collision energies (eV) |
|---|---|---|---|---|
| Testosterone acetate | 464 | 163 / 357 / 385 | 3.4 | 40 / 30 / 28 |
| Testosterone propionate | 478 | 163 / 371 / 399 | 3.9 | 40 / 30 / 28 |
| Testosterone benzoate | 526 | 163 / 419 / 447 | 4.6 | 42 / 31 / 29 |
| Testosterone valerate | 506 | 151 / 399 / 427 | 4.7 | 40 / 31 / 29 |
| Testosterone isocaproate | 520 | 163 / 413 / 441 | 4.9 | 42 / 32 / 30 |
| Testosterone enanthate | 534 | 163 / 427 / 455 | 5.2 | 42 / 32 / 30 |
| Testosterone cypionate | 546 | 163 / 439 / 467 | 5.3 | 44 / 34 / 31 |
| Testosterone decanoate | 576 | 151 / 469 / 497 | 5.9 | 43 / 34 / 31 |
| Testosterone undecanoate | 590 | 163 / 483 / 511 | 6.0 | 45 / 34 / 33 |
| Testosterone undecanoate-D3 (IS) | 593 | 514 | 6.0 | 32 |

**Table 1.** SRM transitions for testosterone esters in DBS

## Results and Discussion

The ITP of testosterone esters in Tasso M-20 DBS was validated for the following parameters: selectivity, reliability of detection, LOD, carry-over and extract stability. Table 2 summarizes the validated method parameters for each target included in the ITP.

The method's selectivity for each target was evaluated using 10 different DBS matrices. For most testosterone esters, adequate selectivity was demonstrated, with no significant interferences detected. Testosterone enanthate and testosterone cypionate showed matrix-related interferences which complicate target detection below 0.5 ng/mL. When confirming ITP results, improved selectivity of testosterone enanthate and testosterone cypionate can be achieved using LC-HRMS or by using a different derivatization reagent, such as methoxyamine [6].

The reliability of detection was evaluated by spiking ten DBS matrices at 0.5 ng/mL. At the time of validation, no MRPL for testosterone esters was available, but a limit of 1 ng/mL has since been suggested by WADA (personal communication). Under these conditions, all testosterone esters were reliably detected at 0.5 ng/mL, as illustrated in Figure 2. The LOD for each target was evaluated using 6 different DBS matrices and a five-point calibration curve (50 pg/mL to 1000 pg/mL) which was extracted in two different validation batches. The LOD was estimated visually based on the signal obtained for all SRM transitions of each analyte. Detection was confirmed when the signal-to-noise ratio exceeded 3.

Carry-over was evaluated by injecting two blanks immediately after a DBS sample that had been spiked with 10 ng/mL of the target compound. Following the 10 ng/mL injection, no contamination of the blanks was observed for any of the target compounds. The stability of derivatized DBS extracts was confirmed for up to 96 hours when stored at 4°C. Long-term use of the method showed no clogging of the C18 column, LC tubing, or MS source contamination after more than one year of direct injection of Girard-P derivatized extracts.

| Compound | LOD (pg/mL) | Selectivity (n=10) | Reliability at 0.5 ng/mL (n=10) | Carry-over (at 10 ng/mL) |
|---|---|---|---|---|
| T-Acetate | 100 | Adequate | Yes | Not observed |
| T-Propionate | 100 | Adequate | Yes | Not observed |
| T-Benzoate | 50 | Adequate | Yes | Not observed |
| T-Valerate | 100 | Adequate | Yes | Not observed |
| T-Isocaproate | 100 | Adequate | Yes | Not observed |
| T-Enanthate | 250 | Inadequate below 500 pg/mL | Yes | Not observed |
| T-Cypionate | 500 | Inadequate below 500 pg/mL | Yes | Not observed |
| T-Decanoate | 250 | Adequate | Yes | Not observed |
| T-Undecanoate | 100 | Adequate | Yes | Not observed |

**Table 2.** Validation results of the ITP for the different testosterone esters in DBS

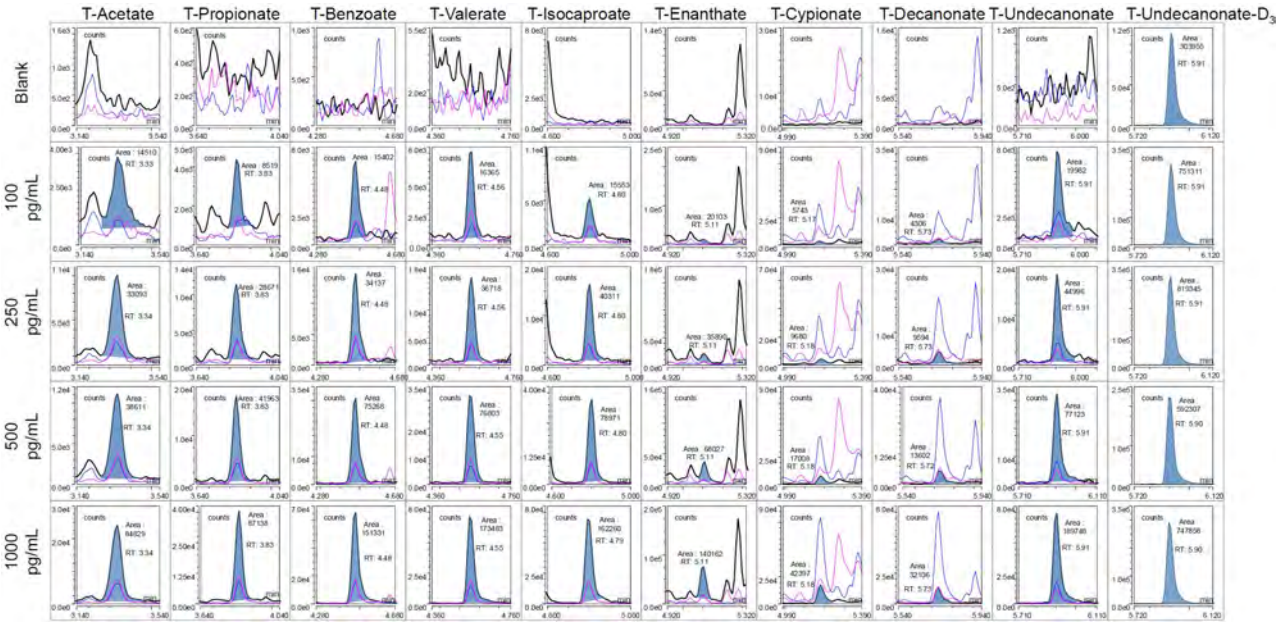


**Figure 2.** SRM chromatographs on TSQ Altis Plus for an extracted blank DBS at various spiked concentrations of testosterone esters

## Conclusions

This study establishes a robust ITP for detecting nine testosterone esters in Tasso-M20 DBS samples. However, further development of more selective confirmatory procedures is underway for compounds such as T-enanthate and T-cypionate, particularly using LC-MS-HR. Preliminary development of boldenone esters in DBS has already shown that methoxyamine derivatization is necessary as these esters cannot be reliably detected using Girard-P at the MRPL. Ultimately, it will not be possible to cover the full steroid ester panel with a single derivatization strategy. While some steroid esters respond better to Girard-P, others require a different derivatization reagent. Future work will aim to incorporate boldenone and nandrolone esters.

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## Acknowledgements

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Stojanovic B, Geisendorfer T, Gmeiner G

# Detection of metandienone in paired DBS and urine samples after transdermal administration

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## Abstract

In sport drug testing it is important to cover every possible doping scenario. This is why new matrices, new analytical methods and combined approaches are of increasing interest. In the present study, paired samples consisting of dried blood spots (DBS) and urine were collected after transdermal administration of metandienone to show the potential and significance of complementary testing of different matrices. For this purpose, one male volunteer administered 20 mg of metandienone transdermally on palm skin. As a collection device for DBS samples a TASSO M20 system was used. Tasso samples were collected up to 30 h while urine samples were collected for 17 days after administration. The collected TASSO samples were analyzed using a liquid chromatography - high resolution mass spectrometry (LC-HRMS) method for the presence of metandienone while urine samples were analyzed using a gas chromatography - mass spectrometry method (GC-MS/MS) for the presence of metandienone metabolites. The results obtained indicated early detection of metandienone in DBS samples while urine samples show metandienone misuse after 5 h until at least 11 days.

## Introduction

Testing of DBS samples in sport drug analysis increased significantly in the last years [1,2]. To gain experience and collect scientific data it is important to understand the correlation between concentration of marker analytes in urine and DBS samples. An additional challenge is to target prohibited substances or its metabolites after transdermal administration in both relevant matrices – urine and DBS. Doping scenarios after transdermal administration of 4 anabolic agents including metandienone are already published for urine samples [3]. Further on, excretion pattern of urinary metabolites of metandienone after transdermal application is presented in a publication [4]. On the other side, the presence of metandienone in paired urine and DBS samples is confirmed in samples collected in Danish fitness centers [5]. However, there are no administration studies with simultaneous collection of DBS and urine samples in a controlled period after transdermal administration of metandienone.
The aim of this study was to compare the results for metandienone in paired DBS and urine samples after transdermal administration in an administration study.

## Experimental

The protocol for the determination of metandienone in Tasso samples using a liquid extraction (LE) - LC-HRMS method is presented in Figure 1.

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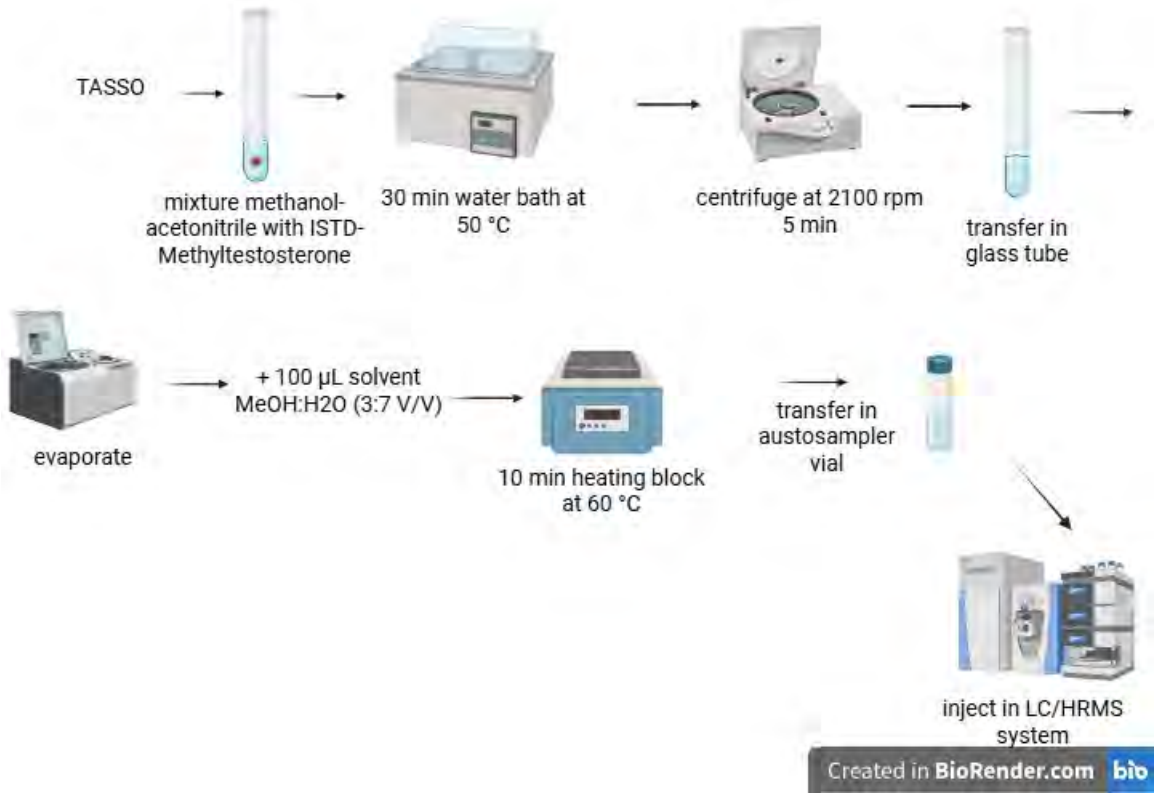


**Figure 1.** The protocol for the determination of metandienone in Tasso samples

The protocol for the determination of metandienone metabolites in urine samples using a liquid-liquid extraction (LLE) – GC-MS/MS method is presented in Figure 2.

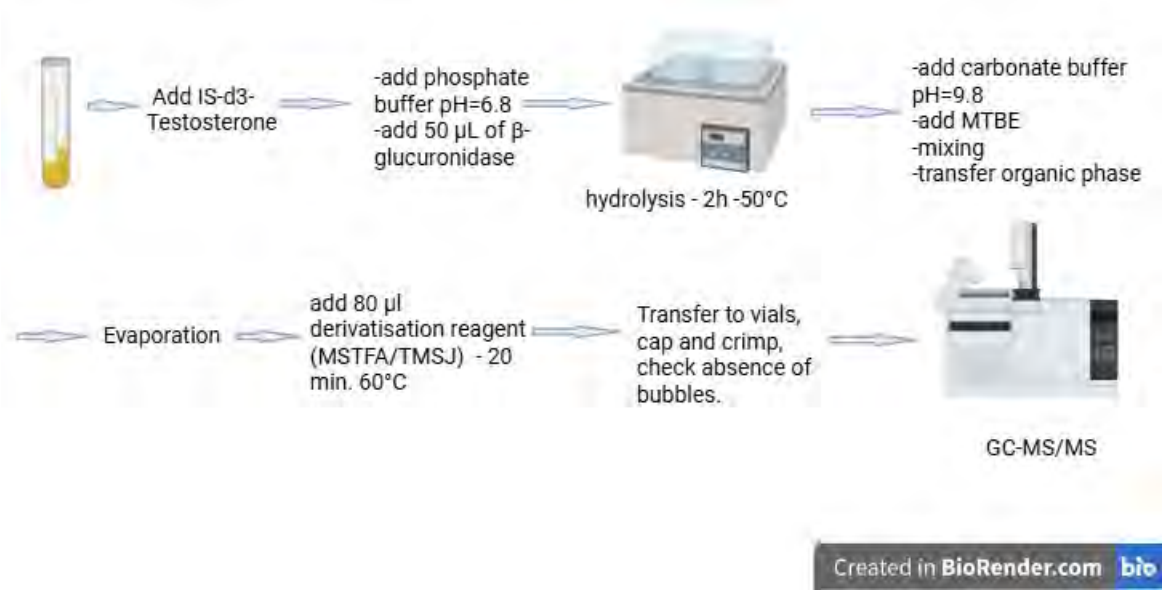


**Figure 2.** The protocol for the determination of metandienone metabolites in urine samples

**Administration study**

One male volunteer (age 51) administered 20 mg of metandienone, dissolved in dimethylsulfoxide, transdermallyon his palm skin. Tasso samples were collected 0 h, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 24 h and 30 h after administration. Urine samples were collected up to 17 days after administration: in the first 48 h, all urine samples were collected, 2-3 samples per day were collected on the third and the fourth day, while morning urine samples were collected until the end of the collection time. Samples were kept frozen at -20 °C until analysis.

## Results and Discussion

Many publications showed that DBS testing undoubtedly offers many possibilities as a complementary matrix in sport drug testing [1,2,5]. Nevertheless, there are still many open questions, and the most comprehensive way to understand drug disposition between DBS and urine is an administration study with simultaneous collection of DBS and urine samples. In this study, metandienone was the target analyte in DBS samples and the limit of detection (LOD) with the LC-HRMS method used for samples analysis is 5 ng/mL. The GC-MS/MS method for urine sample analysis uses the following metandienone metabolites as targets: epimetendiole (17β-methyl-5β-androst-1-ene-3α,17α-diol), 6ß-hydroxymetandienone (6β,17β-dihydroxy-17α-methylandrosta-1,4-dien-3-one), tetrahydromethyltestosterone (17α-methyl-5β-androstane-3α,17β-diol) and metandienone long term metabolite (LTM; 17β-hydroxymethyl-17α-methyl-18-norandrost-1,4,13-triene-3-one) with LODs of 0.2 ng/mL, 0.25 ng/mL, 1 ng/mL and 0.25 ng/mL, respectively.

Concentrations obtained from metandienone in DBS samples are graphically presented in Figure 3.

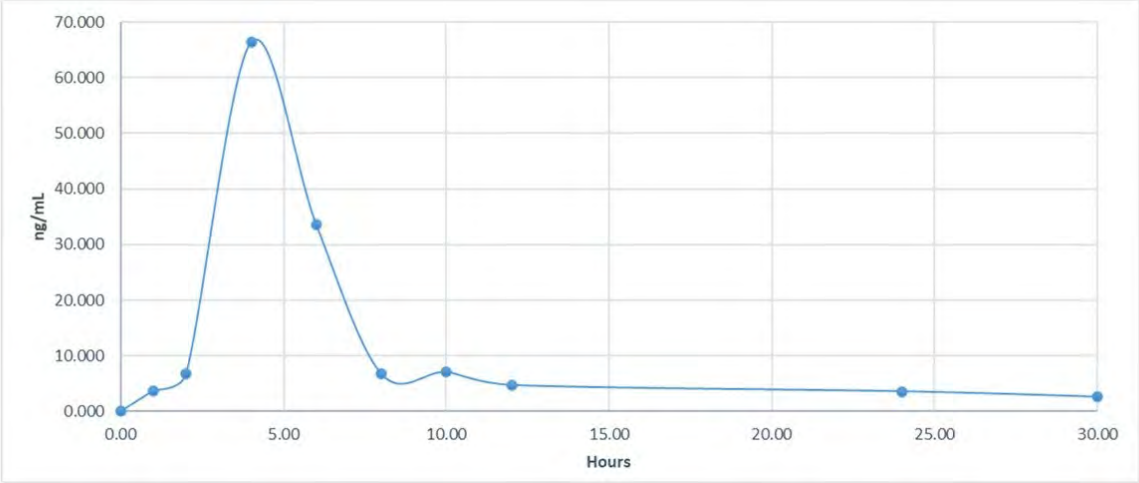


**Figure 3.** Concentrations obtained from metandienone in DBS samples

Estimated concentrations of metandienone metabolites in urine samples are presented in Table 1.

| Metabolite | The first detected | | Sample with the highest concentration | | The last detected | |
|---|---|---|---|---|---|---|
| | Time after adm. | Conc. (ng/mL) | Time after adm. | Conc. (ng/mL) | Time after adm. | Conc. (ng/mL) |
| Epimetendiole | 18 h | 0.23 | 44 h | 6.9 | 5 days | 1.3 |
| 6ß-hydroxymetandienone | 5.5 h | 1.9 | 44 h | 57 | 11 days | 0.5 |
| Tetrahydro -methyltestosterone | 44 h | 6.9 | 68 h | 9.6 | 6 days | 4 |
| Metandienone LTM | 44 h | 2.4 | 68 h | 2.6 | 10 days | 0.2 |

**Table 1.** Estimated concentrations of metandienone metabolites in urine samples

Metandienone at a concentration of approx. 3.7 ng/mL was found in the first DBS sample, collected 1 h after administration, while the highest concentration of approx. 66 ng/mL was found in the DBS sample obtained 4 h after administration. In the last DBS sample collected 30 h after administration, the presence of metandienone was also confirmed. On the other side, the first urine sample indicating administration of methandienone was collected 5.5 h after transdermal application. As expect, metandienone metabolites are detectable much longer in urine samples – the last detected metabolite was found in urine collected 11 days after administration. The main advantage of the DBS testing is the ability to detect the presence of metandienone in the bloodstream at the very beginning of the administration. However, the detection window of metandienone in DBS is narrower compered to urine. In addition, urine is the preferred matrix for the detection of metandienone doping due to the detection of a variety of metabolites, while only the parent is targeted in DBS. Finally, the optimum scenario for the detection of metandienone doping would be the simultaneous collection of DBS and urine samples, covering the different stages of drug resorption and elimination.

## Conclusions

The presented study showed that collection of DBS samples after transdermal administration could bring evidence for metandienone misuse in the first hours, while urine samples detected metandienone misuse after 5 h until at least 11 days. The results obtained additionally indicated that simultaneous collection of both DBS and urine could bring benefits in the fight against doping. Finally, to confirm the obtained results, it is necessary to conduct a more extensive study.

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Ericsson M

# Laboratory operations during the Paris 2024 summer Olympic and Para-Olympic Games

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## Abstract

The organization and operation of the French Anti-Doping Laboratory (LADF) during the 2024 summer Olympic and Para-Olympic games in Paris, France are summarized together with its findings. During the games the lab analyzed 6849 urine samples, 916 serum samples, 329 dried blood spot samples and 718 whole blood samples. The samples were delivered from the different venues to a sample hub close to the laboratory. Once daily, all the collected samples were transferred to the laboratory and the reporting time was 36 h for negative samples with an allowance of 48 h for suspicious findings and to conclude ATF or AAF. The laboratory used 102 staff members to do the work during the Olympic Games: 42 permanent, 19 students and 41 WADA lab experts both technicians and certifying scientists. For the Para-Olympics the external staff numbers were reduced; 15 students and 26 WADA lab, in total 83 staff members. The operation was conducted at the laboratory’s new permeant home in Orsay-Ville south of Paris, France.

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# Detection of Turkesterone in dietary supplements, human urine samples and its use by Olympic and elite athletes

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## Abstract

The consumption of ecdysteroids as dietary supplements by athletes to enhance their sport performance led to it being placed on the World Anti-Doping Agency Monitoring Program. Turkesterone, an analogue of 20-hydroxyecdysone (20HE), may have greater potency than 20HE, and therefore is more likely to be abused. The present study aimed to: i. Develop a method for the detection of Turkesterone, ii. Detect and quantify it in dietary supplements, iii. Determine the elimination of Turkesterone in healthy volunteers after oral ingestion of it, and iv. To estimate the prevalence of its use by elite and Olympic athletes.

A method was successfully developed for the detection of Turkesterone (2 ng/µL, CV 0.11%). However, while 20HE was detected in all tested supplements, Turkesterone was only seen in one of these and at concentrations far below that claimed on the label (detected versus label: 33.8 mg/gm versus 500 mg). Turkesterone (1 M: 1F, 5 mg dose each) showed peak levels in urine within 10 hours for the male volunteer and 6-8 hours for the female following oral ingestion. Analysis of samples from elite athletes suggested a prevalence of 0.9% (n=9 out of 1000 samples) for the use of 20HE; none showed the presence of Turkesterone, while the use of supplement-derived ecdysteroids as per declaration on Doping Control Forms during the Olympics was 0.4%.

In conclusion, a novel ecdysteroid, Turkesterone, was detected in supplements and is consumed by elite athletes, albeit at lower levels than 20HE, and may have a presumed gender difference in its pattern of excretion.

**Keywords:** Turkesterone, 20-hydroxyecdysone, assay development, antidoping, prevalence of use, elite athletes, Olympics

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## Detection of tramadol misuse in sports

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Integrative Pharmacology and Systems Neuroscience Group, Hospital del Mar Research Institute, Barcelona, Spain [4];
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### Abstract

Tramadol is a centrally acting synthetic opioid agonist and serotonin/norepinephrine reuptake inhibitor used in the treatment of acute and chronic pain. Since January 2024, tramadol is prohibited in sports competition and is included in the Prohibited List. Tramadol misuse is detected using a minimum reporting level (MRL) of 20 µg/mL for the parent compound (excreted free in urine) and a 24 h washout period to avoid false positives due to out-of-competition treatments close to competition is recommended. The criterion was established with preliminary data obtained in the present study. This work presents the complete data from a clinical study of single-dose oral tramadol administration.

After oral administration, tramadol is extensively metabolized. The main analytes detected in urine are unchanged tramadol and the metabolites, O-desmethyltramadol (ODMT) and N-desmethyltramadol (NDMT). Formation of ODMT is catalyzed by CYP2D6 and it is subjected to a genetic polymorphism with different groups of metabolizers from poor to ultra-fast metabolizers, being extensive metabolizers the most common genotype.

A clinical study was performed with 23 male and female volunteers. The volunteers were stratified following CYP2D6 genotypes. The distribution was as follows: 5 poor metabolizers, 15 extensive and intermediate metabolizers, and 3 ultra-fast metabolizers. A single oral dose of 100 mg or 75 mg (for the ultra-fast metabolizers) was administered, and urine, plasma and DBS samples were collected up to 36 h after administration. The doses of tramadol administered in the study were justified in the clinical protocol and they are aligned with the recommendation not to exceed 100 mg of tramadol per dosage. A quantitative method based on liquid chromatography mass spectrometry technology was developed and validated to determine tramadol, ODMT and NDMT. Tramadol and ODMT are the main urinary metabolites.

The conversion from tramadol to ODMT is different across phenotypes. Tramadol is a suitable marker for poor and extensive-intermediate metabolizers and allows a low detection of ultra-fast metabolizers. ODMT may increase the sensitivity of the administration of tramadol in ultra-fast metabolizers. Although a criterion including both markers could be an effective strategy for detecting tramadol misuse, the implemented criterion targets only tramadol. This approach was chosen due to the low prevalence of the ultra-fast metabolizer population and to simplify method development in laboratories.

In plasma, tramadol showed the highest concentrations in extensive and poor metabolizers, while ODMT levels were highest in ultra-fast and extensive metabolizers. A strong correlation was observed between DBS and plasma concentrations. Data obtained in the present study demonstrates that an MRL could also be established to detect tramadol misuse in DBS samples.

The evaluation of the complete dataset obtained in this project demonstrates that the MRL at 20 µg/mL together with a washout period of 24h ensures no false positives due to OOC treatments close to competition, as well as a proper detection of the administration during the first 8h.

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# Synthetic applications in analytical chemistry

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## Abstract

Identifying and analyzing drug candidates with potential performance-enhancing properties is a key aspect of our profession. To establish a proactive doping control system, such substances must be investigated as early as possible to allow for their detection and, thereby, deterrence of misuse as doping agents. However, such substances and associated internal standards are rarely available. In these cases, chemical synthesis might be the only option to address this issue. Through in-house synthesis, new and potentially doping-relevant substances can be synthesized and analyzed on-site.
Further analysis of their analytical characteristics and their metabolic behavior is then enabled, supporting the development of suitable methods for detecting these compounds and their associated metabolites in doping controls. Therefore, *in vitro* incubation and/or synthesis of potential metabolites are crucial for a comprehensive anti-doping system.
This work aims to illustrate the use of organic synthesis for doping control analysis, with an emphasis on its application and practicality in daily lab work. The substances investigated in this study range from selective androgen receptor modulators (SARMs) to ryanodine channel complex stabilizers (Rycals) and estrogen-related receptor agonists (pan ERR agonists). Additionally, SARM 2f, as well as the Rycal compounds S107, JTV 519, ARM036, and ARM210 were metabolized *in vitro* to identify potential metabolites. Selected metabolites of SARM 2f and the various Rycal compounds were synthesized to confirm their structure and provide reference materials for anti-doping control purposes.
Through this presentation, we hope to encourage others to synthesize reference standards and demonstrate that these synthetic methods can be applied in any lab. The results of this study contribute to expanding our knowledge of new substances and their metabolic properties, and the compounds synthesized here can serve as reference materials in routine anti-doping controls.

*The details of this study will be published elsewhere.*

Korsmeier L[1], Krombholz S[1], Alhalabi H[1], Thomas A[1], Thevis M[1,2]

# Exploring transdermal SARMs exposure: Analysis of the elimination profiles and metabolism for doping control purposes

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

## Abstract

Transdermal drug delivery has been a focus of pharmaceutical research for decades and is increasingly relevant in sports drug testing, as athletes' exposomes play a crucial role in case result management by doping control authorities. Previous studies demonstrated, that an (unintentional) oral ingestion of trace amounts of the prohibited selective androgen receptor modulators (SARMs), e.g. due to product contamination, can lead to an adverse analytical finding (AAF) in doping controls. Another contamination site is presented by the skin, as it provides a large surface for drug penetration. However, the extent of diffusion through various layers of the skin and into the blood vessels depends, among other things, on the physicochemical and biological properties of a drug substance. The aim of this project was to investigate the skin penetration of microdoses of three common SARMs (LGD-4033, RAD140 and S-23), as well as their metabolism following transdermal administration. For this purpose, an administration study was conducted, in which 10 and 50 µg of the substances were applied to the lower forearm of 5 volunteers each. The collected urine samples were analyzed via LC-MS/MS following enzymatic hydrolysis and solid-phase extraction. This methodical approach is distinguished by its high sensitivity, enabling the detection of at least 5 pg/mL for LGD-4033 and S-23. After 10 µg administration, LGD-4033 and S-23 as well as associated metabolites were detected, while RAD140 was only detected in urine samples of one subject (n=5). Following 50 µg application, RAD140 was detected in all subjects (n=5) and additional metabolites of LGD-4033 and S-23 were identified. The long-term metabolite of LGD-4033 (M5b) showed a detection time of up to 12 days after 10 µg administration, and even up to 25 days after 50 µg administration.
It was demonstrated for all three SARMs that they penetrate the skin and may – even in trace amounts – cause an AAF when administered transdermally. Information on urinary concentrations and metabolism following transdermal administration of SARMs may assist in the interpretation of AAFs, particularly when dermal contamination or intentional doping via the skin is discussed.

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# Determination of haematocrit measurements on dried blood spots Tasso-M20 using near-infrared spectroscopy

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University of Lausanne, Ecole des sciences criminelles, Lausanne, Switzerland[2]

## Abstract

In recent years, the use of dried blood spots (DBS) has attracted significant attention in the field of anti-doping due to their numerous logistical advantages, including enhanced sample stability, transport at ambient temperature, minor space requirements for long term storage and reduced susceptibility to environmental degradation compared to traditional liquid blood samples. However, despite these benefits, quantitative analyses in DBS are still challenging. Specifically, the measurement of haematocrit (HCT), the ratio of red blood cell volume to total blood volume is crucial for interpreting measured concentrations in DBS. Furthermore, Luginbühl *et al.* suggested that HCT variations can impact analyte extraction efficiency from DBS, potentially introducing biases. Precise HCT measurement is therefore essential for bias correction, accurate conversion of blood analyte concentrations to plasma or serum equivalents, and reliable validation of quantitative analytical methods.

While HCT measurement methods are well-established for standard cellulose card -based DBS, they are not applicable to polymeric Tasso-M20 DBS mainly due to its unique cylindrical design. To address this challenge, we developed a novel methodology for predicting HCT values using near-infrared (NIR) spectroscopy, taking advantage of an algorithm developed by NIRLAB (NIRLAB AG, Switzerland). This technology offers significant advantages, including simplicity, speed, portability, and non-destructiveness, making it ideal for this purpose.

The NIRLAB model demonstrated strong performance, with a coefficient of determination ($R^2$) of 0.935 and a root mean square error of calibration (RMSE C) of 0.96. These values reflect good accuracy and a solid correlation with the reference values obtained from EDTA, measured by the Sysmex XN 1000. A mean relative error of ± 1.66% was observed, with 98% of the samples falling within a relative error of 5% (ranging from -5.35% to +6.34%).

Additionally, the stability of HCT in DBS was assessed over a one-month period under three different storage conditions: room temperature (RT), 4°C, and -20°C, to evaluate the impact of storage conditions on HCT measurement reliability.

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# Biochemical impacts of synthetic compounds: Risks of doping and hormonal contraceptives on mental health

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## Abstract

**Purpose:** Hormonal contraceptives are linked to a higher prevalence of depressive symptoms. Given their popularity in Western countries, understanding the biochemical effects on neuronal cells is crucial to minimizing mental health risks.

**Experimental design:** Neural progenitor cells were treated with ethinyl estradiol (EE) and levonorgestrel (LNG), two synthetic sex hormones commonly used in oral contraception, and S-23, a selective androgen receptor modulator developed as a potential synthetic sex hormone for male hormonal contraception. Label-based quantitative proteomics with the TMTpro 16plex tandem mass tags was used to assess protein expression changes between treated and untreated cells.

**Results:** Treatment of human neural progenitor cells with EE, LNG, EE + LNG, and S-23 led to distinct and overlapping proteomic changes, with enrichment in pathways related to inflammation, oxidative stress, transcriptional regulation, and cell death. Disease association analyses linked these changes to neurodegenerative and psychiatric conditions, including mechanisms relevant to depression.

**Conclusions and clinical relevance:** These findings suggest that hormonal compounds used in contraception and performance enhancement may influence molecular pathways implicated in mental health, particularly depression. While not directly translatable to clinical outcomes, the results support the need for further investigation into the neuropsychiatric effects of hormonal treatments.

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# Using ambient ionization mass spectrometry for rapid screening of prohibited substances in medicinal and dietary products

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Waters Corp, Wilmslow, UK[3]

## Abstract

Mass spectrometry (MS)-based assays are mainstay in sports anti-doping for detection of prohibited substances in biological and non-biological samples. However, the necessity for dedicated laboratory space, highly trained personnel, and costly instrumentation means that alternative and complementary approaches are warranted. Many ambient ionisation MS (AIMS) setups utilise smaller footprint instruments, offer high-throughput workflows, and/or require reduced technical expertise for routine use. Therefore, the application of AIMS provides a tangible approach to increase measurement throughput at a reduced cost, serving as a potential screening mechanism to act as a prior and additive step in anti-doping protocols.

For the current work, atmospheric solids analysis probe (ASAP)-MS was used to develop a proof-of-concept project to rapidly (i.e. within minutes) and objectively identify the presence of prohibited substances in a series of performance enhancing medicinal products purchased from the black market. These included 16 products which had previously been tested and confirmed for the variable presence of eight different prohibited substances (categories S1 and S4) by LC-MS/MS. To maintain a rapid and reduced complexity approach, all products were either crushed (for tablets) or diluted in methanol (for gel capsules) and analysed directly by loading onto a glass capillary rod and inserting into the ASAP-MS source enclosure. The MS was set to capture ion spectra at increasing cone voltages (to induce in-source fragmentation) and completed in both positive (pos) and negative (neg) ionisation mode. The resultant data were investigated manually (i.e. subjectively) for presence of prohibited substances through the identification of known precursor and fragment ions. In addition to black market products, 11 products of differing formats (e.g. gels, powders, etc.) that had previously been batch tested via the UK's Informed Sport process were analysed to assess the risk of false positive results. Following this, pure reference standards were analysed and used to produce a spectral search library for each of the prohibited substances. The data files from the product analyses were assessed using the automatic identification software to assess the capability to provide a correct qualitative identification of one or more of the eight prohibited substances.

Subjective analyses of the acquired spectra correctly identified the presence of a prohibited substance in 88% (pos) and 69% (neg) of the black market products. Three products had multiple prohibited substances present in a mixture, with not one of these tests resulting in the correct subjective identification for all substances present. Subjective analyses of the Informed Sport batch-tested products resulted in the reporting of no positive matches for the eight investigated prohibited substances. Inclusion of the objective library searching slightly decreased the correct identification of a prohibited

substance within the sample (75% and 56% for pos and neg) but did allow for 2/3 of the products with substance mixtures to be fully identified. Not one false positive was identified through objective analysis of batch-tested products. The reduction in positive identification could be overcome through alteration of search parameters to allow inclusion of lower abundant ions within the search function. Predictive quality of the automated search function (including all black market and batch-tested products) assessed by prohibited substance yielded an average sensitivity of 78% and 50% (pos and neg), with specificity at 96% and 98% (pos and neg). When considered by sample, sensitivity values were reported at 80% and 60% (pos and neg), and specificity at 92% for both ionisation modes. Interrogation of data highlighted the success for identification depended on ionisation mode and a search function was built to utilise the preferential ionisation mode for each analyte. From this, an average sensitivity of 86% and specificity of 96% when considered by analyte, and sensitivity of 88% and specificity of 100% when considered by sample were reported. Importantly, when all black market product analyses were considered, only one product (6%) yielded a result which failed to correctly identify the presence of a prohibited substance in either positive or negative ionisation mode.

This proof-of-concept study demonstrated the capability to rapidly analyse products for the presence of prohibited substances applying a low complexity, minimal-preparation approach. This utilised ASAP-MS as an exemplar technique falling under the AIMS umbrella. This approach offers promise to reduce the risk of inadvertent doping through positive identification of contaminated or adulterated products that are not batch tested. Future work to develop comprehensive and widespread libraries of prohibited substances is necessary. Additionally, the small footprint of the instrumentation affords the opportunity to deploy the instrumentation into the field (e.g. in an event stadium) for on-site analyses.

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

# Investigations into the metabolism and elimination of tesofensine in urine

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

## Abstract

Tesofensine ((1R,2R,3S,5S)-3-(3,4-dichlorophenyl)-2-(ethoxymethyl)-8-methyl-8-azabicyclo-[3.2.1]octane; NS-2330) is an active pharmaceutical ingredient that has shown a positive effect on weight loss in obese patients in clinical studies. The active ingredient is currently still in the approval process for marketing as a medicinal product. However, food supplements containing tesofensine can already be ordered on the Internet. These are advertised as “Weight Management & Metabolic Boost”. Weight-controlling substances are also of interest to competitive athletes. This mainly affects sports with weight classes and, for example, in cases where the “power to weight” ratio is decisive. Tesofensine is subject of class “S6 stimulants” of the WADA Prohibited List and is therefore only prohibited in-competition. For the development of the necessary detection methods, excretion studies with these substances are required. These are essential for effective implementation of anti-doping measures to protect the health of athletes and ensure fair competition.

To date, the effect and elimination of tesofensine and a dealkylated metabolite have been investigated and described in the literature. However, the structure of the metabolite has not been described in detail. To gain an initial idea of which potential metabolites could be detectable in an excretion study, an *in-vitro* assay was performed with enzymes from human microsomes and S9 fraction. The metabolism and elimination profiles of tesofensine and its metabolites in urine were investigated subsequently with six male and female volunteers. Following ingestion of a 410 µg dose of tesofensine in the form of a dietary supplement, urine samples were collected over 500 h (3 weeks).

The urine sample preparation was conducted by solid-phase extraction (SPE) with Oasis® HLB cartridges (3 mL, 60 mg). After conditioning with 1 mL of methanol and 1 mL of $H_2O$, the urine samples (2 mL) were fortified with internal standard ($D_3$-testosterone) and loaded for SPE. The samples were washed with 1 mL of $H_2O$ and the analytes were eluted with 1 mL of MeOH. Following evaporation to dryness, the residues were reconstituted in 100 µL of $H_2O$/ACN (50:50; v/v).

The LC-HRMS analysis of the target analytes was conducted using a Vanquish UHPLC chromatograph coupled via a HESI source (3.0 kV in positive mode) to a ThermoScientific Exploris™ 480 mass spectrometer. The chromatographic separation was achieved using an Agilent® poroshell 120 EC-C18 analytical column (50 x 3 mm; 2.7 µm particle size). The mobile phase was 0.1% FA (aq.) (solvent A) and 0.1% FA in ACN (solvent B). Gradient: 100% A at injection, decrease to 0% A over 5 min, the column was then flushed at 0% A for 1 min and subsequently re-equilibrated at 100% A for 4 min. The flow rate was set to 0.3 mL/min, the injection volume was 5 µL.

Both, the *in*-vitro assay and excretion urines showed four main metabolites, three dealkylated metabolites and one subsequently hydroxylated and glucuronidated metabolite were detected.

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M1: Desmethyl-tesofensine; M2: Desethyl-tesofensine; M3: Desethyl-desmethyl-tesofensine; M4: Desmethyl-hydroxytesofensine-glucuronide. The $MS^2$-product ion mass spectra showed dissociation patterns attributable to the cleavage of the tropane structure for tesofensine and its dealkylated metabolites. Furthermore, product ions originating from the loss of the aliphatic side chain and, in case of desmethyl-hydroxytesofensine-glucuronide, also of the loss of glucuronic acid were observed (*m/z* 312).
The method was validated for the quantification of tesofensine in urine. Results were e.g. recovery of 34%, an LOD of 0.01 ng/mL, and an interday imprecision of 8% at a concentration of 1 ng/mL.
Regarding the elimination profiles of target analytes, it was found that the interindividual $C_{max}$ values and the maximum detection windows were in a wide range. The $C_{max}$ values of tesofensine (1-4 ng/mL) were reached after 4 to 46 h, and the maximum detection window was found between 238 and 500 h. Similarly extensive variabilities were also observed for the monitored metabolites. Additionally, for M3, there might be differences of the levels in male and female subjects.
A quantitative method for the detection of tesofensine in urine was developed and validated. In addition, new metabolites of tesofensine were detected, their structure elucidated and their urinary elimination described. The study therefore makes a contribution to the expansion of analytical procedures and to the assessment of possible anti-doping offenses. In consideration of the long detection windows of both the intact drug and its metabolites, careful result interpretation is indicated in case of an AAF. In consideration of the currently applicable MRL for stimulants, the herein found urinary levels of tesofensine suggest that the commonly recommended dosing of the drug might rarely (if at all) result in concentrations that constitute an AAF.

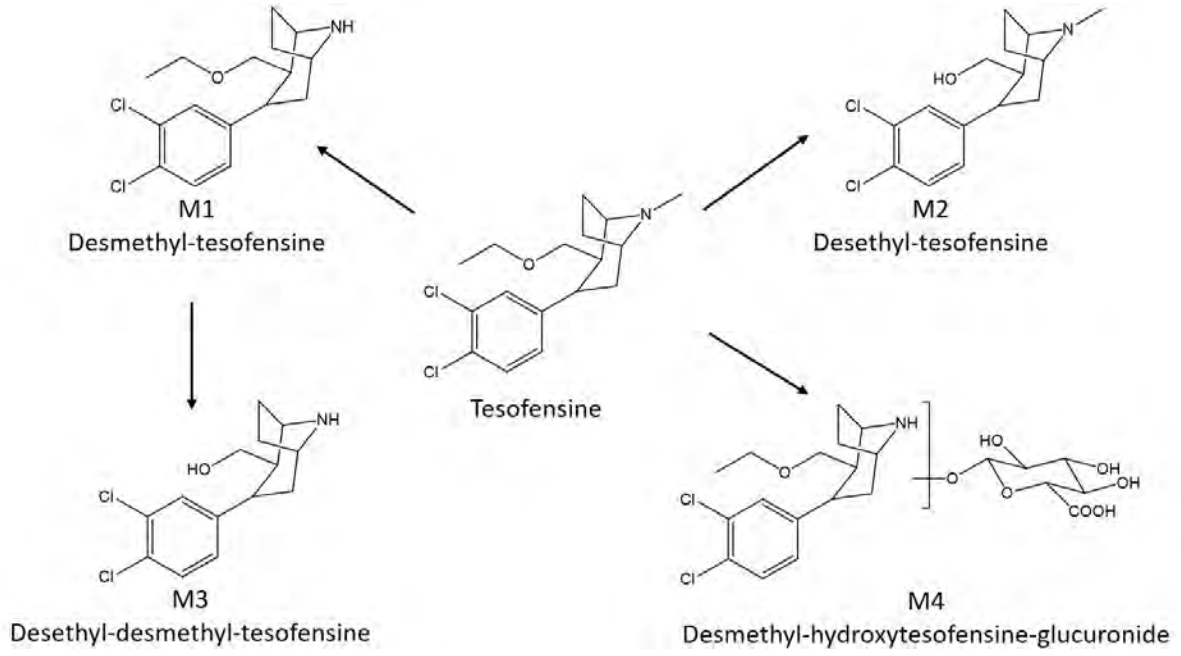


**Figure 1.** In-vitro and in-vivo produced tesofensine metabolites

The full manuscript has been submitted to *Drug Testing and Analysis*.

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Peters I[1], Naumann N[1], Thevis M[1,2]

# Characterization of the HepaRG 3D liver cell model for *in-vitro* prediction of doping-relevant substance metabolism by measuring CYP enzyme activities and expression levels

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

## Abstract

In preventive doping research, the prediction of potential metabolites of banned substances is of crucial importance, especially for non-approved substances classified under category S0 of the World Anti-Doping Agency (WADA) Prohibited List. To predict these metabolites, the Cologne laboratory is investigating the use of a 3D liver model composed of human HepaRG cells and primary human hepatic stellate cells, as well as its integration into an organ-on-a-chip system (TissUse, Berlin, Germany).
The objective of this study was to further characterize the 3D cell model by evaluating key cytochrome P450 (CYP) enzyme activities and their mRNA expression levels. Enzyme activities were assessed using a cocktail of seven model substrates corresponding to the major CYP isoforms 1A2 (phenacetin), 2B6 (bupropion), 2C8 (amodiaquine), 2C9 (tolbutamide), 2C19 (S-mephenytoin), 2D6 (propafenone) and 3A4 (midazolam), applied at 0, 7, 14 and 21 days of cultivation. The resulting supernatants were analyzed by HPLC-MS/MS with regard to the main metabolites formed by each CYP enzyme. To assess the functionality of the system by testing the inducibility of CYP enzyme expression, the 3D liver model was exposed to rifampicin for 72 hours, after which the substrate cocktail was administered again. Additionally, mRNA expression levels of six CYP enzymes (1A2, 2B6, 2C9, 2D6, 3A4 and 2E1) were determined by digital polymerase chain reaction (dPCR).
Enzyme activities were detected for all seven CYP isoforms, as indicated by the formation of their respective main metabolites. These activities remained detectable even after three weeks of cultivation in the organ-on-a-chip system, demonstrating the persistent metabolic activity of the system over the entire experimental period. Rifampicin treatment resulted in an induction of CYP activity, particularly for CYP3A4, and these findings were corroborated by increased expression levels measured by dPCR. CYP2C9 induction was primarily observed at the transcriptional level. For CYP2B6 and CYP2C19, an increase in enzyme activity was detected through metabolite formation, though this induction was less pronounced compared to CYP3A4.

*The details of this study will be published elsewhere.*

Krombholz S[1], Piper T[1], Lagojda A[2], Kühne D[2], Thevis M[1,3]

# The influence of alternative routes of drug administration on the metabolic profile of trenbolone in doping control urine samples

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## Abstract

**Objective:** In recent years, there has been an increasing number of cases in which adverse analytical findings (AAFs) in doping controls have been attributed to an unintentional dermal exposure of athletes to prohibited substances in their environment. Furthermore, it has been reported, that the transdermal or buccal absorption of anabolic androgenic steroids (AAS) for performance enhancement could be deliberately used to undermine analytical approaches in routine doping controls. Therefore, the extent to which alternative routes of drug administration might influence the detectability of doping substances or their (long-term) metabolites has become an important aspect of anti-doping research. Within this study, the AAS trenbolone (Tren) was used as a model substance to investigate the detectability and the urinary metabolite profile after oral, transdermal and buccal drug administration.

**Methodology:** An excretion study was conducted with a total of 12 healthy male volunteers. Each volunteer was administered 10 mg of Tren either orally in a capsule (n = 4), transdermally in the form of a cream containing DMSO on the lower forearm (n = 4) or buccally, by swirling around an alcoholic solution of trenbolone in their mouth (n = 4). Both before and up to 7 days after administration, urine samples were collected by the volunteers. The study was approved by the local ethics committee (#119/2024) of the German Sport University Cologne (Germany), and all participants provided written consent. Following enzymatic hydrolysis with ß-glucuronidase, the samples were concentrated by solid-phase extraction (SPE) and screened for previously identified, as well as novel *in silico* generated metabolites of Tren using high performance liquid chromatography-high resolution tandem mass spectrometry (HPLC-HRMS/MS). The method used for the quantitative determination of Tren and 17α-trenbolone (Epitren) was validated based on the WADA ISL with additional parameters, where deemed appropriate. Trenbolone-diol conjugated to cysteine (cystein-S-yl-trendiol) was synthesized and purified by SPE and semi-preparative HPLC. The synthetic product was comprehensively characterized by LC-HRMS/MS and nuclear magnetic resonance (NMR) spectroscopy.

**Results:** The method used for the quantitative determination of Tren and Epitren was validated and found to be selective and linear from 10 to 500 ng/ml with a LOD of 0.12 ng/ml for Tren and 0.06 ng/ml for Epitren. Precision was determined to be < 10 % and the sample extracts proved to be sufficiently stable for 72 h at 10 °C. No carry-over effects were observed at 500 ng/ml. Application of trenbolone via the skin resulted in prolonged detection windows of the parent compound and its metabolites, although the measured signals in the collected urine samples were significantly lower compared to oral or buccal drug administration. In contrast to that, the detection windows following buccal application were

markedly shorter than after oral ingestion. Irrespective of the route of administration, Epitren, cysteine-conjugates of Tren and a hydroxy-Tren derivative (M5) were identified as the main urinary metabolites. Following transdermal and buccal administration, increased formation of a cysteine-conjugated Tren derivative (M15-cys) was observed, resulting in an elevated peak area ratio of M15-cys/Tren as depicted in Figure 1. To confirm the structure of the analyte organic synthesis of Trendiol-cys was conducted and analyzed by LC-HRMS. In addition, the synthetic product was characterized by $^{1}H$ and $^{13}C$-NMR, including 2D techniques, which determined the structure to be 12α-cystein-S-yl-estra-5,9-dien-3x,17ß-diol.

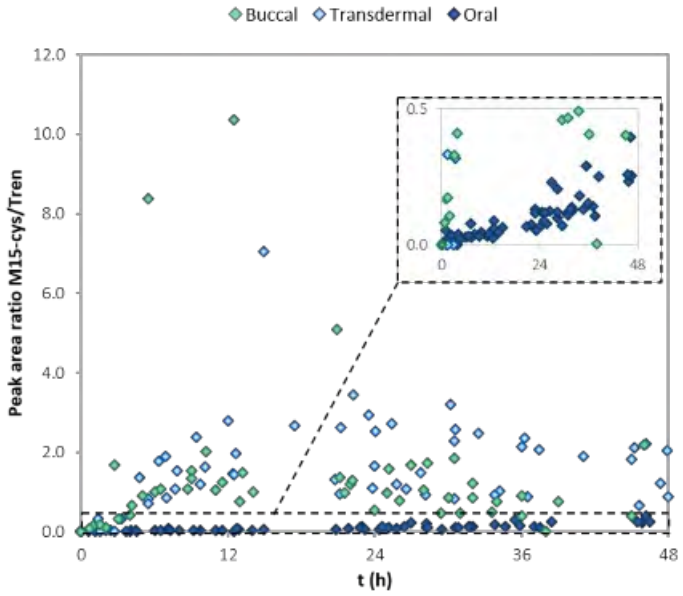


**Figure 1.** Scatter plot of M15-cys/Tren peak area ratios calculated for all samples collected until 48h post-administration. Values obtained after oral administration are shown in dark-blue, values after transdermal administration in light blue, values after buccal administration in green

**Conclusion:** While oral and parenteral steroid administration remain most common, alternative ways of drug administration like transdermal or buccal are becoming increasingly important in the interpretation of doping control findings. This study demonstrated, that Tren and its major metabolites were detectable in urine samples regardless of the administration route, supporting robust anti-doping efforts. However, detection times varied significantly. Notably, a metabolite (M15-cys) was identified, which could serve as a marker to indicate application of the steroid via the skin/mucosa. Future research should be dedicated to the question as to how these results can be transferred to other AAS, as potential biomarkers that provide a closer evaluation of the route of administration (e.g. transdermal/oral) could help to assess the plausibility of (presumed) dermal contamination scenarios.

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# Identification of metabolites of the novel 5α-reductase inhibitor epristeride *in vitro* and *in vivo* and its potential impact on doping testing: A computational chemistry approach

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Research Institute for Doping Control, Shanghai University of Sport, Shanghai, China[2]

## Abstract

Epristeride, a novel non-competitive inhibitor of type II 5α-reductase, has emerged as a potential therapeutic alternative for benign prostatic hyperplasia (BPH). Given that other 5α-reductase inhibitors, such as finasteride and dutasteride, are already monitored for their potential impact on doping detection, comprehensive metabolic studies of epristeride are crucial for doping control applications. This study aims to investigate the metabolic characteristics of this novel inhibitor drug and elucidate its metabolic pathways from the perspective of computational chemistry, *in vitro* microsomes, and *in vivo* zebrafish models, thereby providing a scientific basis for assessing its potential risks to athletes and doping detection. Metabolite profiling was performed using liquid chromatography-high resolution mass spectrometry (LC-HRMS), with data acquisition facilitated by Xcalibur 4.2 software and metabolite identification facilitated by Compound Discoverer 3.3. By employing network pharmacology and integrating high-throughput data, the potential targets of thiazolidinedione are predicted. The binding energy of the molecular docking between the compound and the target protein is calculated using AutoDock Vina software to predict its impact on steroid metabolism. The study identified three primary metabolites of epristeride: two phase I oxidation products (M1 and M2) and one phase II glucuronidation product (M3). Pathway analysis revealed that among the five CYP450 isoforms examined, CYP3A4 played a dominant role in epristeride metabolism, highlighting its significance in the compound's biotransformation. Additionally, network pharmacology and molecular docking studies were employed to predict epristeride’s potential impact on steroid metabolism. The docking results identified five key target proteins (ESR1, CYP19A1, STAT3, AKR1C3, and CYP17A1) with low binding energies, indicating stable interactions. Notably, phase I metabolites (M1 and M2) showed significant binding potential with these targets, suggesting their possible involvement in modulating steroid pathways, whereas the phase II metabolite (M3) exhibited lower binding stability. These findings provides a detailed understanding of epristeride’s metabolic pathways and its potential biological impacts, offering valuable insights for monitoring its presence in athletes and preventing its misuse in sports.

**Keywords:** epristeride, 5α-reductase inhibitor, microsome, zebrafish models, CYP450 enzyme, molecular docking

Alhalabi H, Korsmeier L, Thomas A, Thevis M

# Investigations into the urinary metabolite elimination profile of the selective androgen receptor modulator S-23 in studies mimicking contaminated product ingestion for doping control purposes

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## Abstract

Selective androgen receptor modulators (SARMs) have repeatedly been reason of adverse analytical findings (AAFs) in routine doping controls. Among these, S-23 has been identified in five AAFs reported in 2022. In addition to intentional doping, inadvertent exposure through contaminated dietary supplements has emerged as a significant concern, purportedly as well as evidently contributing to AAFs involving SARMs. Thus, the differentiation of inadvertent intake and intentional abuse of S-23 is of growing relevance.

This study aimed at investigating the urinary concentration profile of microdosed S-23 and to characterize the elimination pattern of the compound and its metabolites over time. Single- and multi-dose administration studies with 1, 10, and 50 µg of S-23 were conducted, and collected urine samples were analyzed by LC-MS/MS following enzymatic hydrolysis and solid-phase extraction. The analytical method was validated for a semi-quantitative detection of S-23, and characterized by a limit of detection of 1 pg/mL. A total of 18 metabolites was detected in human *in vivo* samples following oral administration of microdosed S-23. Moreover, the study demonstrated that a single dose of 1 µg can be detected for an average of up to 253 hours, while a single dose of 50 µg can be detected up to 544 hours on average. Data on urinary metabolite elimination patterns can contribute to assess the plausibility of an inadvertent drug intake through contaminated dietary supplements.

Sakellariou P[1], Walpurgis K[1], Thomas A[1], Marchand A[2], Miller G[3], Dellanna F[4], Thevis M[1,5]

# Multiplexed detection of inhibitors of the activin receptor signalling pathways in human samples by means of (immuno-) affinity purification, tryptic digestion and LC-HRMS/MS

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## Abstract

Members of the transforming growth factor-β (TGF-β) superfamily such as activin A (ActA), myostatin (MSTN) and growth differentiation factor-11 (GDF-11) are dimeric cytokines signalling through activin type II receptors A & B (ActRIIA/B) and regulate key physiological processes including skeletal muscle development, tissue repair and erythropoiesis. Inhibition of these pathways enhances muscle mass and red blood cell production. Therefore, inhibitors of the activin receptor signalling pathways (IASPs) have attracted interest both as therapeutics for muscle wasting diseases and disorders of erythropoiesis, but have also gained attention for their potential to promote muscle growth and improve athletic performance and are prohibited under sections S2 and S4 of the World Anti-Doping Agency (WADA) Prohibited List. The neutralization and interception of the receptor ligands are two of the different strategies that can be adopted by the misuse of humanized/fully human therapeutic antibodies (Garetosmab, Apitegromab, Landogrozumab, Stamulumab, and Domagrozumab) or fusion proteins from the extracellular domain of activin receptors and a human immunoglobulin Fc fragment (Sotatercept, Elritercept, Luspatercept and Ramatercept). Within the last years, doping control detection methods using electrophoretic techniques, Western blotting, immunoassays or liquid chromatography - high resolution tandem mass spectrometry (LC-HRMS/MS) have been developed for several drug candidates. This is particularly crucial because, even in the absence of clinical approval, reference materials for these emerging drugs are often accessible for research or can be traded on the black market.

Within this research project, a multiplexed detection assay for the nine different IASPs mentioned above from doping control serum/plasma samples was developed (**Figure 1**). A combined extraction of the target analytes was achieved by using NHS magnetic sepharose beads coupled to different TGF-β cytokines (ActA, MSTN, proMSTN and GDF-11) and a goat anti-ActRIIB antibody, with a preceding ammonium sulfate precipitation step. Following elution, samples extracts were proteolytically cleaved into proteospecific tryptic peptides which were monitored by LC-HRMS/MS. Unique peptides were selected *in silico* and confirmed experimentally, allowing differentiation even between closely related fusion proteins such as Luspatercept, Ramatercept and Elritercept.

The method was validated according to WADA guidelines and showed high selectivity and sensitivity. Limits of detection (LODs) ranged from 10 to 50 ng/mL depending on the analyte, with linear response up to 500 ng/mL and stable extracts for at least three days at 10 °C. Carryover was not detected after

high-concentration injections, and beads could be re-used after rigorous washing without measurable cross-contamination. Authentic Luspatercept and Sotatercept post-administration serum samples were successfully analysed as proof of concept. Luspatercept was detected in serum up to 70 days after the first administration and seven weeks after the second, while Sotatercept was detected at in serum collected 43 hours post-injection. Additionally, the method was modified to allow for the use of urine instead of serum, and Luspatercept post-administration urine samples were analysed. Luspatercept was also measurable in urine up to 70 days post-dose in three volunteers and 28 days in one, albeit at concentrations 100–1000 times lower than in serum.

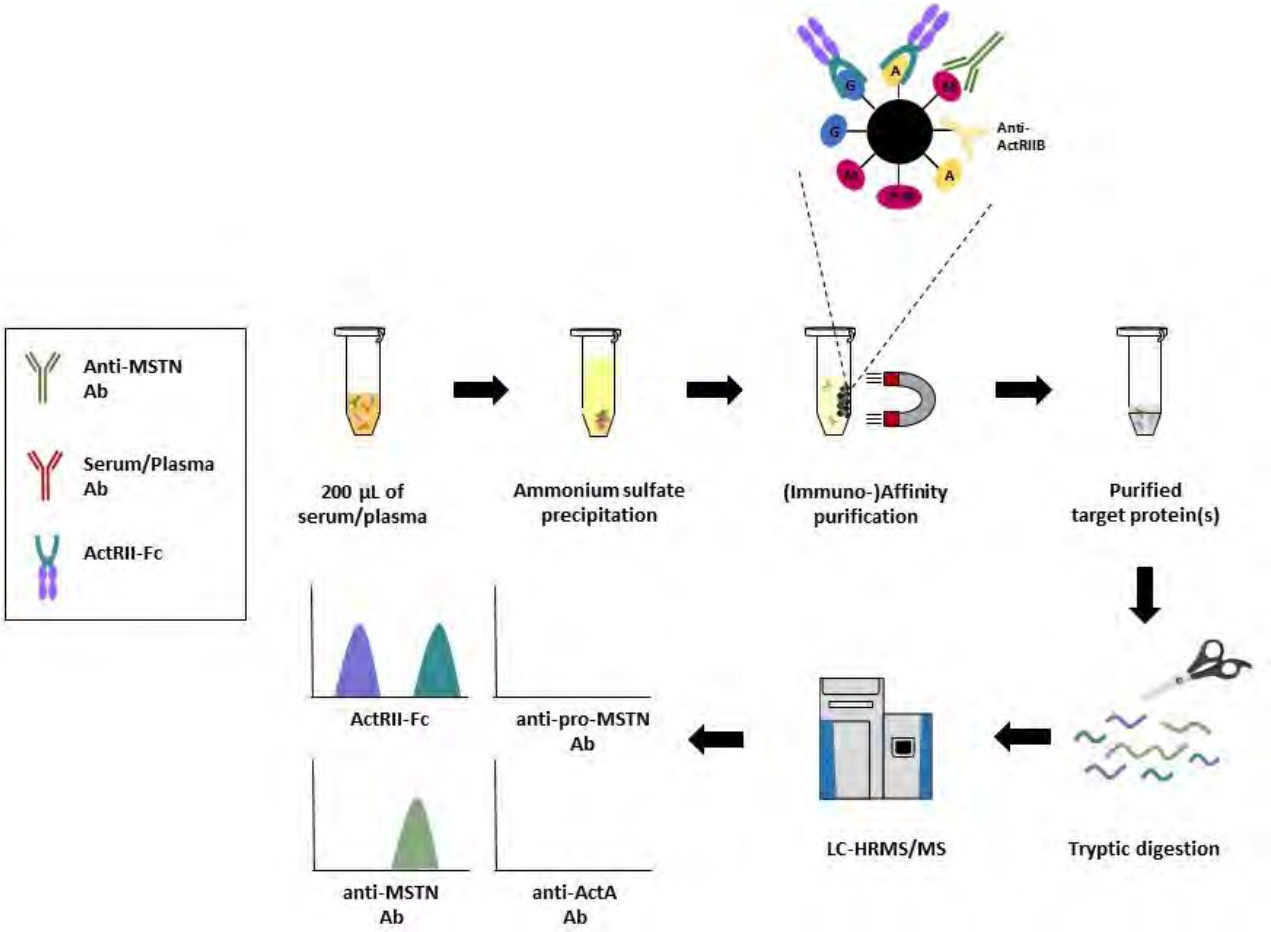


**Figure 1.** Method overview

The present method can be employed in doping control routine analysis as an initial testing procedure enabling both the clear identification of the target analytes at the amino acid level and a retrospective evaluation of the samples for the presence of novel IASPs binding to one of the TGF-β cytokines or the anti-ActRIIB antibody used during (immuno-)affinity purification.

Data published in:
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Piper T[1], Fusshöller G[1], Thevis M[1,2]

# Investigations into the human metabolism of trestolone (7α-methyl-19-nortestosterone, MENT) – metabolites suitable for routine

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

## Abstract

Already in the 1960s, the anabolic properties of MENT (7α-methyl-19-nortestosterone) were investigated in the context of cancer research, and MENT was found to be 10-times more potent regarding its anabolic properties compared to testosterone. The human metabolism of MENT was only investigated once in an anti-doping context, and three urinary metabolites were identified, corroborating earlier findings from *in-vitro* and animal experiments. Based on these metabolites, no doping control sample was reported to contain MENT or its metabolites in the last two decades albeit MENT is readily available via online distributors. One reason for the lack of adverse analytical findings in doping controls could be analytical challenges originating from the chromatographic properties of MENT and its urinary metabolites. Therefore, the human metabolism of MENT was reinvestigated employing an excretion study with deuterated MENT and metabolite detection based on hydrogen isotope ratio mass spectrometry in combination with high accuracy/high resolution mass spectrometry. Considering unconjugated, glucuronidated and sulfated metabolites, 50 potential candidates were detected. In order to identify those metabolites suitable for sports drug testing, three volunteers administered a single oral dose of non-deuterated MENT, and all post-administration samples were investigated using triple quadrupole mass spectrometry-based determinations routinely employed in doping controls. From the 50 metabolites detected, two showed promising results with respect to their detection windows and suitability under routine measurement conditions. The specificity of the novel metabolites was ensured by the reanalysis of 200 routine doping control samples demonstrating the absence of potential co-eluting compounds.

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Sakellariou P[1], Thomas A[1], Garzinsky A[1], Schröder S[1], Thelen J[1], Thevis M[1,2]

# Simultaneous detection of testosterone, nandrolone and boldenone esters in combination with non-MRL substances in dried blood spots by LC-HRMS/MS as a comprehensive approach for doping control purposes

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European Monitoring Center for Emerging Doping Agents, Cologne, Germany[2]

## Abstract

Anabolic androgenic steroids (AASs) have been the most frequently detected class of prohibited substances in sports, according to the statistics of the World Anti-Doping Agency (WADA), largely because of their capacity to increase muscle mass, enhance power and endurance, and shorten recovery times. Testosterone, nandrolone and boldenone are usually administered for doping purposes as commercially available esterified derivatives to prolong the half-life of the steroids and reduce the frequency of injections. In the bloodstream, the esters are hydrolysed by blood esterases, becoming fully active. The direct detection of steroid esters leads to unequivocal proof of doping since such esters are not of endogenous origin. However, since ester concentrations in blood are relatively low and the intact esters are barely excreted via the kidneys and therefore cannot be directly detected in urine, their detection requires highly sensitive analytical strategies and appropriate biological matrices. The elimination of humidity via dehydration and the inactivation of enzymatic degradation make dried blood spots (DBS) a more appropriate sample matrix for the detection of esters. Within the last years, doping control analytical methods for the detection of several steroid esters in blood or DBS by liquid chromatography high resolution tandem mass spectrometry (LC-HRMS/MS) have been presented.
Depending on the type of the absorbent device, three to four spots per athlete's sample are available for the initial testing procedures (ITP) and the possible confirmation procedures (CP), thus, the spot number is the limiting factor of multi-analyte testing. In this study, a method for the simultaneous detection of 15 esters (9 testosterone, 3 nandrolone and 3 boldenone) along with 85 non-threshold substances without minimum reporting level (MRL) substances in DBS by LC-HRMS/MS was developed. After extract splitting, the esters were derivatised as oximes to improve stability and ionization efficiency, and combined sample preparation enabled parallel analysis of both esters and non-MRL substances, by using the same single spot followed by three different injections in the LC-HRMS/MS system. The ITP-method was validated in two types of absorbent devices, DMPK-C cellulose and a polymer resin (Tasso M20), according to WADA guidelines, and was found to be selective and robust for all the target analytes with limits of detection (LODs) from 0.1 to 0.5 ng/mL (for all esters). As a proof-of-concept, testosterone undecanoate was detectable up to 5 h after administration in DBS samples from a male volunteer who received 80 mg oral testosterone undecanoate. Validation results demonstrate that this method can be employed in doping control routine analysis, simplifying the analytical workflow, reducing sample volume requirements, and maximizing information obtained from each sample.

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Goodrum J, Nair V, Eichner D, Miller G

# Evaluating the utility of the serum T/LH ratio compared to the serum T/A4 ratio and the urinary T/E ratio

The Sports Medicine Research and Testing Laboratory (SMRTL), Salt Lake City, United States

## Abstract

Testosterone (T) is one of the most commonly abused performance enhancing drugs despite being banned for use by athletes. A common route of administration involves the intramuscular injection of a testosterone ester of which there are numerous varieties, including longer acting “long chain” esters and shorter acting “short chain” esters. While the detection of testosterone abuse can be challenging, both direct and indirect methods are utilized with varying degrees of success depending on factors such as testosterone preparation type, the route of administration, and individual factors. Current indirect testosterone detection methods include the urinary testosterone/epitestosterone (T/E) ratio in the Steroidal Athlete Biological Passport module and the testosterone/androstenedione (T/A4) ratio in the Serum Athlete Biological Passport module. Additionally, the longitudinal measurement of luteinizing hormone (LH) and a T/LH ratio shows promise in indirectly inferring testosterone use. In order to evaluate the ability of these methods to indirectly detect testosterone ester abuse, fifteen recreationally active, male individuals were administered a single dose of either a short chain testosterone ester (testosterone propionate, n = 8) or a long chain testosterone ester (testosterone cypionate, n = 7). Blood and urinary steroid profiles were generated for each individual, including the urinary T/E ratio, serum T/A4 ratio, and the serum T/LH ratio. This talk will explore and compare the effectiveness of these tools used for detecting testosterone ester usage. Data from other transdermal, oral, and multi-dose testosterone studies performed at our lab will also be shown for comparison.

**This year's Manfred-Donike-Award for the best oral presentation recognized the excellent work by Dr. Jenna M. Goodrum and her co-authors from the Sports Medicine Research and Testing Laboratory, Salt Lake City, Utah, USA, which advances the scientific understanding of endocrine markers in anti-doping analysis. By evaluating the serum testosterone-to-luteinizing hormone ratio using both a controlled transdermal testosterone administration trial and field-collected serum samples, the authors bridge experimental research with real-world anti-doping practice. Their findings demonstrate the added value of the serum T/LH ratio as a complementary biomarker, contributing to more robust and evidence-based screening strategies for the detection of exogenous testosterone use.*

 ISBN 978-3-9828334-0-8

Fedoruk M[1], Lewis L[1], Nicoli R[2], Sobolevsky T[3], Ahrens E[3], Bohl A[1]

# Managing dorzolamide adverse analytical findings: Trends and solutions

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UCLA Olympic Analytical Laboratory, Los Angeles, United States[3]

## Abstract

Carbonic anhydrase inhibitors (CAI), including dorzolamide and brinzolamide, are listed on the WADA Prohibited List and prohibited in sport except when administered by topical ophthalmical route of administration. The results management process requires that anti-doping organizations confirm that the athlete was using the CAI by the permitted route of administration. This can be time consuming and cause athletes anxiety to receive notification of an adverse analytical finding. The majority of athletes are using CAIs to treat legitimate medical conditions. Since 2018, USADA has collected almost 8 years of data on almost 70 dorzolamide AAFs, primarily in para sports, involving 20 male and female athletes. Some athletes have multiple AAFs which make it informative as to the variability of urinary concentrations observed over time. CAIs also accumulate in erythrocytes, therefore their excretion can be prolonged over time. This presentation will share USADA's observations and offer some suggestions as to how to improve the management of CAIs. Specific case examples will also be presented in urine and DBS.

 ISBN 978-3-9828334-0-8

Nair V, Goodrum J, Eichner D, Miller G

# Stability of testosterone esters in serum after administration and increased detection windows relative to dried blood spots

The Sports Medicine Research and Testing Laboratory (SMRTL), Salt Lake City, United States

## Abstract

There is increased focus on dried blood spots (DBS) as a matrix for antidoping. One of the targets of DBS is testosterone (T) doping by direct detection of the administered ester. Dried blood spots have been suggested as a superior matrix compared to serum for this test since it has been postulated that serum esterases may cause hydrolysis of the ester and reduce detectability. On the other hand, DBS provides limited sample volume which may challenge the sensitivity of the detection method. An in-vitro experiment was carried out by spiking known amounts of T enanthate in male and female serum samples and monitoring for decrease in concentration over different storage conditions. Further, an administration study was conducted with T cypionate in male subjects. Serum was collected in SST tubes, while plasma was isolated from whole blood collected in sodium fluoride tubes which have been demonstrated to minimize esterase activity. The samples were subjected to different storage conditions and monitored over time for decrease in the ester concentration. Finally, detectability in post administration samples was compared between serum and simultaneously generated dried blood spots. Data from these studies will be presented.

Sternberg J[1], Peters I[1], Naumann N[1], Thomas A[1], Thevis M[1,2]

# Preliminary results on the *in vitro* metabolism of doping agents (stanozolol, LGD-4033, anastrozole, GW1516, trimetazidine) by seminal vesicle and pooled human liver fractions

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

## Abstract

Recent studies have shown that many doping and clinically relevant substances and their metabolites are transferred into seminal fluid. This circumstance might necessitate consideration in the context of Adverse Analytical Findings (AAF) in anti-doping cases where female athletes claim contamination of their doping control urine sample by seminal fluid residues. Some metabolites, indicative of specific routes of administration, help to distinguish contamination from intentional drug use. In vitro experiments with subcellular fractions such as human liver cellular fraction (HL-S9) and seminal vesicle cellular fraction (SV-S9) can provide insight into tissue-specific drug metabolism and thus identify specific metabolites.

This study investigated the metabolic activity of SV-S9 (donor source: single individual) compared to HL-S9 (donor source: pooled, n=50) using substances frequently implicated in doping, including stanozolol, LGD-4033, GW1516, trimetazidine, and anastrozole via liquid chromatography high-resolution mass spectrometry (LC-HRMS). Findings suggest that SV-S9 exhibits low metabolic activity for LGD-4033, trimetazidine and GW1516. No metabolism of stanozolol and anastrozole was observed in SV-S9, in contrast to the well-documented liver metabolism. In addition to the activity of the CYP450 proteins of the S9 fractions, the cellular expression of CYP450s (CYP2D6, 2E1, 2C9, 3A4, 1A2) in HL-S9 and SV-S9 was be determined using digital polymerase chain reaction (dPCR). CYP2C9, CYP2E1 and CYP2D6 were detected in HL-S9 and SV-S9, whereas CYP3A4 and CYP1A2 could only be detected in HL-S9.

These findings are consistent with previous studies and case reports on this topic and emphasise that, as expected, the role of the seminal vesicle in biotransformation is minor. Although there may be some low-level metabolic activity, so far, there are no metabolic markers to distinguish contamination of urine with seminal fluid.

Published as:
Sternberg J, Peters I, Naumann N, Thomas A, Thevis M. In Vitro Metabolism of Doping Agents (Stanozolol, LGD-4033, Anastrozole, GW1516, Trimetazidine) by Human Seminal Vesicle and Liver Fractions. *Metabolites*. 2025 Jul 4;15(7):452. doi: 10.3390/metabo15070452.

Eleftheriou D[1,2], Neocleous T[2], Piper T[3], Thevis M[3,4]

# Enhancing doping control analysis in athletes' steroid profile: A multivariate Bayesian learning approach

Leiden Academic Centre for Drug Research, Leiden University, Leiden, The Netherlands[1];
School of Mathematics and Statistics, University of Glasgow, Glasgow, United Kingdom[2];
Institute of Biochemistry, German Sport University, Cologne, Germany[3];
European Monitoring Center for Emerging Doping Agents (EuMoCEDA), Cologne/Bonn, Germany[4]

## Abstract

Doping has been widely discussed in recent years and remains a challenging topic in the athletic world. In competitive sports, anabolic androgenic steroids refer to the most frequently detected drug class in doping controls according to the World Anti-Doping Agency. In order to detect the administration of such prohibited substances like testosterone, the steroidal module of the Athlete Biological Passport has been developed. Therefore, biomarker analysis of athletes' urinary steroid profiles is crucial for the anti-doping efforts. Current statistical analysis methods generate personalised limits for each athlete based on univariate modelling of longitudinal biomarker values from the urinary steroid profile. However, simultaneous modelling of multiple biomarkers has the potential to further enhance abnormality detection. In this work, we propose a multivariate Bayesian adaptive model for longitudinal data analysis, which extends the established single-biomarker model in forensic toxicology. The proposed approach employs Markov chain Monte Carlo sampling methods and addresses the scarcity of confirmed abnormal values through a one-class classification algorithm. By dynamically adapting decision boundaries as new measurements are obtained, the model provides robust and personalised detection thresholds for each athlete. We tested the proposed approach on a database of professional athletes, which includes longitudinal steroid profiles containing samples classified as normal, atypical, or confirmed abnormal. Our results demonstrate improved detection performance, highlighting the potential value of a multivariate approach in doping detection.

**Keywords:** Anti-doping, Bayesian adaptive model, forensic toxicology, longitudinal biomarker data, multivariate analysis, one-class classification, sports, urinary steroid profile

Aguilera R[1], Wolff K[1], Ventura R[2], Coll S[2], Ramirez R[2]

# The steroid mass balance. Complementary approach to the steroid adaptive model and the IRMS analysis

Drug Control Centre King's College, London, Great Britain[1];
Barcelona Antidoping Laboratory, Barcelona, Spain[2]

## Abstract

In 2014 WADA implemented the steroidal module of the Athlete Biological Passport (ABP) for the longitudinal monitoring of urinary testosterone and its metabolites to identify suspicious samples reflecting the use of synthetic forms of endogenous anabolic androgenic steroids (EAAS). This model consists of six urinary markers involved in the metabolism of endogenous steroids – testosterone (T), epitestosterone (E), 5α-androstane- 3α,17β-diol (5αAdiol), 5β-androstane-3α,17β-diol (5βAdiol), androsterone (A) and etiocholanolone (Et) and the ratios T/E, A/T, A/Et, 5αAdiol/5βAdiol and 5αAdiol/E. Today, the steroid profile is reported in the Anti-Doping Administration and Management System (ADAMS), where a sample is matched to other samples collected from the same athlete and a longitudinal profile is generated. If a T/E ratio is outside the reference limits, the system will generate an atypical profile notification sent to the laboratory as an ATPF. If the T/E is confirmed to be atypical, an GC/C/IRMS analysis should be performed on the sample. However, 98.7 % of the samples triggered by the adaptive model for confirmations of the Steroid Profiles and the IRMS analysis are negative. Moreover, many samples analysed fall below the limit of quantitation (LOQ) where the GC/C/IRMS values cannot be obtained during the analysis. To improve the efficiency of the process a new complementary approach is proposed related to the mass balance of the six urinary markers that could help to rationalise the confirmation procedures that trigger the confirmation analysis and IRMS test.

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Nair V, Howa J, Campbell T

# Trends in IRMS analysis of boldenone and 19NA

The Sports Medicine Research and Testing Laboratory (SMRTL), Salt Lake City, United States

## Abstract

The existence of nandrolone preparations with pseudoendogenous values has documented extensively. It has been noticed that over the last four years, the distribution of delta values for 19NA in samples analyzed in our laboratory has changed slightly, which has resulted in a slight increase in IRMS positivity. On the other hand, the isotope values of boldenone and boldenone metabolite are consistently in the exogenous range. However, there has been an increase in positivity in samples arising from certain geographical locations. This supports the possibility of these findings resulting from unintentional food contamination.

Retrospective data from four years of IRMS analysis for boldenone and 19NA will be presented. Finally, the incidence of samples with unusually enriched isotope values for the primary endogenous reference compound (pregnanediol) will also be presented.

Piper T[1], Drewes J[1], Thevis M[1,2]

# Accelerating the sample preparation of sports drug testing samples by employing supercritical fluid chromatography for sample clean-up – first results

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

## Abstract

Doping control samples showing elevated urinary concentrations of testosterone or testosterone metabolites are forwarded to isotope ratio mass spectrometry-based determinations in order to differentiate between naturally elevated concentrations and doping offenses. The sample preparation encompasses liquid-liquid and solid phase extraction steps and enzymatic deconjugation of steroid glucuronides. In order to separate all steroid of interest from the biological matrix and to obtain sufficiently clean urinary extracts, high performance liquid chromatography (HPLC) with fraction collection is currently employed in routine doping controls. This preparation step is very time consuming as each HPLC run requires approx. 45 min, and the evaporation of collected fractions containing water and acetonitrile lasts for up to 90 min.

Supercritical fluid chromatography (SFC) employs liquid carbon dioxide as eluent combined with other organic solvents like methanol or acetonitrile as modifiers. The unique physiochemical properties of liquid carbon dioxide enable to accelerate the chromatographic separation of different compounds and subsequently collected fractions only contain the modifier and additional methanol as make-up solvent which enables a very fast evaporation within 10 minutes.

Considering the potential benefits, a method was developed and is going to be validated in-line with current World Anti-Doping Agency-based regulations for doping control purposes encompassing testosterone, epitestosterone, dehydroepiandrosterone, androsterone, etiocholanolone, 5α- and 5β-androstanediol as target analytes and cholesterol, pregnanediol, 16-androstenol, and 11-oxo-etiocholanolone as endogenous reference compounds. An additional focus was set on potential isotopic fractionation during the SFC-based separation and the fraction collection process. Investigations into reference population derived carbon isotope ratios especially of cholesterol will finalize this approach.

*The full article will be published elsewhere.*

de La Torre X, Colamonici C, Botrè F

# Confirmation of AICAR by isotope ratio mass spectrometry

Laboratorio Antidoping FMSI, Rome, Italy

## Abstract

AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside) is an AMP-activated protein kinase activator. In addition to the recognized potential uses in therapeutics, some activities as its involvement in glucose uptake stimulation and increase of protein kinases α and β in skeletal muscle tissue, make them attractive in sports as doping agents. This finally led to its inclusion on the WADA list of prohibited compounds and methods in section S4.4.1 Activators of the AMP-activated protein kinase (AMPK).
Since this is an endogenous compound involved in several metabolic routes, its mere detection in urine does not allow a definitive proof of its use as a doping agent. Some detection limits based on population studies have been proposed for its use at the ITP level based on the absolute concentration or the ratio with SAICAR (AICAR/SAIAR), its metabolic precursor. However, a definitive confirmation by IRMS seems necessary as for other endogenous compounds (i.e testosterone and precursors).
A review of the methods by GC-C-IRMS proposed so far and the introduction of a new IRMS analytical approach will be presented showing that some alternatives worth to be investigated exist to produce a robust confirmation method for this substance.

Walpurgis K[1], Thomas A[1], Rauer A[1], Majer B[1], Sato M[2], Okano M[2], Al-Jaber M[3], Abushareeda W[3], Arsene C[4], Geyer H[1,5], Thevis M[1,5]

# Detection of the GH analogue somatrogon in doping control urine samples by means of LC-HRMS/MS

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Anti-Doping Laboratory, LSI Medience Corporation, Tokyo, Japan[2];
Anti-Doping Laboratory Qatar, Doha, Qatar[3];
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European Monitoring Center for Emerging Doping Agents (EuMoCEDA), Cologne/Bonn, Germany[5]

## Abstract

Somatrogon is a synthetic growth hormone (GH) analog, whose misuse in sports is prohibited at all times. As analytical approaches complementary to existing immunological GH detection methods were considered beneficial for its detection in doping control serum samples, a sensitive and specific qualitative mass spectrometric assay was recently developed, comprehensively characterized, and successfully employed to analyze authentic *in vivo* study samples. Within this follow-up project, this approach based on affinity purification, tryptic digestion, and LC-HRMS/MS was modified by implementing an additional ultrafiltration step to allow for the extraction of the intact drug and/or its metabolites from urine. Method validation demonstrated its specificity and sensitivity (LOD: 1 ng/mL), and the subsequent analysis of post-administration urine samples showed for the first time that intact (or at least GH receptor binding) somatrogon is excreted into urine and can be detected in most subjects for at least 96 h following injection. Consequently, anti-doping laboratories can also use urine to confirm the presence of somatrogon in athletes, where the GH differential isoform assay yielded atypical results in the corresponding serum specimens. Moreover, the latter assay also proved to be valuable as screening tool for the identification of urine samples potentially containing the GH analog.

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Walpurgis K, Thomas A, Rauer A, Majer B, Sato M, Okano M, Al-Jaber M, Abushareeda W, Arsene CG, Geyer H, Thevis M. Detection of the GH analogue somatrogon in doping control urine samples by means of LC-HRMS/MS. *Sci Rep*. 2025 Apr 16;15(1):13160. doi: 10.1038/s41598-025-96361-4.

Miller G, Goodrum J, Eichner D

# Implementing Tasso+ into routine testing: observations with ABP, BSP, and EPO analyses

The Sports Medicine Research and Testing Laboratory (SMRTL), Salt Lake City, United States

## Abstract

Much focus in recent years has been placed on alternative matrix testing and collection techniques, especially involving blood sampling. The Tasso+ device has become a popular choice as an alternative blood collection technique due to the potential of collecting relatively large volumes of blood from capillary sites with the ability to collect multiple tubes from a single puncture. Recent work has shown strong analytical correlation between venous and Tasso+ capillary collections for both whole blood and serum analytes.

Over the past year, SMRTL has implemented Tasso+ analysis into routine testing procedures, performing ERA, blood steroid profile, and ABP testing in over 1000 Tasso+ serum and whole blood samples. Benefits, areas for improvement, and other experiences with this technology will be shared in this one year retrospective.

Requena-Tutusaus L[1,2], Anselmo I[1], Coll S[1], Goodrum JM[3], Miller GD[3], Ventura R[1]

# Detection of recombinant erythropoietin single micro-dose using one dried blood spot: A feasible alternative to urine samples in doping control

Catalonian Antidoping Laboratory, Hospital del Mar Medical Research Institute, Barcelona, Spain[1];
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Sports Medicine Research and Testing Laboratory, Salt Lake City, USA[3]

## Abstract

The use of dried blood spot (DBS) microsampling devices is gaining prominence in anti-doping controls, also for the detection of erythropoietin receptor agonists (ERAs), substances widely analyzed in urine or blood. Detecting recombinant erythropoietin (rEPO) micro-doses, however, has been a persistent challenge in doping control and this difficulty is magnified with the use of DBS Tasso-M20®, where only 3 spots of 17.5 µL are available for the A sample analysis.

To comply with the new technical document TD2024EPO of World Anti-Doping Agency (WADA) establishing the requirements for the analysis of ERAs in DBS, firstly, two different MAIIA immunopurification methods using one single spot (cellulose and polymeric) have been fully validated. The stability of all analytes in polymeric DBS at different storage temperatures was also evaluated. Secondly, the methods were tested to detect rEPO (Retacrit®) 15 IU/kg single mico-dose in four healthy volunteers, comparing the detection window of one single polymeric DBS (Tasso-M20®) with the standard method using 15 mL of urine. Following immunopurification, SAR-PAGE electrophoresis was applied in all cases.

Limits of detection lower to those required by WADA for DBS were achieved using one polymeric or cellulose spot and all ERAs were stable up to at least 90 days at room temperature and at -20°C. Detection of rEPO micro-doses was also possible using one DBS and comparison of detection windows in DBS and urine will be presented.

The high stability of ERAs in DBS samples together with the high sensitivity of the actual electrophoretic techniques makes this matrix suitable to detect rEPO micro-doses using a single spot, applying the same SAR-PAGE electrophoresis method typically used in routine urine and blood analysis.

Salamin O, Ramic L, Nicoli R, Kuuranne T

# Discovery of candidate blood biomarkers of recombinant human erythropoietin administration using targeted metabolomics

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

## Abstract

The detection of erythropoiesis-stimulating agents (ESAs), including recombinant human erythropoietin (rhEPO), relies on two complementary approaches: direct detection via SAR-PAGE and indirect detection through the Athlete Biological Passport (ABP) hematological module. While the ABP has remained a cornerstone of anti-doping strategies for over 15 years, its basis on a fixed set of biomarkers limits its sensitivity and specificity. To address these boundaries, anti-doping research has shifted towards identifying complementary biomarkers that can enhance the effectiveness of indirect detection methods. Metabolomics has emerged as a promising approach for uncovering metabolic signatures associated with physiological processes, including erythropoiesis. Especially, polar metabolites offer a largely unexploited resource of information linked to cell and energy metabolism, and hypoxia adaptation, which all are essential pathways modulated by rhEPO dosing. To explore this potential, we developed a targeted metabolomic method using HILIC LC-MS/MS to investigate the effects of rhEPO administration on a broad spectrum of endogenous polar metabolites. This method was applied to plasma samples of two independent cohorts from rhEPO administration studies, leading to the identification of hypoxanthine and inosine as highly responsive candidate biomarkers. Following rhEPO administration, inosine exhibited up to a 20-fold increase with CERA and a 10-fold increase with Dynepo, while hypoxanthine increased between 3.5- and 5-fold, respectively. Notably, the elevation of these metabolites coincided with the peak in reticulocyte percentages, reflecting maximal erythropoietic activity. As intermediates in purine metabolism, their increases are likely tied to enhanced purine turnover during red blood cell production.
These candidate biomarkers offer several advantages, including their substantial amplitude of response and the ability to detect changes with as little as 25 µL of plasma. Additionally, we observed in dried blood spots clear increases in both metabolites following NESP administration in a male volunteer, demonstrating their applicability across sample matrices.
The reproducibility of these findings across three independent rhEPO administration studies highlights the robust potential of hypoxanthine and inosine as biomarkers for targeting of rhEPO doping. However, critical validation steps are still to be carried out, including the development of a quantitative method for plasma and dried blood spots, assessments of intra- and inter-individual variability, and evaluations of potential confounding factors, such as altitude or other erythropoiesis-stimulating conditions.

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Salamin O, Ramic L, Nicoli R, Rudaz S, Guillarme D, Kuuranne T. Identification of Candidate Blood Biomarkers of Recombinant Human Erythropoietin Administration Using Targeted Polar Metabolomics by HILIC-MS/MS. *Drug Test Anal.* 2025 Dec;17(12):2323-2332. doi: 10.1002/dta.3943.

Sastry K[1], Hendawi H[1], Al-Mohannadi I[1], Al-Jaber M[1], Al-Maadheed M[2,3], Mohamed-Ali V[2,3]

# Camelid VHH nanobodies:
# A novel approach to improving EPO detection

Research Department, Antidoping Lab Qatar, Doha, Qatar[1];
Doping Analysis Lab, Antidoping Lab Qatar, Doha, Qatar[2];
University College London, London, United Kingdom[3]

## Abstract

Recombinant human erythropoietin (rHuEPO) continues to be a widely abused performance-enhancing agent in endurance sports, despite its strict inclusion on the WADA Prohibited List. Current detection methods, such as Isoelectric Focusing (IEF) and SAR-PAGE, rely on antibodies like AE7A5. However, these techniques face significant challenges, including cross-reactivity, limited sensitivity and specificity, lengthy assay times, and difficulties in distinguishing endogenous EPO from exogenous rHuEPO. To overcome these limitations, advanced diagnostic tools are essential for the precise detection and confirmation of EPO or Erythropoietin Receptor Agonists (ERAs) misuse in athlete samples.

Camelid-derived VHH fragments, or nanobodies, present a promising solution due to their exceptional specificity, stability, and ability to detect post-translational modifications, such as glycosylation. This study aims to develop VHH-based assays to revolutionize anti-doping testing for EPO, focusing on three key objectives:

1. Generating camel-derived VHH antibodies against four frequently abused rHuEPO isoforms and endogenous human EPO and evaluating their binding performance.
2. Assessing the ability of VHH to distinguish exogenous rHuEPO from endogenous EPO.
3. Engineering VHH fusions to enhance assay sensitivity and specificity, integrating them into platforms like ELISA and SAR PAGE, and reducing assay duration via HRP conjugation.

Camels were immunized with EPO variants and immune VHH libraries created. Screening of over 500 clones from EPO-alpha VHH library identified 5E2 VHH, which demonstrated robust recognition of all tested EPO forms while excluding irrelevant proteins. Notably, the binding characteristics of 5E2 VHH align with the widely used AE7A5 antibody. Ongoing work focuses on isolating additional VHH fragments capable of differentiating endogenous EPO from exogenous forms as well as VHH for various EPO isoforms.

This innovative VHH-based approach offers a transformative advancement in EPO detection, providing superior specificity, sensitivity, and efficiency. These developments hold immense promise for strengthening anti-doping protocols and ensuring fair competition in sports.

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Leuenberger N[1], Chappuis J[1], Bækken L[2], Salamin O[1], Kuuranne T[1]

# Comparative analysis of CERA detection and stability in blood and urine

Laboratoire Suisse d'Analyse du Dopage Centre Hospitalier Universitaire Vaudois et Université de Lausanne, Lausanne, Switzerland[1];
Nordic Athlete Passport Management Unit, Oslo, Norway[2]

## Abstract

Continuous erythropoietin receptor activator (CERA), a third-generation erythropoiesis-stimulating agent (ESA), is misused in endurance sports for its performance-enhancing effects. CERA's detection in anti-doping analyses remains challenging due to its high molecular weight and stability issues in certain biological matrices. While blood is the preferred matrix for CERA detection, urine is also widely targeted matrix for ESA analysis. World Anti-Doping Agency's (WADA) technical document for erythropoiesis-receptor agonists (TD2024EPO) outlines the minimum required performance levels (MRPL) for CERA as 150 pg/mL and 5 pg/mL in blood and urine, respectively.

This study presents comparative data on CERA detection in serum and urine samples, highlighting Adverse Analytical Findings (AAF) in serum that were undetected in corresponding urine samples. Direct detection methods demonstrated longer detection window compared to indirect approaches, such as the Athlete Biological Passport (ABP), emphasizing the need to prioritize direct CERA analysis in blood matrix.

Stability studies were conducted on urine, serum, and dried blood spot (DBS) samples under varying conditions. CERA exhibited significant instability in urine when stored at 37°C, whereas serum and DBS samples maintained exceptional stability, even under harsh conditions.

In conclusion, the greater stability and detectability of CERA in blood-based matrices (serum and DBS) compared to urine stress the need for anti-doping authorities to include blood sample collection in their test distribution plans. This shift would significantly improve the efficiency and sensitivity of CERA detection in doping control, ensuring more effective doping control programs.

 ISBN 978-3-9828334-0-8

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

# Comprehensive evaluation of commercial antibodies for high sensitivity detection of EPO-doping on Western blots OR About finding the perfect antibody to detect EPO doping on immunoblots

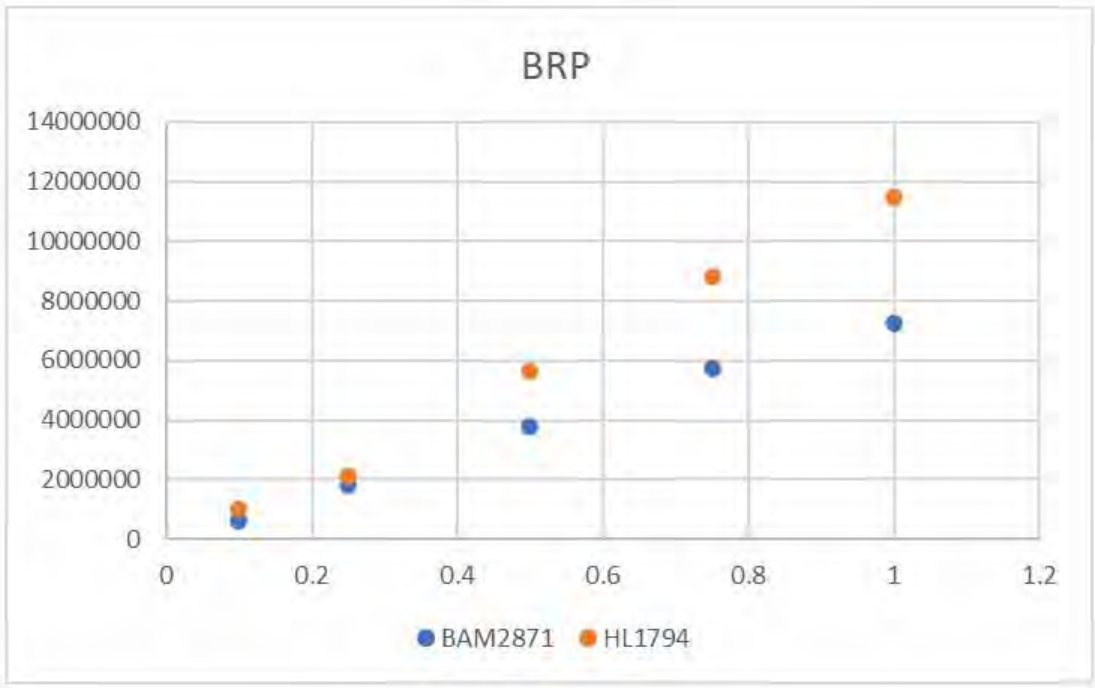
Seibersdorf Labor GmbH, Chemical Analytics - Doping Control Laboratory, Seibersdorf, Austria[1];
German Sport University Cologne, Institute of Biochemistry, Cologne, Germany[2]

## Abstract

Currently, all TD2024EPO electrophoretic detection methods for EPO-doping are relying entirely on the superior sensitivity of clone AE7A5 monoclonal EPO antibody as no alternative antibody with similar performance characteristics is known [1]. One drawback of this antibody is that it non-specifically binds some proteins, which are present in human urine and blood [2-4]. The non-specific binding is caused by the fact, that the peptide used as immunogen contained two errors in the amino acid sequence. Hence, all doping control samples have to be first immunoaffinity-purified with another EPO-specific antibody before separation by electrophoresis. Additionally and in accordance with TD 2024 EPO, the confirmation procedure (CP) has to differ from the initial testing procedure (ITP).
This difference may be achieved e.g. by
(1) employing a second IP-method with a different capture antibody,
(2) using a different electrophoretic separation technique,
(3) double-blotting in case single blotting was applied for the ITP, or
(4) the application of a different detection antibody.

In search of an alternative detection antibody, we screened about 160 commercial EPO antibodies regarding their sensitivity and selectivity in comparison with the highest sensitivity version of clone AE7A5 EPO antibody (i.e. the biotinylated BAM2871 from R&D Systems, which we developed in close collaboration with the manufacturer in 2018). Results of this study are shown and detailed data of a new monoclonal detection antibody are presented. The new antibody is not only equal to the old one but even surpasses it regarding sensitivity, specificity and costs.

The following Figures show a few selected results obtained for the evaluation of clone HL1794 anti-EPO antibody. For more details, please refer to the forthcoming full article in *Drug Testing and Analysis* journal.



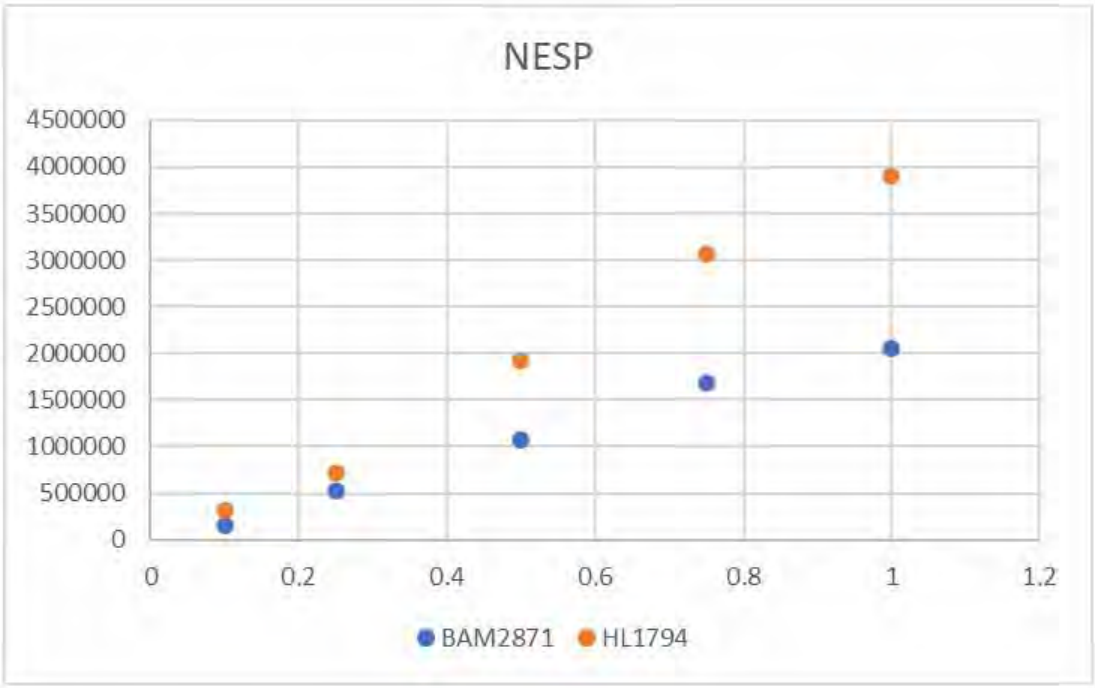




**Figure 1.** Sensitivity comparison of clone AE7A5 (BAM2871) antibody (*blue*) vs. clone HL1794 (*orange*) for rEPO (BRP) and NESP on SAR-PAGE and chemiluminescent detection. SAR-PAGE with chemiluminescent detection (substrate: SuperSignal West Atto).

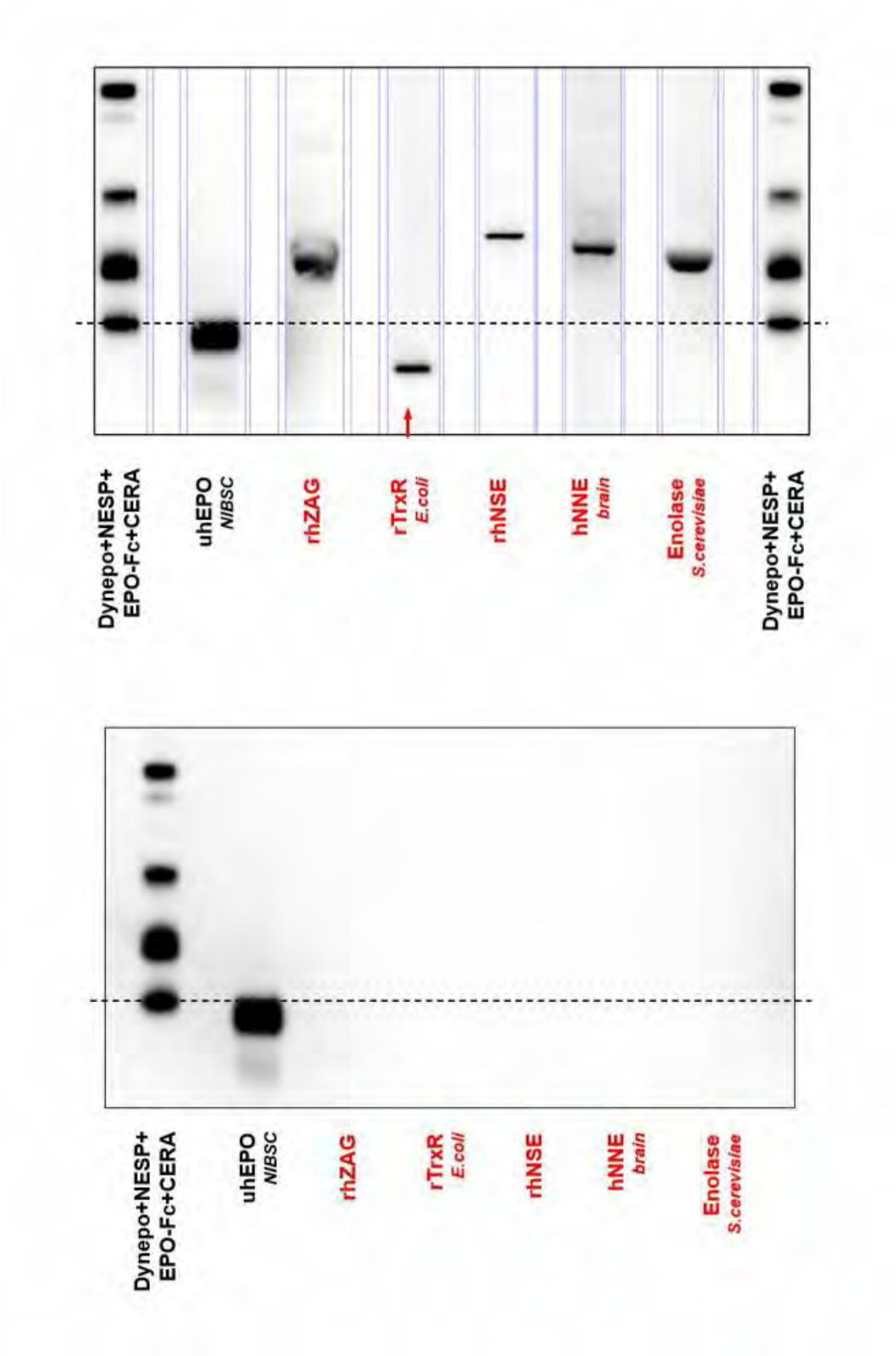



**Figure 2.** Compared to clone AE7A5 (*upper* image) the new antibody (*lower* image) does not bind to proteins, which AE7A5 non-specifically detected on Western blots. *rhZAG* (recombinant human zinc alpha 2-glycoprotein), *rTrxR* (recombinant *E. coli* thioredoxin reductase), *rhNSE* (recombinant human neuron-specific enolase), *hNNE* (human brain non-neuronal enolase), *uhEPO NIBSC* (reference standard for human urinary EPO). SAR-PAGE with chemiluminescent detection (substrate: SuperSignal West Atto).

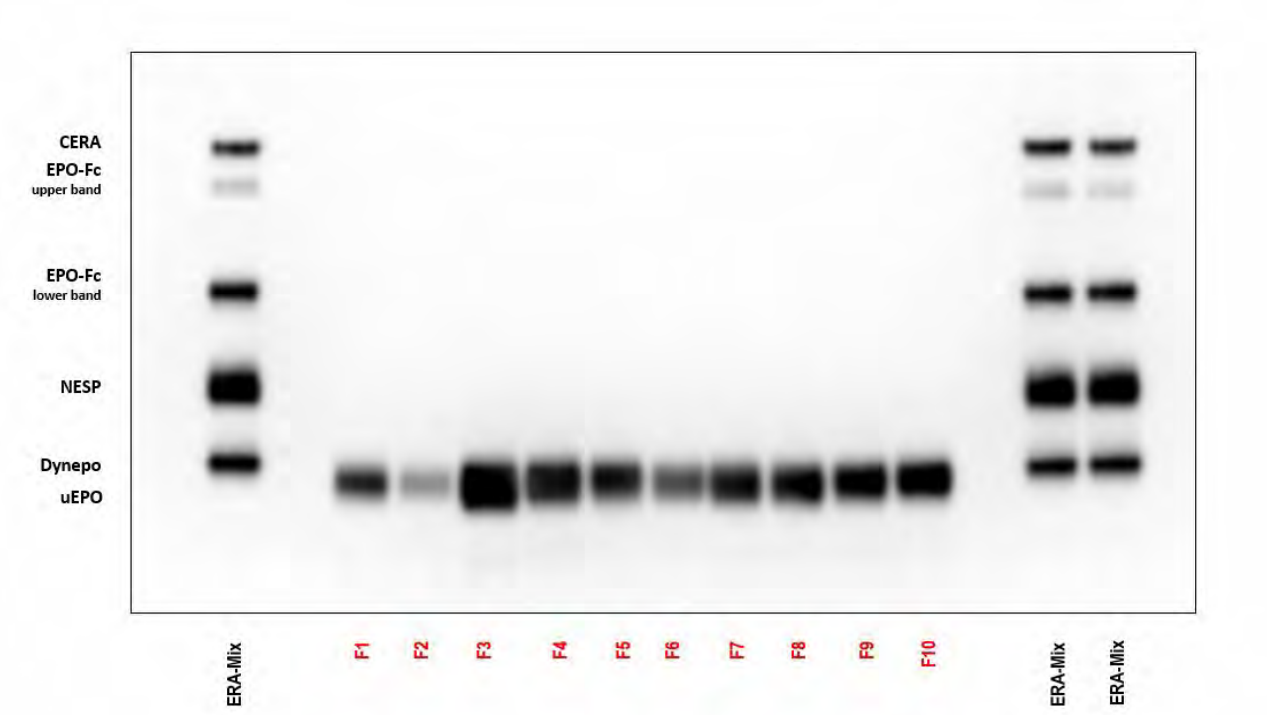



**Figure 3.** Validation results of clone HL1794: Selectivity for urine samples. Shown are 10 female samples (F1-F10) after immunopurification with Stemcell ELISA. Non-specific bands were not observed. SAR-PAGE with chemiluminescent detection (substrate: SuperSignal West Atto). *ERA-Mix* (Dynepo/NESP/EPO-Fc/CERA), *uEPO* (urinary EPO).

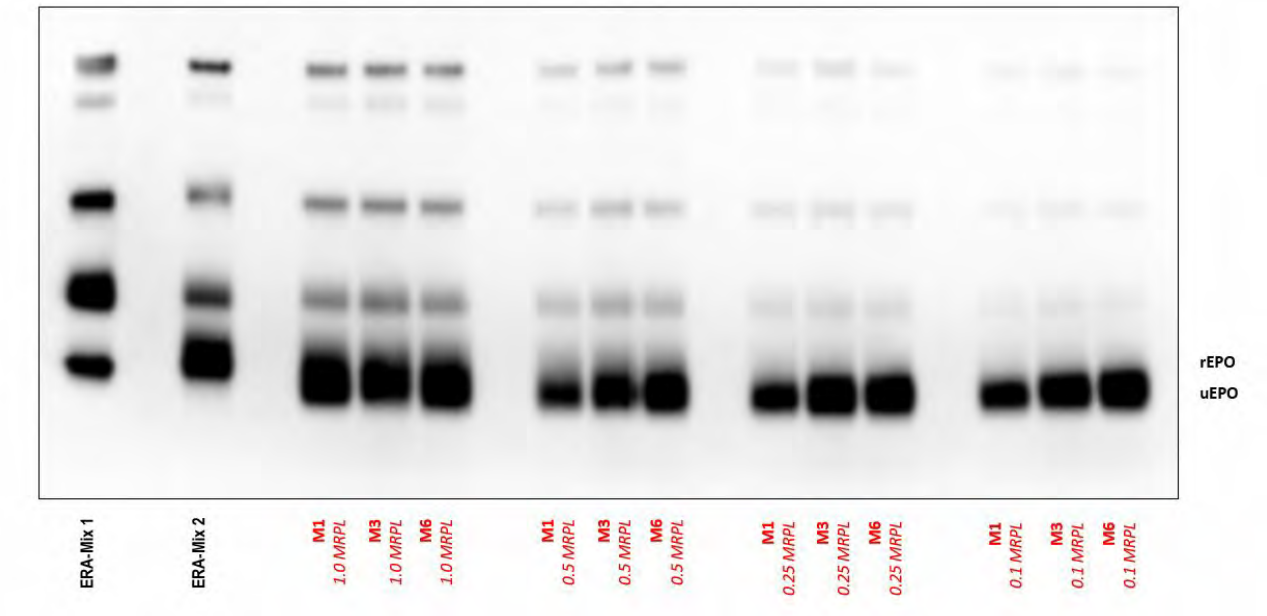



**Figure 4.** Validation results of clone HL1794: LOD in urine samples. Shown are 3 male samples (M1, M3, M6) spiked with an ERA-Mix (rEPO/NESP/EPO-Fc/CERA) at 1.0/0.5/0.25/0.1 MRPL and after immunopurification with Stemcell ELISA. The antibody detected all ERAs down to 0.1 MRPL. SAR-PAGE with chemiluminescent detection (substrate: SuperSignal West Atto). *ERA-Mix 1* (Dynepo/NESP/EPO-Fc/CERA), *ERA-Mix 2* (rEPO/NESP/EPO-Fc/CERA), *rEPO* (recombinant EPO), *uEPO* (urinary EPO).

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## Acknowledgements

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Reihlen P, Blobel M, Weiß P, Thevis M

# 3-year experience after implementation of an internal standard in ERA SAR-PAGE analysis of doping control samples of various matrices

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## Abstract

In October 2021, the Cologne laboratory implemented an internal standard (ISTD) to routine ERA SAR-PAGE analysis, in form of a customized EPO-polyethylene glycol (PEG)-conjugate to monitor sample preparation procedures of each individual doping control specimen. With the TD2024EPO coming in force in June 2024 additional sample preparations for each matrix were validated to fulfill the requirement of a mandatory difference in procedure between Initial Testing Procedure (ITP) and Confirmation Procedure (CP). The introduced ISTD has been shown to successfully co-elute with ERAs when employing the Immunoaffinity Isolation Plate from StemCell, and both the 3F6 and 7D3 antibodies utilized in the Purification Kits from MAIIA. Three years after ISTD implementation, 17500 human urine, 1700 human blood and 120 equine blood samples have been analyzed. Human and equine blood samples usually do not show signs of enzymatic activity, this is different for human urine samples. Therefore, it has been especially informative to observe possible impairments of the ISTD in enzymatically active urine specimens, which may alter the (apparent) molecular weight of analytes and ISTD. Any interference with the analysis or interpretation of the obtained SAR-PAGE data due to the implemented ISTD would be detrimental to the method. While the ISTD was shown to have impairments in certain urine samples, these did not interfere with analysis or evaluation. Some urine samples could induce a minimal shift in electrophoretic mobility of the ISTD. These samples have been found to be prone to ERA degradation. While not all samples showing EPO degradation also show an ISTD mobility shift most of the samples with a shift in ISTD mobility show ERA-degradation. Over the last three years the ISTD has been a valuable tool in monitoring both ITP and CP preparations and was found to be compatible with all prevalent immunopurification methods. Additionally, the ISTD can flag some urine samples with degrading properties.

Magalhães Jr. G[1], da Cruz SMS[2], Lopes GR[2], Oliveira DS[3], Carneiro GRA[3], Padilha M[3], Pereira HMG[3]

# Enhanced interpretation of HPLC-MS results: A support tool for analysts based on fuzzy logic and nonlinear regression

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## Abstract

There is great potential for the application of novel data-centric technologies in the routine of anti-doping laboratories, in addition to analytical advancements. LC-HRMS systems are widely used in several fields, including Anti-Doping Science. Considering the large volume of datasets to be analyzed, special attention has been given to intelligent and automated result interpretation tools. Colleagues from the Seoul Laboratory were pioneers in our community by suggesting this type of tool for routine anti-doping analysis (PARK et al., 2023). Indeed, the application of mathematical science, such as the coefficient of determination ($R^2$) in nonlinear regression, combined with computational intelligence techniques like fuzzy logic, has emerged as a powerful alternative to facilitate the evaluation of chromatograms (ZADEH, 1965). In this context, recognizing the potential positive impact of automatic result interpretation, our study (M. B. JUNIOR et al., 2024) describes the stages of the conceptual pipeline: i) Injection of samples into HPLC-MS equipment, ii) Generation of .RAW files, extraction of retention time parameters, and substance concentration, iii) Data engineering, involving structuring, cleaning, and tabulating chromatogram data, iv) Calculation of $R^2$ determination coefficients on the datasets to perform a comparison similar to that conducted by a human analyst, v) Application of Fuzzy Logic to represent a non-boolean conclusion using Python and other libraries (PyMsFileReader, NumPy, Pandas, Scikit-learn, Scikit-fuzzy, Matplotlib, PubChemPy, RDKit, and Flask) to analyze *.raw files generated by an LC-HRMS (Q-Exactive system from Thermo Scientific). This approach enables the rapid and automatic detection of doping agents at trace concentration levels (pg/ml) with high mass accuracy, displaying errors below 3 ppm. The approach was based on widely used chromatography coupled with mass spectrometry parameters, such as retention time (RT), diagnostic fragments, peak intensity, smoothing type, and scan filters. The coefficient of determination compares chromatograms of Positive Quality Controls (PQC) with target samples, classifying the degree of similarity using fuzzy logic. Structural information about the analytes is retrieved via PubChem Compound, providing data and metadata such as CAS numbers, IUPAC names, and molecular structures. Available through Flask, the web interface allows interactive processing and visualization of results. The pipelines were tested in three experimental rounds, each consisting of 500 samples and 10 controls, totaling 600,000 analytical records. Building a user-friendly interface for end-users was one of the key objectives. So far, 125 target substances have been incorporated. The data from these analytes were interpreted in each round to evaluate the tool's performance under different experimental conditions, and the interpretation took 20 seconds per sample. Three phases of the project have already been completed: i) Positive samples vs. PQC, providing a controlled scenario focused on model validation. In this scenario, a performance of 100% (accuracy/

precision/F1-Score) was achieved; ii) Typical anti-doping samples vs. PQC. The model showed a slight decrease in performance but still achieved excellent results, with 98% accuracy and precision, and 99% F1-Score; iii) To increase the challenge, an experiment was designed by mixing spiked and negative samples to create a more complex environment to test the model's robustness. Despite the higher level of difficulty, the results remained consistent, with 98% accuracy, 98% precision, and 99% F1-Score, demonstrating the model's robustness in flagging presumptive findings. Fuzzy logic enabled qualitative interpretations closer to human judgment, while $R^2$ provided a solid quantitative foundation. The proposed pipeline offers an integrated and scalable approach to LC-HRMS analysis, with promising results in both controlled and routine scenarios. The inclusion of a complete selection of analytes and the extrapolation of the GC-MS analysis concept have already been established as future stages of the project.

 ISBN 978-3-9828334-0-8

Ponzetto F[1], Marinelli L[1], Leoni L[1], Settanni F[2], Parasiliti-Caprino M[1], Mengozzi G[2], Ghigo E[1]

# Development of an LC-MS/MS platform for serum targeted steroidomics and its application to a population with gender incongruence

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Clinical Biochemistry Laboratory, City of Health and Science University Hospital, Turin, Italy[2]

## Abstract

Acquired disorders of androgen deficiency and excess typically do not result in differences in sex development, but they can still significantly impact pubertal development, fertility, and overall health. Among the clinical conditions in which androgens may play a role, gender incongruence (GI) should also be included. GI is defined as a condition of marked and sustained clinical incongruence between the experienced gender and the sex assigned at birth. Detailed investigations of steroid metabolism in this population have not yet been conducted, with laboratory measurements often limited to a restricted panel of circulating steroid hormones.

In this work, a novel LC-MS/MS platform was developed for the detection and quantification of 200 steroidal compounds (146 free hormones and 54 phase II metabolites) in serum/plasma samples. Two separate sample preparation procedures were optimized for free and conjugated steroids, employing supported liquid extraction and solid-phase extraction, respectively. The ideal chromatographic separations for the two groups of analytes were assessed by testing six different LC columns with a variety of stationary phase chemistries, and by employing ammonium fluoride as an aqueous mobile phase modifier to both enhance steroid ionization and improve the peak shape of sulphated compounds.

The developed methods were applied to the analysis of the steroidome in a cohort of patients with GI, recruited during routine clinical activity at the Division of Endocrinology, Diabetes, and Metabolism at the City of Health and Science University Hospital of Turin. A total of 27 assigned females and 19 assigned males were enrolled in the study, and steroidomic analysis allowed the identification and quantification of 78 steroidal compounds (49 free steroids and 29 phase II metabolites). The measured concentrations of a restricted panel of 26 hormones and metabolites were then compared with those obtained from healthy controls in a previously published study, without revealing any significant differences between GI patients and controls of the same assigned sex.

This study represents a first step towards the implementation of a targeted steroidomic workflow in a clinical context and an important attempt to provide an extensive hormonal report on adult transgender individuals. The results obtained further confirm that, at present, it is not possible to identify a detectable hormonal pattern specific to the transgender population, regardless of whether they were assigned male or female at birth.

Park H, Son J

# Optimising AI models for doping detection using LOOCV to address data imbalance in mass spectrometry

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## Abstract

The application of artificial intelligence (AI) in analytical chemistry has advanced significantly, improving data processing, pattern recognition, and decision-making in complex datasets. In doping analysis, the continuous expansion of the World Anti-Doping Agency (WADA) list of prohibited substances poses increasing challenges to conventional screening and reviewing results, particularly in maintaining analytical efficiency and accuracy. To address these limitations, this study applies AI-based methodologies to enhance the detection of banned substances in mass spectrometry (MS) data. A major obstacle in doping analysis is the extreme class imbalance, with positive cases comprising only approximately 1% of total samples. Training machine learning models on such highly skewed datasets often results in poor sensitivity to rarely positive cases. To overcome this issue, we systematically evaluated multiple machine learning models and employed robust validation strategies to improve model generalizability. Specifically, six machine learning algorithms – logistic regression, K-nearest neighbor (KNN), support vector machine (SVM), Gaussian Naive Bayes, random forest (RF), and extreme gradient boosting – were trained and assessed using both K-fold cross-validation and leave-one-out cross-validation (LOOCV). Comparative analysis demonstrated that LOOCV outperformed K-fold cross-validation by improving sensitivity to positive cases in an imbalanced dataset. Notably, the RF and KNN models trained with LOOCV achieved 100% in all classification metrics, highlighting the effectiveness of LOOCV as a validation strategy for doping datasets with extreme class imbalance. Furthermore, the implementation of this AI-based framework reduced manual intervention, improved processing efficiency, and enhanced consistency and reliability in doping detection workflows.

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Zhou G, Yan K, Liu L, Wang Z

# A stability study of buthiazide in urinary and aqueous matrices

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## Abstract

In Beijing Anti-doping Laboratory, there were two cases in which buthiazide and ACB were simultaneously detected in 2024, investigations found that some weight-loss medicines contained buthiazide without notice on the label. We'd taken enormous care for it has been rarely reported except one case in 2017 WADA Anti-doping testing figures.

It was published that thiazide substances including Hydrochlorothiazide, Chlorothiazide, etc, are unstable in urine matrices, which could easily hydrolysis resulting in the formation of the degradation product 4-amino-6-chloro-1,3-benzenedisulphonamide (ACB). Buthiazide, which has the similar chemical structure with these mentioned above thiazide diuretics, but it has not been published whether buthiazide degrades during the storage process.

This study evaluated the stability of buthiazide under different pH environment, temperatures and light conditions using actual urine and artificial urine, respectively. Meanwhile, the degradation rate under different conditions was visually analyzed through a single point-quantitative method. Buthiazide and ACB could be detected under both Day light and Lab light at pH 2.6, pH 5.0, pH 7.0 and pH 8.8; while could be detected only at pH 2.6 under UV light. Under all pH conditions, the degradation product could be detected after incubation at 20, 40, 60°C for 48 h. At higher pH value and temperature, the parent compound could rarely be detected. For the long-term stability under 4°C, buthiazide is unstable within 1 month, and could rarely be detected within 3 months. For -20°C, buthiazide is stable within 1 month, and could be partial resolved into ACB within 3 months. When the degradation rate was compared under different temperatures and pH values using the single point-quantitative analysis method, it is revealed the fastest degradation was observed at 60°C which consistent with the results in qualitative analysis. This study confirms that buthiazide could produce degradation products during storage process, thus, storage condition should be taken into serious consideration when B sample confirmation is delayed.

Martinez Brito D, Leogrande P, Botrè F, de La Torre X

# Short term stability of 6-bromo-androstenedione assessed by LC-MS and GC-MS

Laboratorio Antidoping FMSI, Rome, Italy

## Abstract

Short term stability studies are generally carried out simulating conditions during transport or storage or use, among others. For this purpose, storage under different conditions is assessed during relatively short periods of time regularly from 3 to 6 months. Samples stored at various temperatures (e.g. room temperature, +4°C and –20°C) should be analyzed at regular time intervals. In general, protic (protogenic) solvents show the presence of O-H or N-H substituent groups able to easily form hydrogen bonds, which are a powerful intermolecular force. Aprotic (non-protogenic) solvents may also have hydrogens on their structures, but the lack of O-H or N-H groups prevents the formation of hydrogen bonds with other structures. Depending on the chemical structure of the compound and the interactions with the solvent, degradation products may be formed. Also, for the instrumental analysis of steroids by gas chromatography, the formation of degradation products may be influenced by the derivatization reactions that normally occur in an acidic medium. The aim of the work was to study the short-term stability of 6β-bromo-androst-4-ene-3,17-dione (6-Br-androstenedione, 6-BrAED). The analyte was dissolved in methanol and dimethylsulfoxide (DMSO) and the solutions were stored at room temperature, +4°C and –20°C for 3 months. The analysis was performed by GC-qTOF and LC-MS/MS. 6β-br-androst-4-ene-3,17-dione showed no stability at RT and +4°C. The main degradation products were androstene-dione, 6-ene-androstenedione, 6α/β-hydroxy-androstenedione and 6-oxo-androstenedione. The most unexpected result was the stability in DMSO. According to the Compendium of Chemical Terminology of the IUPAC, DMSO is a dipolar aprotic solvent, that shows high dielectric constant and a considerable permanent dipole moment that cannot donate hydrogen atoms to form hydrogen bonds. Such solvents are usually not aprotic but protophilic and at most weakly protogenic. A notable number of studies described the reaction mechanisms of DMSO with secondary alcohol and halogenated compounds. The oxidation of hydroxyl to form ketones with DMSO mediation or the reactions of DMSO with chlorine and bromide are appropriately published under dissimilar purposes and conditions respect to temperature and pressure, for instance. Reactions like Swern oxidation, Kornblum oxidation, or Torrsell mechanism are examples of the reactivity of DMSO. Besides, DMSO has been used in the direct syntheses of N- and O- heterocycles as carbon, oxygen, and sulfur sources and as an oxidizing agent.

This work was supported by a grant from the World Anti-Doping Agency Grant (22A05XT) and it is part of the published manuscript:
Martínez Brito D, Leogrande P, de la Torre X, Botrè F. Evaluation of the short-term stability of 6α-chloro-testosterone, 6β-bromo-androstenedione and 6-oxo-androstenedione in dimethylsulfoxide and methanol using liquid and gas chromatography - mass spectrometry. *Steroids*. 2025 May;217:109597. doi: 10.1016/j.steroids.2025.109597.

Kwiatkowska D[1,2], Peta B[2], Grucza K[1], Drapala A[1], Konarski P[1], Wojtkowiak K[1], Bedecka M[1], Stanczyk D[1], Sytniczuk A[2], Grela K[2], Wicka M[1]

# New psychoactive substances – Challenges in correct interpretation: A case study

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Biological and Chemical Research Centre, University of Warsaw, Warsaw, Poland[2]

## Abstract

The issue of using new psychoactive substances (NPS), also known as recreational drugs in Europe and other regions, has been recognized for several years. These substances represent a rapidly evolving group of compounds. Due to the inability to obtain ethical approval from the Bioethics Committee for administering drugs containing NPS, research on their metabolism is significantly hindered. The Polish Anti-Doping Laboratory analyzed an in-competition urine sample using the UPLC/MS-MS method and detected a metabolite of pentedrone norephedrine. According to the literature, this metabolite could be derived from pentedrone, α-pyrrolidinopentiophenone (α-PVP), or N-ethylpentedrone (NEP)[1-4]. Since a certified reference material (CRM) for the pentedrone norephedrine metabolite is not available, the reliability of this result has been questioned. In response, the Polish Laboratory successfully synthesized the metabolite, which was then compared with the compound previously identified in the sample.
Conclusions:

- The synthesis of the new reference material allowed the unambiguous identification of 2-amino-1-phentan-1-ol present in the AAF sample.
- The newly synthesized reference material allowed the confirmation of the presence of both diastereomers in the AAF sample.
- Due to the increasing popularity of NPS, especially NEP, on the black market, it seems advisable to add 2-amino-1-phentan-1-ol into ITPs.

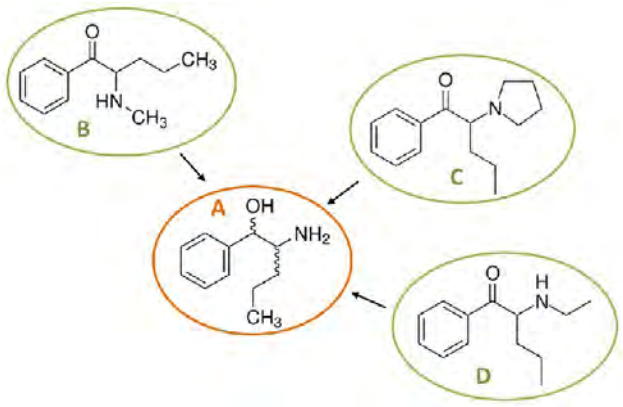


**Figure 1.** Chemical structures of (A) PNM, (B) pentedrone, (C) PVP, and (D) NEP

*The details of this study will be published elsewhere.*

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Zhu S, Wang S, Liu X, Zhang L

# A "Pheraplex" capsule labeled as desoxymethyltestosterone from the market turned out to be 17,17-dimethyl-18-nor-5α-androst-13-en-3β-ol

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

## Abstract

In an effort to conduct a desoxymethyltestosterone (DMT) administration study to replenish excretion urine inventory of Beijing Anti-Doping Laboratory for quality assurance purpose, a product labeled as "Pheraplex" was purchased from the internet. The product's label indicated that each capsule contained 10 mg of 17α-methyl-etioallocholan-2-ene-17β-ol (DMT), along with medicinal corn starch and gelatin. To verify the product's contents, gas chromatography tandem quadrupole mass spectrometry (GC–MS/MS) was employed to analyze the active ingredient and compare it with the reference materials of DMT. Surprisingly, the results revealed that the product did not contain DMT or any other steroids monitored in the initial testing procedure of our laboratory. Subsequently, nuclear magnetic resonance was utilized to identify the compound's structure, which was determined to be 17,17-dimethyl-18-nor-5α-androst-13-en-3β-ol. This compound was referred to as M10 of 17α-methyltestosterone in a literature. This finding highlights the potential discrepancies between product labeling and actual contents in the supplement market, which deserves attention from the anti-doping community.

Published as:
Zhu S, Wang S, Liu X, Zhang L. A "Pheraplex" Capsule Labeled as Desoxymethyltestosterone From the Market Turned Out to Be 17,17-Dimethyl-18-nor-5α-androst-13-en-3β-ol. *Drug Test Anal.* 2025 Oct; 17(10):2032-2035. doi: 10.1002/dta.3912.

Olgaz Bingöl S[1], Akin S[2], Gungor Orhan I[2], Demirel AH[3]

# Detection of the performance-enhancing substance BGP-15 in biological samples

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## Abstract

BGP-15, a nicotinic amidoxime derivative ($C_{14}H_{22}N_4O_2 \cdot 2HCl$) originally developed for insulin resistance, has gained attention as a potential performance-enhancing compound due to its effects on mitochondrial function, muscle preservation, and endurance capacity. It stimulates heat shock protein (Hsp72) expression, enhances stress response, and increases cellular energy supply. Additionally, BGP-15 inhibits poly(ADP-ribose) polymerase (PARP), reduces reactive oxygen species (ROS) production, and improves membrane fluidity. Studies indicate that BGP-15 may enhance exercise performance by restoring diaphragm force capacity after immobilization, preserving muscle fiber integrity, and protecting skeletal muscle from oxidative damage. Furthermore, BGP-15 upregulates peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), promoting mitochondrial biogenesis, increasing ATP availability, and enhancing resistance to fatigue. By inhibiting PARP and activating SIRT1, BGP-15 further improves mitochondrial function. Given these properties, BGP-15 holds promise as an endurance-boosting agent.

Although BGP-15 is not explicitly listed on the World Anti-Doping Agency (WADA) Prohibited List, its potential for misuse as a performance enhancer highlights the need for an analytical detection method. In this study, serum and urine samples were collected from 20 Wistar rats, with 2 serving as controls and the remaining receiving a 15 mg/kg intraperitoneal injection of BGP-15. Samples were obtained at 3, 6, 12, 24, 36 and 48 hours post-administration (n=3 per time point). BGP-15 ion transitions (279>142, 279>194, positive mode) were successfully detected in both matrices using UPLC-MS/MS (TQD, Waters, Acquity UPLC BEH C18 1.7 µm column) coupled with a liquid-liquid extraction (LLE) method. The results indicate that BGP-15 is excreted in its free form and can be effectively quantified using this method. While detectable at all time points, BGP-15 levels declined to trace amounts after 36 hours.

These findings support the feasibility of analytical detection of BGP-15 in doping control and reinforce the need for further research on its potential ergogenic effects.

Park H, Son J

# Vaccine for Foodborne Doping (VFD): Comprehensive LC-MS/MS quantitative method to prevent unintentional doping from foods and dietary supplements

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## Abstract

Unintentional doping caused by the ingestion of contaminated foods and dietary supplements remains a critical issue in anti-doping efforts. While various analytical methods have been developed, there is no validated approach capable of simultaneously screening and quantifying hundreds of sports prohibited substances across diverse food matrices. To address this limitation, we introduce a "foodborne vaccine" concept – a robust analytical platform designed to proactively mitigate the risks of foodborne doping through the rapid and accurate quantification of over 300 prohibited substances using LC-MS/MS combined with a universal QuEChERS-based sample preparation method. This method was validated across 10 diverse food matrices, assessing matrix effects and recovery rates to confirm its broad applicability. Furthermore, three representative matrices – pork, oats, and beverages – were selected to develop a matrix-specific quantification approach, ensuring accuracy across different food compositions. The method was subjected to rigorous validation for selectivity, limits of detection (LOD), limits of quantification (LOQ), linearity, intra- and inter-day accuracy, and precision, demonstrating full compliance with Codex guidelines. Compared to existing methods, this approach offers significantly reduced run time, enhanced specificity, high sensitivity, and superior accuracy and precision, making it a powerful tool for doing analysis in food and dietary supplement matrices. By functioning as a foodborne vaccine, this analytical framework provides a proactive and preventive solution against unintentional doping, ultimately strengthening anti-doping measures and ensuring integrity in sports.

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Vonaparti A[1], Scheiff A[2], Berg C[2], Anielski P[1], Voss S[1]

# Analysis of different homeopathic medicinal products for prohibited substances – preliminary results

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NADA Deutschland, Bonn, Germany[2]

## Abstract

Homeopathic medicines often consist of diluted mother tinctures of plant extracts, inorganic chemical compounds or animal organ extracts. Especially for plant and organ extracts the exact chemical composition is difficult to identify and for most homeopathic medicines practically unknown. In case the homeopathic medicine contains substances prohibited by WADA, this can bear the risk of unintentional Anti-Doping rule violations for athletes. While the presence of the stimulant strychnine, prohibited in-competition according to WADA's Prohibited List, was investigated in ayurvedic herbal preparations and in Nux vomica preparations of other countries, there is no data available on German homeopathic medicinal products within the context of doping analysis. Therefore, the aim of this pilot study was the qualitative and quantitative examination of the content of selected homeopathic medicinal products available in Germany. Seven different homeopathic medicinal products were examined in this pilot study using GC-MS and LC-MS methods. Two substances, Strychnine and Methylprednisolone, were detected in concentrations from 50 – 660 ng/mL. These preliminary data confirm previous studies conducted in other countries and show that also homeopathic products in Germany can contain substantial amounts of prohibited substances.

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# Investigations on Ashwagandha as potential confounding factor of the steroidal module of WADA's Athletes Biological Passport

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Institute of Doping Analysis and Sports Biochemistry (IDAS) - Dresden, Kreischa, Germany[2];
NADA Deutschland, Bonn, Germany[3]

## Abstract

Ashwagandha is an herbal dietary supplement that is used in traditional medicine as a remedy with calming, strengthening and stimulating properties. The plant contains various substances with a steroid structure and a significant increase in testosterone levels after intake of Ashwagandha in men has been reported by Chauhan *et al.* Due to the regular occurrence of Ashwagandha intake being declared on doping control forms and the potential use as an excuse for suspicious steroid profiles, it was decided to investigate if the supplement could potentially affect the markers of the urinary and/ or serum steroidal module of WADA's Athletes Biological Passport. 31 healthy and strength-trained participants participated in a randomized, double-blind, placebo-controlled trial. Participants received either 1,000 mg Ashwagandha (5% withanolides) or placebo daily for 14 weeks. No effect on any of the parameters of the urinary or serum steroid profile could be observed. Ashwagandha supplementation can be excluded as confounding factor of the urinary or serum steroid profile.

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Liao L, Ge Y, Liu M, Fang X, Sun H, Deng X

# A novel analytical strategy based on gas chromatography-orbitrap high resolution mass spectrometry combined with solid-phase extraction for the monitoring of stanozolol misuse in human urine

Shanghai Anti-Doping Laboratory, Shanghai, China

## Abstract

**Rationale:** Stanozolol, an anabolic androgenic steroid listed in Part S1 of the WADA Prohibited List, exhibits a low response and significant matrix interference in urine samples when using LLE-GC-MS. Enhancing sample preparation techniques remains essential for the effective detection of stanozolol and its metabolites.

**Methods:** A method for determining stanozolol and its metabolites (3'-OH-stanozolol, 4β-OH-stanozolol, and 16β-OH-stanozolol) in human urine was developed and validated using GC-Orbitrap-HRMS combined with optimized mixed-mode SPE. This method was applied to urine samples from two volunteers who orally administered a single dose of stanozolol, with samples collected over a 30-day period post-administration.

**Results:** The optimized mixed-mode SPE method reduced matrix interference and achieved satisfactory extraction efficiency and high sensitivity, enabling confident identification of all targets in human urine. Validation showed extraction recovery of 74% to 81% and LODs from 0.1 ng·mL$^{-1}$ to 0.25 ng·mL$^{-1}$. The method was successfully applied to detect urinary excretion profiles of stanozolol and its metabolites in positive volunteer samples.

**Conclusions:** This study presents a novel detection protocol for stanozolol and its metabolites, enhancing the monitoring of stanozolol abuse and contributing to the integrity of sports competitions. This protocol offers a robust tool for anti-doping laboratories, aiding in the accurate detection of stanozolol misuse and supporting the enforcement of fair play in athletics.

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Zhu S, Wang S, Liu X, Wang C, Zhang Y

# Validation for detection of anabolic androgenic steroids in human urine with a "SPE after enzymolysis" sample preparation system by GC-MS/MS

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

## Abstract

Anabolic androgenic steroids (AAS) are prohibited substances in sports, and accurate and efficient detection in human urine is crucial for anti-doping efforts. In this study, a practical "solid phase extraction (SPE) after enzymolysis" sample preparation system was established and validated for the detection of AAS in human urine using tandem-gas chromatography-mass spectrometry (GC-MS/MS). The SPE extraction cartridges and solvents were optimized. The method's performance in terms of selectivity, reliability of detection at MRPL, limit of detection (LOD), carryover and sample extract stability was evaluated and met the requirements of WADA. The key sample preparation steps were automated, and the most AAS detection windows were enhanced compared to existing methods. Results indicated that this method is reliable and valid for AAS detection in human urine, facilitating more effective anti-doping control in sports.

*The details of this study will be published elsewhere.*

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Harps LC[1], Molaioni F[2], Liu L[1], Barone G[2], de La Torre X[2], Parr MK[1], Botrè F[2]

# (Semi-)Targeted approach to uncover methylclostebol misuse

Institute of Pharmacy, Freie Universität Berlin, Berlin, Germany[1];
Laboratorio Antidoping FMSI, Rome, Italy[2]

## Abstract

Potential misuse of methylclostebol (chloromethyltestosterone, ClMT) is commonly monitored by the two isomeric urinary metabolites 4-chloro-17α-methylandrost-4-ene-3α,17β- and 3β,17β-diol. Transferring the knowledge on the metabolism of 17-methyl steroids we expected that also 17β-hydroxymethyl-17α-methyl-13-ene metabolites are excreted. Four of these metabolites have been detected after administration of the analogues steroid dehydrochloromethyltestosterone (DHCMT), which is the 1,2-unsaturated analogue of ClMT (Sobolevsky “I”, “M2”, “M3”, and “M4”).

For further investigation, a controlled administration of ClMT (10 mg, p.o.) was performed in seven healthy male volunteers. Urine samples were collected for 30 days. Sample preparation was adapted from the routine ITP protocol of the Rome laboratory, including enzymatic deglucuronidation and per-TMS derivatization. Samples were analyzed by GC-QQQ-MS after integrating relevant ion transitions for the hypothesized metabolites. For analytical method optimization, reference materials of probable metabolites and/or their diastereomers were synthesized in-house and characterized by HRMS and NMR.

As expected, all volunteers excreted the parent compound and 4-chloro-17α-methylandrost-4-ene-3α,17β-diol. Additionally, the fully A-ring reduced metabolite 4α-chloro-17α-methyl-5α-androstane-3α,17β-diol, 4α-chloro-17α-hydroxymethyl-17β-methyl-5α-androst-13-en-3α-ol, and 4α-chloro-17β-hydroxymethyl-17α-methyl-5α-androst-13-en-3α-ol (‘Sobolevsky’s M3’) were detected and excretion kinetics are determined.

Additional diastereomeric metabolites were tentatively detected due to extended retention time windows used for the included transitions. Comparison of the retention times with several synthesized diastereomeric 4ξ-chloro-17α-methyl-5ξ-androstane-3ξ,17β-diols suggests the occurrence of epimerized D-ring configuration as well. Further confirmation of detailed structures will be performed in the near future.

Barone G[1], Harps LC[2], Liu L[2], Parr MK[2], Botrè F[1], de La Torre X[1]

# Methylclostebol metabolism: An untargeted approach

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Institute of Pharmacy, Freie Universität Berlin, Berlin, Germany[2]

## Abstract

This project introduces an alternative experimental approach by integrating high-resolution mass spectrometry with multivariate statistical analysis to maximize information extraction from data.
This approach was evaluated by analyzing data obtained from an excretion study in healthy volunteers for methylclostebol, an anabolic substance generally administered orally. Analyses were carried out on a GC-HRMS system (Agilent 8890 GC coupled with 7250 GC/QTOF), utilizing low-energy electron ionization (< 18 eV) to preserve the native molecule's carbon skeleton, simplifying mass spectrum interpretation. Mass spectra were acquired in full-scan mode and statistical analysis was conducted using Agilent *MassProfiler Professional* software.
Two statistical approaches were evaluated to analyze the data. The first approach used an ANOVA test, while the second one applied a supervised analysis method (PLS-DA) to construct a predictive classification model. The mass spectra of the most important features were compared with in-house reference standards. Several metabolites linked to methylclostebol consumption were identified by matching retention times, and the metabolic modification positions were determined. One unknown metabolite was also detected, showing a unique retention time that did not match those of all available standards.
This approach demonstrated the effectiveness in the identification of markers without any *a priori* assumptions, improving the drug discovery potential of the analytical method.

Martinez Brito D[1], Colamonici C[1], Montes de Oca R[2], Botrè F[1], de La Torre X[1]

# Markers to distinguish between oral and transdermal clostebol administrations

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Antidoping Laboratory, Havana, Cuba[2]

## Abstract

The aim of this work was to search for specific clostebol metabolic markers or concentration thresholds that may help to distinguish between a transdermal and an oral administration, helping to set up adequate criteria to be adopted by the antidoping community, to support the ADOs in their investigations and results management activities.

Urine samples were collected after the administration of a single oral dose of clostebol (n=3, male), a single transdermal dose (n=1, male) and multiple transdermal administration (n=3 male and n=3 female). After enzymatic hydrolysis, liquid-liquid extraction and the formation of trimethylsilyl derivatives, the samples were analyzed by gas chromatography coupled to tandem mass spectrometry and time-of-flight mass spectrometry.

The metabolism of clostebol after oral and transdermal applications was described. Ten metabolites were detected after oral administration, 5 of which could not be detected after transdermal under the assay conditions here applied. Absolute concentration levels of the metabolites seem to be not discriminating of the different administration routes, due to the variability in the individual absorption, metabolism and/or excretion processes. On the contrary, the ratios between M4, M3 and M2 to M1 (3α-hydroxy-4-chlroro-4-androsten-17-one) showed very promising results to distinguish between oral vs. transdermal administration, under the conditions here described, mainly when the concentrations of M1 are low.

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# Comparative analysis of oxandrolone metabolism through *in vitro* and *in vivo* metabolic prediction models

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Institute of Biophysics Carlos Chagas Filho, IBCCF-UFRJ, Rio de Janeiro, Brazil[2];
Laboratory for the Evaluation and Synthesis of Bioactive Substances (LASSBio), ICB-UFRJ, Rio de Janeiro, Brazil[3];
Bazilian Doping Control Laboratory - LBCD, Rio de Janeiro, Brazil[4]

## Abstract

The study of the metabolism of doping agents is a hot topic in anti-doping science. Here, three different models were compared using the steroid oxandrolone as molecular subtract. The first model was the classical rat liver microsome (RLM), using the NADPH regeneration cofactor solution to induce phase I metabolites. Already presented in the Manfred Donike Workshop, the Zebrafish Water Tank (ZWT) was the second model. Finally, a HepG2 cell culture model in human liver 3D scaffolds was evaluated. Untargeted metabolomics based on Global Natural Product Social Molecular Networking (GNPS) platform was applied to identify putative metabolites. When identified, the suspected peaks were confirmed using the IDCR criteria, when the reference material was available. Both GC-MS and LC-HRMS were used in the detection and identification process. Several hydroxylated metabolites, including the already reported in horses, the 17-hydroxymethyloxandrolone, was detected in the RLM. Without the necessary co-factor, no phase II metabolites or derivatives were detected, as expected. The hydroxylated metabolites detected are not the main analytical targets for routine oxandrolone detection. In a different way, the ZWT produced the oxandrolone sulphate – LC-HRMS detection. As consequence, the target analytes derived sulphate metabolite were also observed, including the epioxandrolone and 17-hydroxymethyl-17-methyl-18-nor-metabolites. This is the first report of the 18-nor metabolites in ZWT, opening the possibility to increase the scope of application of the ZWT to investigate the metabolism of steroids. The 17α-hydroxymethyl-17β-methyl-18-nor-2-oxa-5α-androsta-13-en-3-one (LTM2) was confirmed based on the IDCR criteria. The confirmation of the 17β-hydroxymethyl-17α-methyl-18-nor-2-oxa-5α-androsta-13-en-3-one metabolite (LTM1) was hampered by an interferent eluting just before the expected retention time. Further investigation becomes necessary to elucidate the interferent structure. The hypothesis that this co-eluted peak comes from an isomer of the LTMs formed by zebrafish needs further investigation. HepG2 cell culture model in human liver 3D scaffolds is a promising tool, which can be understood as an artificial human liver. For the first time, oxandrolone was exposed to this model. Despite sulphate metabolite having not been detected, epioxandrolone was confirmed by GC-MS-MS. In addition, 16-hydroxylated metabolites (two epimers) were detected by LC-HRMS. From the experiments developed so far, the 17-hydroxy-17-methyl-18-nor metabolites were not detected. Issues regarding the low production of the sulfate in the model are put forward as a hypothesis. From the comparison, ZWT presents great simplicity regarding the experimental design, forming the targets currently used for oxandrolone detection. Considering the novelty of HEP, complementary studies are necessary for the natural evolution of the model.

Liu B, Deng X, Xu S, Zhuo Y

# Metabolic characteristics of vamorolone in silicon/liver microsomes/ mouse and zebrafish models: Aiding anti-doping strategies

Shanghai Anti-Doping Laboratory, Shanghai University of Sport, Shanghai, China

## Abstract

Vamorolone, as a potential alternative to conventional glucocorticoids, shows significant promise in sports medicine due to its reduced side effects and superior pharmacodynamic properties. This study aims to investigate the metabolic characteristics of this novel synthetic cyclodextrin-steroid anti-inflammatory drug and elucidate its metabolic pathways from the perspective of computational chemistry, *in vitro* human liver microsomes (HLMs), and *in vivo* rat and zebrafish models, thereby providing a scientific basis for assessing its potential risks to athletes and doping detection. All compounds were detected by liquid chromatography-high resolution mass spectrometry (LC-HRMS), and metabolite identification was performed using Compound Discoverer 3.3 software. In the computational theoretical analysis, the drug was shown to be significantly associated with the steroid metabolic pathway; in the HLMs model, 12 metabolites of Vamorolone were successfully identified, including 10 phase I metabolites and 2 phase II metabolites; in the rat and zebrafish models, sulfate-conjugated phase II metabolites were identified for the first time. Among these, the reduction metabolite M1 exhibited the highest peak area, indicating it as one of the primary metabolic pathways. This study systematically identified the structures of Vamorolone's metabolites both i*n vitro* and *in vivo*, providing key data for the pharmacokinetics and biomarker research of this drug. These findings not only enhance the understanding of its metabolic mechanisms but also offer a scientific basis for evaluating its safety and efficacy in sports medicine. Meanwhile, these discoveries can contribute to better regulation and control of Vamorolone's use in competitive sports, ensuring fairness in competitions.

Hou J, Wang Z, Yang S

# Pilot study of new metabolites of enarodustat by *in vitro* human liver microsome and characterized using liquid chromatography/high resolution mass spectrometry

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## Abstract

This study aimed to investigate the in vitro metabolism of Enarodustat, a WADA prohibited substance of hypoxia-inducible factor-prolyl hydroxylase inhibitor (HIF-PHI), by human liver microsomes to identify and characterize its phase I and phase II metabolites for anti-doping applications. Enarodustat was incubated with human liver microsomes for phase I and phase II metabolic reactions. The incubation samples were extracted and injected to Liquid Chromatography/High Resolution Mass Spectrometry (LC-HRMS). And detection was carried out in positive ionization mode using parallel reaction monitoring (PRM). A phase I metabolite (M1) and a phase II metabolite (M2) were found. M1, which is a hydroxylation product, exhibited a quasi-molecular ion at *m/z* 357.11935, 16 Da higher than the parent compound, while M2, a glucuronide conjugate, with a *m/z* of 517.15652, consistent with the anticipated elemental composition. Mass errors for both metabolites were less than 3 ppm. The study of new metabolites of Enarodustat in human liver microsome cultures highlights the efficacy of the LC-HRMS approach for metabolite detection, which has the potential to enhance anti-doping analysis. However, further synthesis of reference materials is necessary to substantiate the structures of these metabolites. This study establishes a foundational understanding of HIF-PHI metabolism in doping control.

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# Detection and evaluation of a novel early-stage excreted metabolite of LDG-4033

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Cyprus Anti-Doping Authority (CyADA), Nicosia, Cyprus[3];
European Monitoring Center for Emerging Doping Agents, Cologne/Bonn, Germany[4];
Natural Products Synthesis & Bioorganic Chemistry Laboratory, Institute of Nanoscience and Nanotechnology, National Centre for Scientific Research "Demokritos", Athens, Greece[5]

## Abstract

LGD-4033 (Ligandrol) is a non-steroidal Selective Androgen Receptor Modulator (SARM) listed under section S1 (“Anabolic Agents”) of the World Anti-Doping Agency (WADA) Prohibited List. This substance has been repeatedly detected in doping controls, resulting in an increasing number of Adverse Analytical Findings (AAFs). In recent years, several SARM-related AAFs have been linked to contaminated dietary supplements, highlighting the need for analytical methods capable of distinguishing between unintentional doping and abuse of SARMs for performance enhancement in sports. The human metabolism of LGD-4033 has been extensively characterized and several long-term metabolites have been described. The presence of a long-term metabolite may reflect either the late excretion of an administered substance or a contamination scenario. A more comprehensive strategy for monitoring LGD-4033 would therefore combine the detection of long-term metabolites with early-stage excreted metabolites, enabling laboratories to better associate an analytical finding with the athlete’s time of exposure.

In the present study, a previously unreported metabolite of LGD-4033 was synthesized and detected by liquid chromatography–high-resolution tandem mass spectrometry (LC-HRMS/MS) for up to six days post-administration after enzymatic hydrolysis and liquid–liquid extraction. The findings reveal an unexpected structure involving double dehydration of the parent compound via an unknown pathway, producing an aromatic pyrrole derivative with distinctive mass-spectrometric features, which can arise metabolically through oxidative aromatization of the LGD-4033 pyrrolidine ring (**Figure 1**).

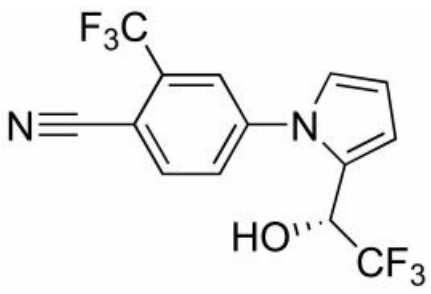


**Figure 1.** Structure of pyrrole derivative metabolite

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This new metabolite is excreted exclusively in the glucuronide fraction and can be detected in negative electrospray ionisation (ESI) mode, displaying a characteristic acetate adduct and a diagnostic aryl phenol fragment.

Additionally, analysis of post-administration samples from an excretion study simulating supplement contamination led to the detection of the pyrrole derivative metabolite in low-dose samples. The aryl phenol fragment proved much more sensitive than the acetate adduct, which was only detectable following high-dose administration. The aryl phenol fragment was detected up to 192 h after administration in the 5×50 µg study, up to 120 h after the administration of 50 µg of LGD-4033, and from 72 h to 120 h in the 5×10 µg study, indicating a bioaccumulation phenomenon.

Pyrrole derivative metabolite represents a novel metabolite, expanding the current knowledge of LGD-4033 metabolism. Although it is a minor, early-stage metabolite, its presence can provide valuable timing information to doping control laboratories and testing authorities. With environmental contamination becoming increasingly significant in anti-doping, and samples with very low concentrations being reported as AAFs, this new metabolite could become an important marker for distinguishing unintentional exposure from misuse. Furthermore, monitoring this metabolite along with established long-term metabolites, such as the dihydroxylated metabolites and other late-stage excreted metabolites, could assist testing authorities in distinguishing between findings of late-stage excreted metabolites associated with abuse and cases of unintentional doping caused by supplements contaminated with LGD-4033, ultimately protecting clean athletes in the anti-doping context.

Data published in:

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# Investigation structures of *in vitro* phase I and *in vivo* rat metabolites of a novel 20-keto-steroid (S42) by GC-MS analysis and chemical synthesis

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Institute of Biochemistry, German Sport University, Cologne, Germany[2]

## Abstract

The synthesis of S42, a new 20-keto-steroid, was published in 2006 by *Uyanik et al*. [1]. Later, S42 was found to be a candidate for Selective Androgen Receptor Modulator (SARM) [2]. Effectively, S42 shows not only anabolic function but also displays tissue-specific transcriptional activity, which can minimize side effects in the prostate [3]. Therefore*, Thevis et al.* expressed concerns on the potential of S42 for illicit sports doping [4].

To enable control and investigation of S42 abuse, an *in vitro* phase I study and *in vivo* metabolism experiments in rats were conducted. Resulting products were analyzed with GC-EI HR $MS^2$ on orbitrap instruments. Additionally, a S42-d7 isotopologue was synthesized with $^2$H-labels at carbon atoms C1, C2, C3, C6 and C7 [5]. The use of stable isotope derivatives was instrumental to interpret the GC-EI HR MS data sets and to identify metabolites observed in these studies. The S42-d7 labeled compound was used for the *in vitro* phase I metabolism study. Comparison of the respective mass spectra of labeled and unlabeled reference materials and of specifically mass-shifted fragment ions provided the foundation for the structure elucidation of S42 *in vitro* phase I metabolites. In addition, S42-C20-OH, S42-C6*ß*-OH and S42-C7*α*-OH were synthesized as reference materials to study their exemplary EI-HR (electron ionization-high resolution) mass spectra.

Close inspection of the spectra and comparison with MS data of synthetic reference materials evidence the formation of a benzyl-mono-hydroxylated S42 metabolite. This finding rests on the exhaustive analysis of three isomeric tri-methyl-silyl (TMS) derivatives found in the *in vitro* phase I experiments. The results indicate that S42 is mostly oxidized in A, B or D ring locations. We detected at least 8 individual phase I metabolites of S42 in the *in vitro* study which were analyzed as TMS ether derivatives. In addition, significant fragment ions from *in vitro* metabolites were also discovered in the hydrolyzed rat urine samples.

S42-C20-OH, S42-C6*ß*-OH and S42-C7*α*-OH synthesis are published in:
Wen HC, Wagener F, Piper T, Neudörfl J, Thevis M, Schäfer M. Investigations Into Structures of *In Vitro*-Derived Phase I Metabolites of a Novel 20-Keto-Steroid S42 by GC-EI HR MS Analysis and Chemical Synthesis. *Drug Test Anal.* 2025 Sep;17(9):1822-1833. doi: 10.1002/dta.3890.

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Avliyakulov NK, Sobolevsky T, Ahrens E

# Analysis and identification of *in vitro* metabolites of exercise mimetic SLU-PP-332 ERRα/β/γ receptor agonist for doping control purposes

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## Abstract

SLU-PP-332 is a small synthetic ERR α/β/γ receptor agonist recently developed using a rational drug design approach. SLU-PP-332 is shown to increase oxidative fibers in muscle, increase fatty acid oxidation and to enhance exercise endurance [1]. In mouse models of metabolic syndrome, it increases energy expenditure and insulin sensitivity [2], and it confers cardiac protection against pressure overload-induced heart failure [3]. Exercise mimetics as a drug class mimic physical activities and prevent progression and development of chronic metabolic diseases including obesity and type 2 diabetes [4]. The World Anti-Doping Agency (WADA) prohibits use of exercise mimetics and metabolic modulators in sports [5]. Identification of metabolites of this emerging therapeutic molecule is a critical step towards detecting its misuse.

This study's aim was identification of phase I and II metabolites generated in vitro using pooled human liver S9 fractions. Metabolites were analyzed using liquid chromatography - high resolution mass spectrometry (LC-HRMS/MS). Five monohydroxylated (M1 through M5), three dihydroxylated (M6 through M8) and four reduced, dihydroxylated metabolites (M9 through M12) have been identified. Metabolites M13 and M19 showed direct glucuronidation and sulfation of the parent compound, respectively. Metabolites M14 to M18 and M20 to M22 showed glucuronidation and sulfation with hydroxylation of the naphthalene or phenolic rings, correspondingly. M1, M7, M9, M10, M13, M14, M19 and M20 are most abundant of the twenty-two metabolites in vitro and potentially useful for doping control purposes. Further studies are necessary to fully elucidate structures.

*Details of the study will be published elsewhere.*

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Jin Y[1], Thomas A[1], Garzinsky A[1], Thevis M[1,2]

# Metabolite pattern of the carbonic anhydrase inhibitors brinzolamide and dorzolamide: Potential markers for the route of application

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## Abstract

Brinzolamide (BA) and dorzolamide (DA) are carbonic anhydrase inhibitors (CAIs), and are commonly used therapeutics for glaucoma treatment via topical application. According to the regulations of the World Anti-Doping Agency (WADA), both drugs are classified under "Diuretics and Masking Agents", prohibiting their use in- and out-of-competition, except for ophthalmic application. Despite ophthalmic use being permitted, systemic absorption occurs, enabling BA and DA detection in blood and urine samples after topical administration. Thus, distinguishing between legitimate (e.g. topical) and prohibited (e.g. oral) drug application routes is critical for sports drug testing result management.

This study investigated the elimination and metabolic profiles of BA and DA following topical and systemic administration in male piglets. Three piglets received BA or DA ophthalmic suspensions, while another three received an oral dose. Urine and blood samples were collected over one week, and all samples were analysed using liquid chromatography-high-resolution tandem mass spectrometry (LCsingle bondHRMS MS). In vitro experiments yielded five phase I metabolites for DA and BA. After method validation, the approach was confirmed to detect DA and BA, with Limits of Detection (LODs) of 55 pg/mL and 75 pg/mL in urine, 110 pg/mL and 180 pg/mL in red blood cells, and 380 pg/mL and 910 pg/mL in plasma. BA and DA metabolites were primarily found in the red blood cell fraction, with only trace amounts detectable in plasma. N-desethylation was observed as the main metabolic reaction for both drugs, and metabolite-to-parent drug ratios were determined in all collected post-administration samples alongside drug concentration levels. The combined consideration of analyte ratios and drug concentrations appears to be indicative of time and dose of drug use (under the chosen routes of administration), which might assist in sports drug testing result management.

This study investigated DA and BA metabolic profiles in piglet models following oral and topical administration using a validated LC–MS/MS method compliant with WADA standards, obtaining elimination behavior and detection time. Urine and blood analyses identified 5 metabolites alongside the parent compounds. Both BA and DA exhibit strong affinity for red blood cells, a characteristic that underlies their prolonged detectability in blood matrices, with N-desethyl-BA and N-desethyl-DA representing primary metabolites. Topical administration, in this study ophthalmic applications (eye drops), exhibited different pharmacokinetic and metabolic profiles compared to oral administration, and excretion patterns demonstrated an administration route-dependent correlation between metabolite-to-parent drug ratios and concentration data, highlighting their potential utility in determining the administration pathway. However, this interpretation should be cautiously applied due to study limitations such as the small sample size, the exclusive use of piglet models, lack of repeated dose evaluation, and limited assessment of inter-individual variability. Also, data on other routes of potential

drug exposure (e.g. unintentional dermal contact are desirable.

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Watanabe Y, Miyamoto A, Ota M, Sato M, Okano M

# Detectability of methyltestosterone metabolites in urine after oral and transdermal administration

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## Abstract

Methyltestosterone (17α-methylandrost-4-en-17β-ol-3-one) is typically administered orally. In Japan, it is also available without prescription as a topical hair-growth preparation, and tablets intended for nutritional use can likewise be obtained over-the-counter.

This study evaluated whether transdermal uptake of methyltestosterone can be detected via urine analysis and whether the route of administration can be distinguished based on metabolite profiles. Ten healthy male subjects were enrolled; five received methyltestosterone orally and five transdermally.

Urine samples were analyzed by gas chromatography-tandem mass spectrometry. The urinary ratio of 17α-methyl-5α-androstane-3α,17β-diol to 17α-methyl-5β-androstane-3α,17β-diol was significantly higher after transdermal administration than after oral administration. ($p$<0.001)

This difference is consistent with prior findings indicating that transdermal application of testosterone promotes conversion to 5α-reduced steroids, likely due to high activity of 5α-reductase in the skin. These results demonstrate that transdermal administration of methyltestosterone is detectable in urine and suggest that metabolite ratios can serve as indicators of the administration route.

*The full paper will be published elsewhere.*

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Wang Y, Li W, Deng X

# Development and application of a dual isotopic labeling method for enhanced detection and quantification of stimulants in urine samples using high-resolution mass spectrometry

Shanghai Anti-Doping Laboratory, Shanghai University of Sport, Shanghai, China

## Abstract

Given the critical nature of anti-doping efforts, the detection of stimulant substances is shifting from accurate qualitative analysis to precise quantitative analysis. Additionally, the use of liquid chromatography-high-resolution mass spectrometry (LC-HRMS) in detecting stimulants is becoming more widespread. However, the lack of isotope-labeled internal standards is causing increasing issues with quantitative accuracy. Furthermore, challenges such as the mass spectrometric response of small molecules and the separation of isomers present additional difficulties. We have developed a quantitative method for stimulant substances containing amine or phenol hydroxyl groups, using a dual-label derivatization system. This method offers a new perspective for analyzing and detecting low molecular weight substances, isomers, or those with poor LC-MS response, and proposes a solution to the problem of missing isotope-labeled internal standards. Methodological validation has shown that this approach has promising application potential.

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# Enhanced morphine detection: Combining antibody engineering, UQ-body and CNT-FET sensing

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Department of Biotechnology, Duksung Women's University, Seoul, Korea[6];
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## Abstract

Morphine is a powerful painkiller that is used to treat pain in health care settings. However, the misuse and overdose of morphine has led to serious societal problems, making effective early detection and continuous monitoring of morphine use critical. Early morphine detection methods used precision instruments, but they require expensive equipment and skilled experts. Therefore, antibody-based immunoassays have been developed with improved efficiency and rapidity. However, they can only detect morphine at high concentrations. Morphine can have powerful physiological effects at low levels, so the ability to detect even low concentrations is important. Therefore, research is still needed to detect morphine with high sensitivity.

In this study, we expressed a structurally stable morphine detection antibody using a mammalian cell expression system and confirmed its activity against morphine using a competitive ELISA. We then used molecular docking and alanine screening to identify key residues involved in morphine interaction to design a morphine detection antibody with enhanced binding affinity. We selected the H:Leu101 for further engineering, replacing H:Leu101 with a charged amino acid, hypothesizing that additional hydrogen bonding with morphine would improve affinity. Competitive ELISA showed a 7.5-fold decrease in the $EC_{50}$ of H:Leu101Glu, resulting in improved sensitivity, but the same LOD of 6 nM as the wild type.

Additionally, a fluorescent biosensor strategy named double-labelled ultra-quench (UQ-body) was chosen to efficiently detect morphine screening. The UQ-body employed fluorescence quenching of two dyes appropriately coupled by a unique tryptophan (Trp) residue in the variable region (Fv) of the antibody to enhance the difference in fluorescence signal in the presence and absence of antigen. Our UQ-body was able to detect morphine in less than 5 minutes, but the LOD was as high as 3 µM.

Finally, we constructed a bio-electronic sensor system using a morphine detection antibody. The antibody was immobilized on a floating electrode of a carbon nanotube field-effect transistor (CNT-FET). The constructed sensor detected morphine at a concentration of 1 aM with high selectivity in real time. These results indicate that the antibody-based bio-electronic sensor can be applied to the rapid and efficient detection of morphine.

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Oliveira FA[1], Farias CR[2], Silva CA[2], Silva TR[2], Motta MTR[2], Louzada NV[2], Padilha MC[2], Pereira HMG[2]

# Optimization and validation of a method for simultaneous analysis of 6-oxo metabolites and the steroid profile markers by GC/C/IRMS

Drug Control Centre King's College, London, Great Britain[1];
Brazilian Doping Control Laboratory, Rio de Janeiro, Brazil[2]

## Abstract

The aromatase inhibitor 6-oxo-androstenedione (6-oxo) is prohibited for use by athletes. 6α-Hydroxy-androst-4-ene-3,17-dione (6α-OH-AD) is recognized as the best marker for detecting 6-oxo misuse, as it is found in urine at higher concentrations and with larger detection window compared to other markers following 6-oxo intake. As 6α-OH-AD can otherwise be detected at low concentrations in some samples as a minor metabolite of androst-4-ene-3,17-dione from either endogenous or exogenous origin, WADA requires laboratories to confirm 6α-OH-AD findings by GC/C/IRMS when its concentration exceeds 10 ng/mL. Additionally, WADA recommends that laboratories perform GC/C/IRMS analysis of the steroid profile markers before determining the need to confirm 6α-OH-AD.

For practical reasons, an ideal confirmatory method would enable the laboratory to analyze both 6-oxo metabolites and steroid profile markers from a single sample aliquot. Notwithstanding, the scarce reports on GC/C/IRMS analysis of 6α-OH-AD published to date may not fully comply with the current WADA requirements or may be unsuitable for purifying relatively less polar compounds such as the markers of testosterone misuse, given the characteristics of the HPLC sample cleanup methods described. The goal of this research was to validate a method allowing both confirmations to be performed using a single sample aliquot. Specifically, this study aimed to include 6α-OH-AD and its 6β-epimer, also a 6-oxo metabolite, in the method previously validated by the Brazilian Doping Control Laboratory (LBCD) for GC/C/IRMS analysis of the steroid profile markers. This involved collecting one additional fraction during the first HPLC cleanup, which contains the two metabolites, and optimizing the second HPLC cleanup to purify 6α-OH-AD and 6β-hydroxy-androst-4-ene-3,17-dione (6β-OH-AD) for this method validation.

The second HPLC step was developed using the same stationary and mobile phases employed at LBCD for analyzing the steroid profile markers: a Waters XBridge Shield RP18 column (25 cm, 4.6 mm, 5 µm), and acetonitrile (ACN) and water as mobile phases. A 2-level factorial design of experiments was conducted to optimize peak resolution and retention factor. Three parameters were evaluated: the mobile phase composition at the start of the gradient (ranging from 20% to 45% of ACN), the ACN gradient increase rate (from 0.5%/min to 4.5%/min), and the temperature (from 15°C to 30°C). Data analysis was performed using Chemoface software, version 1.71.

The sample preparation procedure encompassed solid-phase extraction, liquid-liquid extraction, enzymatic hydrolysis and two preparative HPLC purification steps with an acetylation in between. Sample volumes of 25 mL were used. Method validation encompassed all the parameters required by WADA. Six real samples from the LBCD collection containing 6α-OH-AD at concentrations above 5.0 ng/mL were analyzed to demonstrate the method’s fitness for purpose.

The optimal conditions determined for the second HPLC cleanup step were: initial mobile phase

composition of 25% ACN in water, gradient increase rate of 0.5 %/min, and oven temperature of 30°C. These parameters resulted in a peak resolution of 2.6 between the two isomers and a retention factor of 9.6 for 6α-OH-AD. Linearity of the instrument was established in the ranges of 315-4829 mV for 6α-OH-AD and 291-4794 mV for 6β-OH-AD. The other parameters determined for 6α-OH-AD and 6β-OH-AD were, respectively: LOQ, 5.0 ng/mL and 5.0 ng/mL; intermediate precision, 0.2‰ and 0.2‰; bias, 0.6‰ and 0.5‰; combined uncertainty, 0.6‰ and 0.5‰. Three negative samples from the LBCD collection, containing 6α-OH-AD just above 5.0 ng/mL, were analyzed and presented $\Delta\delta^{13}C$ values between 1.4‰ and 2.3‰ against pregnanediol as ERC. Other three samples, obtained from an individual who used intramuscular testosterone therapeutically, presented 6α-OH-AD at 5.9 to 8.4 ng/mL and $\Delta\delta^{13}C$ values between 13.6‰ and 13.8‰ against pregnanediol.

This method was shown to be useful for doping control laboratories, enabling two simultaneous confirmatory analyses by GC/C/IRMS. It allows for more efficient sample management while reducing redundant work. It was fully validated according to the WADA requirements.

Sobolevsky T, Ahrens E

# Custom combustion interface with capillary-in-capillary design for improved chromatography in GC-C-IRMS analysis of steroids

UCLA Olympic Analytical Laboratory, Los Angeles, United States

## Abstract

The combustion interface in gas chromatography–combustion-isotope ratio mass spectrometry (GC-C-IRMS) must ensure a rapid and efficient transfer of vaporized sample components into the hot zone, followed by their complete combustion. Equipment manufacturers achieve this in a variety of ways, each according to their own design. The need to accommodate multiple peripherals usually results in a compromise between versatility and performance. Given that antidoping laboratories usually undertake compound-specific carbon isotope ratio analysis, default configurations can often be tailored to improve robustness and efficiency.

The GC Isolink combustion interface (Thermo Fisher) has been extensively modified. The oxidation reactor was made in-house and contained three Cu/one Pt wires and was operated at 850°C. The multifunctional valve cluster was replaced with a 1/32” 6-port diaphragm valve (Valco) acting as a selector between the backflush and straight modes. Oxygen was distributed via three paths: a small fraction was continuously introduced with the backflush helium (about 0.1%) such that the oxidation reactor did not lose charge while in standby. Additionally, the makeup helium entering the oxidation reactor in straight mode had a low percentage of oxygen, which was also non-switchable. Lastly, for the conditioning of a new reactor, the backflush helium could be supplemented with a much larger quantity of $O_2$ via an MOVPT valve (Trajan/SGE) actuated from within Isodat. Helium flow exited the diaphragm valve into an open split constructed from a 1/32” Valco tee. A short silica capillary opened to the atmosphere, while a sniffing capillary allowed a fraction to be pulled through a Nafion dryer into the ion source. A dedicated helium line connected to the diaphragm valve ensures only pure helium is allowed into the ion source during backflush. The connection of the GC column to the oxidation reactor was implemented such that inside the GC oven the column first passed through a large-bore 1/32” crosspiece (0.5-mm ID) and then through a 0.53-mm ID silica sheath to the oxidation reactor. The two other ports of the crosspiece connected to the backflush MOVP valve mounted outside the GC oven and to the makeup gas flow (He + $O_2$). The column tip with its polyimide coating removed was introduced directly into the combustion reactor and positioned approximately 10 mm from the hot wires. Since the column OD is smaller than the sheath capillary ID, flow could pass in both directions with minimal restriction, allowing the makeup gas in and backflush out. It should be noted that the capillary-in-capillary design decreases solvent backflush efficiency: while non-polar solvents such as cyclohexane are easily managed, PTV solvent split injection mode is required to avoid elevated background when solvents such as toluene and ethyl acetate are employed.

These hardware modifications resulted in robust and trouble-free combustion performance with no dedicated oxidation cycles (except for initial oxidation of a new reactor) as well as in minimal peak tailing not affected by connections between the chromatographic column and oxidation reactor. After more

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than 5500 injections with no column or reactor change, this GC-C-IRMS system shows no drift in $\delta^{13}C$ values, even though peak shape did deteriorate to some extent (more so for underivatized steroids).

*The details of this work will be published elsewhere.*

Sobolevsky T, Cittan M, Ahrens E

# Carbon isotope ratio analysis of steroids based on doSPE, 2D HPLC cleanup and selective formylation

UCLA Olympic Analytical Laboratory, Los Angeles, United States

## Abstract

Carbon isotope ratio analysis GC-C-IRMS) remains the gold-standard method to establish the origin of urinary steroids. To ensure the validity of isotopic data, GC-C-IRMS analysis requires extensive sample cleanup, which is usually based on a combination of liquid-liquid and/or solid phase extraction (SPE) and preparative HPLC.

Formylation was previously shown as a promising alternative to acetylation for GC-C-IRMS analysis of steroids due to an improved chromatographic resolution between 5α- and 5β-isomers [1]. To further evaluate the benefits and limitations of formylation, a new procedure was developed to allow isolation, purification, and analysis of key targets and endogenous reference compounds by GC-C-IRMS within a reasonably short time (40 min per sample). Here, urine was extracted using successive application of strong cation and strong anion exchange SPE with enzymatic hydrolysis in between. This procedure removes most acidic and basic substances, such as metabolites of non-steroidal anti-inflammatory drugs or antidepressants frequently found in athlete urine, while neutral steroids are recovered for HPLC cleanup. After this dual SPE, only the testosterone fraction required a secondary HPLC cleanup achieved using a custom 2D LC system. Eight fractions per sample were collected, four of which were formylated (androstanediols, pregnanetriol, pregnanediol, and testosterone), and the remaining four were analyzed underivatized (11-ketoetiocholanolone with 11-hydroxyandrosterone, etiocholanolone, androsterone, and 16-androstenol).

Formylation was essential for the androstanediol fraction, as not only were the formate derivatives of 5α- and 5β-androstanediol well separated from each other, but they were also sufficiently resolved from the biological background. Acetylation was not adequate and would have necessitated a secondary HPLC cleanup. In the case of pregnanetriol, formylation was the only way to obtain reliable isotopic data, as pregnanetriol dehydrates upon formylation to yield a more volatile product with good chromatographic properties. The method has been validated in accordance with the requirements of the World Anti-Doping Agency and met expectations set forth in TD2022IRMS.

*The details of this work will be published elsewhere.*

## References

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Cavalcanti G, Brito B, Padilha M, Pereira HMG

# Evaluation of gas chromatography – triple quadrupole versus liquid chromatography – triple quadrupole for the analysis of relevant steroids in serum samples

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## Abstract

An effective and comprehensive analytical method is essential in doping analysis, particularly to reduce turnaround times and costs. LC-TQ has been the primary method for quantifying endogenous steroids and identifying exogenous steroids in serum. However, limitations such as ionization issues can compromise the sensitivity and comprehensiveness required for effective analysis. Addressing these challenges often necessitates additional steps in the LC analysis workflow and larger serum volume, making the process more laborious, and non-compliant with WADA requirements.

GC-EI TQ is considered the gold standard for steroid analysis due to its robustness against matrix effects, as the electron ionization (EI) mechanism does not involve ion-molecule reactions. Although previous studies have demonstrated the sensitivity of GC-TQ techniques in blood matrices, these focused on DBS and plasma and did not address the challenges, and the broader analytical scope proposed here.

Recent studies have identified serum as a promising matrix to complement the existing steroidal module in urine. The blood steroid profile (BSP) involves the longitudinal monitoring of testosterone (T) and the T/A4 ratio in serum samples. This approach enhances the sensitivity and selectivity of the steroidal module within the Athlete Biological Passport (ABP), particularly for individuals with low urinary T excretion rates, such as females and those with UGT2B17 gene deletion polymorphisms.

This study aimed to evaluate the analytical performance and equivalence of GC-EI TQ and LC-TQ for detecting relevant steroids in serum samples for doping control. Serum samples underwent protein precipitation, liquid-liquid extraction, derivatization by MSTFA/$NH_4$I/2-mercaptoethanol to obtain the trimethylsilyl derivatives for GC analysis, and mobile phase reconstitution for LC analysis. Both methods were validated following WADA requirements, and their interchangeability was assessed by overlaying concentration measurements of T and Androstenodione (A4) within methods' expanded uncertainty limits.GC-EI TQ demonstrated superior sensitivity for most steroids, including testosterone esters. A testosterone gel (Androgel, 100 mg daily for 8 days) administration study with two female volunteers was conducted. Capillary serum samples were collected by Tasso+. This study revealed significant increases in T levels (30–50-fold) compared to baseline concentration. Dihydrotestosterone (DHT) levels also increased, but in less extension (5–10-fold) during the gel application treatment. DHT levels declined quickly just after one-day treatment withdrawal, but its concentration kept higher (2-3-fold) than baseline level. On the other hand, T level was higher in this period (10-fold) than baseline concentration. Baseline concentration of DHT was below the limit of quantification (0.25 ng/mL). DHT was detectable only with GC-EI TQ, as LC-TQ lacked the necessary sensitivity.

In conclusion, GC-EI TQ provided broader analytical coverage and higher sensitivity than LC-TQ for serum

steroid analysis in single chromatographic run. The GC technique could be useful for incorporating DHT as an additional biomarker in the BSP, alongside T and T/A4. The inclusion of DHT in BSP might be beneficial once this target is very sensitivity regardless of the administration route of T.

Jardines D, Martínez Brito D, de La Torre X, Botrè F

# The matrix effect and its relation with in-source fragmentation on ESI-LCMS analysis. The 5-oxo-mefruside example

Laboratorio Antidoping FMSI, Rome, Italy

## Abstract

Electrospray ionization coupled with liquid chromatography mass spectrometry (ESI-LCMS) has become a cornerstone technique in analytical chemistry. One principal handicap of this technique is represented by the matrix effects, that, arising from sample matrices interfering with analyte ionization and detection, pose a significant challenge in LCMS-based analyses, leading to compromised accuracy and precision in the case of quantitative analyses. Other phenomenon comes up in ESI source, the in-source fragmentation, which is molecule-dependent and it would be directly related to matrix effect. To study this relation, we focused on the 5-oxo-mefruside as the model molecule, analyzed using nine different urine matrices in an initial testing procedure that included the presence of other compounds. As ESI-MS was used (QExactives mass spectrometer with a HESI-II source). Six in-source collision-induced dissociation energies were used. The results demonstrated the implications of in-source fragmentation on matrix effects, and varying the energy of the ion-source collision-induced dissociation were found the best result at 15 eV; matrix effect mitigation and better sensibility. Also, in-source fragmentation provided additional structural information about the analyte studied.

Jardines D, Martínez Brito D, de La Torre X, Botrè F

# GLUCOCORTICOIDS. A multivariate study of the matrix effect and its relationship with in-source fragmentation, on an LC-ESI-HRMS (Orbitrap) platform

Laboratorio Antidoping FMSI, Rome, Italy

## Abstract

Liquid chromatography-mass spectrometry with electrospray ionization source is the analytical technique par excellence used for the qualitative and quantitative analysis of glucocorticoids. These constitute class S9 of the WADA list of prohibited substances and the main criteria to report an adverse analytical finding are based on their urinary concentration levels. The decision to apply a confirmation procedure is directly influenced by one of the main problems of this technique, the matrix effect on the target compounds ionization. Also, these compounds undergo in-source mass fragmentation, rarely investigated or used because its low reproducibility and matrix depended. This work aims to focus on the matrix effects over the S9 class using a multivariate approach and its correlation with the in-source mass fragmentation phenomenon, as obtained on a reference liquid chromatography-mass spectrometry platform, i.e., a LC-ESI-HRMS (Orbitrap) system. The study included 27 glucocorticoids and six in-source collision-induced dissociation energies. It was realized within an initial testing procedure that included the presence of other compounds. Two main results were obtained: the mitigation of the matrix effect and, consequently, the improvement in the initial testing procedures for these compounds, with better reproducibility and accuracy. As part of the study were characterized the in-source fragment ions of 27 glucocorticoids.

Borschel L[1], Thomas A[1], Bally L[2], Thevis M[1,3]

# Method development and validation for the detection of semaglutide in serum and plasma by LC-MS/MS

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Department of Diabetes: Endocrinology, Nutritional Medicine and Metabolism, Inselspital, Bern University Hospital, Bern, Switzerland[2];
European Monitoring Center for Emerging Doping Agents (EuMoCEDA), Cologne, Germany[3]

## Abstract

Semaglutide, a glucagon-like peptide-1 (GLP-1) receptor agonist, is primarily used in the treatment of obesity and type 2 diabetes but has also gained attention in anti-doping research. In addition to its appetite control and insulinotropic effects, animal experiments suggest an enhanced mitochondrial biogenesis stimulated by semaglutide administrations. Currently, semaglutide is subject of the World Anti-Doping Agency's (WADA) Monitoring Program.

The study aims to develop a new analytical method for the detection of semaglutide in plasma and serum samples using liquid chromatography high resolution tandem mass spectrometry (LC-HRMS/MS). For this purpose, tirzepatide, another GLP-1 receptor agonist, was used as an internal standard. Samples were prepared by protein precipitation and the resulting supernatant was analysed by LC-HRMS/MS. The method was validated, regarding e.g. limit of detection (5 ng/mL) as well as selectivity, reliability and stability. The method’s applicability and fitness-for-purpose was confirmed by analysing plasma samples (50 µL) of patients undergoing weekly subcutaneous administrations of 1 mg of semaglutide.

*The details of this study will be published elsewhere.*

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Zhao J, Deng X, Yan R

# Exploratory study on surface-enhanced Raman spectroscopy detection of recombinant human growth hormone in urine based on tyramine signal amplification strategy

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## Abstract

Human Growth Hormone (hGH), a crucial peptide hormone secreted by the pituitary gland, plays an essential role in regulating various physiological processes, including growth, metabolism, and immune function. In clinical practice, hGH is widely employed for the treatment of conditions such as growth hormone deficiency, recovery from burns, and bone fracture healing. Moreover, its potential to enhance muscle development and promote fat metabolism has garnered significant attention in the anti-doping filed. However, the extremely low concentrations of hGH in complex biological matrices such as urine present significant challenges to conventional detection methods, which often exhibit limitations in sensitivity, specificity, and operational complexity. To overcome these challenges, this study presents a Surface-Enhanced Raman Spectroscopy (SERS) technique coupled with a tyramine signal amplification strategy for the sensitive detection of hGH in urine.

In this study, gold nanoparticles (AuNPs) were synthesized via the Frens method and functionalized with 4-mercaptobenzoic acid (4-MBA) to optimize SERS signal enhancement. Tyramine deposition, catalyzed by horseradish peroxidase (HRP), was employed to interact with the nanoprobe, further amplifying the detection signal for hGH. The results demonstrated a detection sensitivity as low as 0.01 ng/mL, with a strong linear correlation over a wide concentration range. The relative standard deviation (RSD) for substrate repeatability and signal uniformity was 5.54%, indicating excellent stability and reproducibility of the method. Furthermore, the spiked urine sample experiments confirmed the method’s high sensitivity and anti-interference capability for hGH detection.

In conclusion, the SERS-based detection method utilizing tyramine signal amplification provides significant improvements in both sensitivity and reproducibility, facilitating rapid and reliable detection of hGH in complex biological matrices. This approach holds substantial promise for applications in the anti-doping filed, clinical diagnostics, and public health surveillance.

Reihlen P, Blobel M, Sakellariou P, Weiß P, Thevis M

# Low-volume urine preparation using an immuno-isolation plate for EPO-SAR-PAGE analysis

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## Abstract

Detection of Erythropoietin Receptor Agonists (ERA; e.g. rEPO, NESP, CERA and EPO Fc) in urine samples is commonly done using a gel electrophoretic approach such as SAR-PAGE followed by an immunoblot. A mandatory immunopurification step needs to be performed on urine samples before SAR-PAGE analysis. Currently, most WADA-accredited anti-doping laboratories utilize a sample volume of about 15 mL of urine in the Initial Testing Procedure (ITP) for the detection of ERAs. The immunopurification is often performed using an EPO Immunoaffinity Isolation Plate from StemCell. Also, it has been common practice to analyze suspicious samples, which showed low analyte signal intensities during ITP detection, with higher volumes of urine during the Confirmation Procedure to mitigate low analyte concentrations. Quality controls and reference standards during ITP are usually applied in amounts adjusted to the signal intensity range anticipated when analysing 15 mL urine aliquots. The procedure is costly and requires a considerable amount of sample volume. Here, a new approach is shown, which reduces the amount of urine sample utilized during ITP preparation to 3 mL. The preparation is based on ultrafiltration and immnopurification using the Immunoaffinity Isolation Plate from StemCell. The amount of quality controls, reference standards and internal standards used during analysis are adapted to the anticipated signal intensity range of 3 mL sample volumes. In order to reduce background and spot formation during blot detection, a mandatory pre-incubation and washing step with chemiluminescence substrate was implemented before substrate incubation and detection. The resulting data are of comparable quality and ERA sensitivity is competitive to methods utilizing 15 mL of sample volume. The use of 3 mL of urine allows for employing smaller ultrafiltration devices, which further increases sample throughput as more urine specimens can be subjected simultaneously to the centrifugation step, and centrifugation time is also reduced. In addition, smaller ultrafiltration devices are commonly cheaper, and less protease inhibitor is needed during sample preparation. Finally, the reduction of the required sample volume used in ERA ITP to as little as 3 mL contributes to ensuring sufficient sample volumes being available for further analyses. A reduction in sample volume and/or adaptation of signal intensities for references and quality controls has also been shown to be beneficial for ERA Confirmation Procedures.

van Haecke N, Bourgeois J, Deventer K, van Eenoo P

# Inclusion of the detection of TGF-β inhibitors luspatercept and sotatercept in EPO/ERAs analysis protocol

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## Abstract

Luspatercept and sotatercept, both Transforming Growth Factor-Beta (TGF-β) signaling inhibitors, block intracellular signaling via the SMAD pathway, and so can stimulate erythropoiesis. Therefore, both substances are included in the WADA Prohibited List of Substances, section S2.1.4. The goal of this research was to evaluate the inclusion of luspatercept and sotatercept in a validated EPO/ERAs detection procedure. Blood serum samples were spiked with ACVR2A-Fc and ACVR2B-Fc standards, immunopurified using specific antibodies and analyzed using SAR-PAGE and immunodetection. The method is sensitive for both substances and easy to implement in the current EPO/ERAs protocol.

Liu L, Wang Z, Zhao L, Zhang L, Zhou X

# Method development of pegmolesatide for doping analysis: A novel synthetic erythropoietin-mimetic agent

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

## Abstract

Pegmolesatide, a novel synthetic erythropoietin-mimetic agent, binds and activates the erythropoietin (EPO) receptor by Hansoh Pharmaceutical Group Co, Ltd (China). In late 2023, it was approved in China for the treatment of anemia in both dialysis and non-dialysis chronic kidney disease (CKD) patients, with the advantages of a reduced immunogenicity and an extended duration of action, compared to recombinant erythropoietin. The aim of this study was to develop a strategy for the detection of pegmolesatide in doping analysis.

Here, we present a bottom-up nano-LC-HRMS/MS approach for the qualitative analysis of pegmolesatide in serum. Extraction from 500 µL of serum was achieved by affinity purification using erythropoietin receptor-coupled magnetic beads, followed by trypsinization of the retained protein content and subsequent detection of characteristic peptides. To identify the tryptic digestion peptides of pegmolesatide, they were first examined by Full Scan and ddMS2 mode and peptides search was preformed via Proteome Discoverer. Then, analytical method using Product ion scan mode was developed to detect the identified characteristic peptides. This developed method was validated for both initial testing procedure (ITP) and confirming procedure (CP) purposes, parameters of selectivity, reliability, limit of detection (LOD), limit of identification (LOI), carryover and stability are evaluated. In this study, two characteristic peptide segments of pegmolesatide were recognized. Both peptide segments successfully identified pegmolesatide in serum for the ITP purpose, while one specific peptide segment was fully assessed for the CP purpose. The negative controls and pegmolesatide samples could be clearly distinguished from each other. The LOD and LOI for pegmolesatide in serum are 2 and 7 ng/mL, respectively. Deduced from the pharmacokinetic parameters indicated by the manufacturer’s prescription instruction, this developed method is considered fit-for-purpose of detecting pegmolesatide for doping control. This method would also expansion multi-analyte testing capability among other erythropoietin mimetic agents and their constructs (e.g.Peginesatide, CNTO-530). Moreover, further research is required to confirm and subsequently optimize this approach in terms of its applicability and efficiency in urine and dried blood spot.

**The 2025 Manfred-Donike-Award for the best poster presentation went to Dr. Lu Liu and her co-authors from the Beijing Anti-Doping Laboratory, Beijing, People´s Republic of China. This exceptional research demonstrates the development and validation of an innovative analytical method for detecting Pegmolesatide, a novel synthetic erythropoietin-mimetic. The poster distinguished itself through scientific depth, innovation and strong relevance to practical anti-doping applications. The study represents a significant scientific contribution to modern anti-doping analysis and advances the efforts to ensure fairness and integrity in competitive sports.*

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Tian T, Chen X, Deng X

# Detection and *in vitro* metabolism of two novel kisspeptin-like dopings by UHPLC-HRMS for doping control

Shanghai Anti-Doping Laboratory, Shanghai, China

## Abstract

Many small peptides are susceptible to enzymatic degradation by peptidases, resulting in their instability and rapid clearance. Hence, structural modifications such as C-terminal modification and amino acid substitution are frequently employed to improve drugability, yielding a series of peptide analogues. Among these, kisspeptins and its analogues have become a new focus in small peptide anti-doping since 2024. Kisspeptins are a group of peptide products encoded by the KISS-1 gene, which have been demonstrated the ability to activate the kisspeptin receptor (KISS1R) and act as the foremost upstream regulator of the hypothalamic-pituitary-gonadal (HPG) axis. Given the effect of testosterone stimulating provoke potential misuse in sports, WADA has included kisspeptins on the 2024 Prohibited List. Since the rapid *in vivo* clearance of kisspeptins limited their application, extensive structure-optimization efforts were dedicated by replacing some of the component amino acids to overcome these pharmacokinetic challenges. Among them, TAK-448 and TAK-683 showed enhanced therapeutic potential and increased biological stability, with a difference in the second amino acid. Their emergence opens new horizons for clinical drug development, but also providing athletes with potential new doping agents to circumvent routine sports drug testing.

As a key means to enhance doping control, comprehensive understanding the metabolic profile of doping agents in human is an essential process. However, the current limited understanding of small bioactive peptide metabolism hinders effective doping control. Up to now, the typical biotransformation pathway that occurs on such substances is considered to be peptidase hydrolysis and resulting in truncated amino acid sequences. A primary challenge lies in distinguishing suspicious features from extensive mass spectral datasets contaminated by biological matrix interference (*e.g.* proteins and macromolecular peptides) and background noise.

In this study, we introduce a strategy integrating in silico prediction and nontargeted data mining to achieve more comprehensive metabolic profiling through ultra-high performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS). The strategy operates by identifying and applying chemical similarity (CSIM) rules of peptides (such as LC/MS behaviors and specific biotransformation) to mine unknown metabolites. With this strategy, a semi-automated workflow utilizing computational software and custom script was constructed and applied to the human liver microsomal metabolism of TAK-448 and TAK-683.

By in-depth data mining from redundant background signals with the proposed strategy, a total of thirteen metabolites (three were validated *via* synthetic standards) and two novel biotransformation pathways (N-terminal vinylation and N-terminal carboxylation) were identified for both investigated compounds. Notably, the two biotransformation pathways offered a new perspective on small peptide metabolism, which was further confirmed in rats, and produced two promising long-term

metabolites for monitoring doping abuse. Furthermore, the inclusion of these two nonapeptides and their in vitro metabolites in the routine analytical method for small peptides in our laboratory showed satisfactory selectivity and LOD (0.04~0.05 ng/mL for TAK-448, TAK-683). The study provided the first comprehensive characterization of the metabolic profiles of TAK-448 and TAK-683, while also offering an effective tool for metabolism research on small peptide doping agents [1].

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Voss S[1], Schwenke D[1], Mirtschink P[2], Wevelsiep A[3], Gaborini L[4], Robinson N[4]

# Comparison of the new Sysmex XR-Series haematology analyser with the XN-Series focusing on Athlete Biological Passport data

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## Abstract

The Athlete Biological Passport (ABP) has been established as an anti-doping tool based on the personalized monitoring of haematological biomarkers. The main aspect for the use as analytical routine method with the introduction of the ABP was the decision to use only one analytical platform as haematological analyser. With the change of the Sysmex XT-2000 to the new Sysmex XN-1000 platform in 2019 it was necessary to adjust the reticulocyte percentage as parameter within the ABP to enable a sufficient integration of the new analytical results into the already existing individual profiles of athletes. With the introduction of the new Sysmex XR-Series in 2021 it is only a matter of time until this instrument type could become necessary to implement. The analytical performance of the XR-series for clinical purposes has been evaluated previously, but data concerning ABP requirements described in the WADA technical documents are not available. Therefore, our goals were to compare the XR series with the XN series and to evaluate the performance of the XR series within an Anti-Doping context, considering the requirements formulated in WADAs technical documents.

Over 300 samples were investigated within this project and results for ABP parameters showed excellent inter-platform comparability. We conclude that a transfer from the XN-1000 platform to the XR-1000 platform does not present a risk of necessary adjustments within WADAs haematological module of the ABP.

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# Physical exercise does not affect the urinary ABP markers in the short term

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## Abstract

Studies show that the steroid ABP module increases the chance to detect testosterone administration as compared to population-based cut-off T/E values in men and women. Nevertheless, more knowledge is warranted for better interpretation of natural variation of the ABP biomarkers. The primary aim is to study the intra-individual variation of ABP markers (T, E, A, Etio, 5βdiol and 5αdiol) in female athletes. The hypothesis is that the markers are excreted in higher urinary amount during rest in comparison to training.

The study has an ethical approval from “Etikprövningsmyndigheten” DNR 2023-00161-01. Study population included urine samples from 26 well trained athletes collected over two days. During the first day the athletes conducted no physical exercise whilst exercising regularly on day 2. All urine was collected and sampled after which they were pooled for a daily total. Each participant provided 4-11 urine samples/day.

Following GC-MS/MS analysis, no apparent variation between day one and day two could be observed in the ABP ratios or the individual concentrations. The daily total concentrations were also compared using a Wilcoxon signed-rank test which agreed with the collectedspot urine samples.

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

# Influence of transportation time and conditions on the results of CSCQ samples in the Antidoping Laboratory of Havana

Antidoping Laboratory, Havana, Cuba

## Abstract

The aim of this work was to study the possible correlation between the results obtained in the CSCQ rounds for the verification of the Laboratory's performance, with the temperature and the time elapsed from their shipment (Switzerland) to their reception in the Laboratory (Cuba).

The CSCQ samples received in the Laboratory were allowed to stabilize for 24-48 hours before their analysis in the Sysmex XN-1000 Hematology Analyzer. Seven consecutive readings were made of each sample and they were immediately stored at 4°C to be reanalyzed after 7 days. The acceptance criteria of the evaluated parameters published in the CSCQ Final Report were compared with the results obtained in the laboratory. According to the manufacturer, prior to the analysis of the samples, the XN CHECK controls (Sysmex Level 1, 2 and 3) were verified and the instrument was calibrated with XNCAL (Sysmex).

The samples received more than 10 days after shipment and reception at the laboratory showed high viscosity and dark color. The scattergram showed an abnormal distribution in the RET channel (RET Abn Scattergram) with the presence of erythrocyte fragments, indicative of cell deformation. Also observed in the RET scattergram were inclusion bodies or foreign bodies in the PLT zone (PLT CLUMPS), which generated cell grouping, affecting the cell count. This behavior was attributed to the transportation time between Switzerland and Havana under non- adequate temperature transportation, which was confirmed during reception of the samples. No significant differences were observed between the measurements made upon arrival at the laboratory and those made seven days later.

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Naumann N[1], Do C[1], Vollmert C[2], Krajina M[2], Thomas A[1], Cheung HW[3], Wong K[3], Ho ENM[3], Wan TSM[3], Thevis M[1,4]

# Multiplex gene doping detection for seven performance-enhancing transgenes using PCR-coupled MALDI-TOF MS analysis – an update

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## Abstract

In the previous Cologne Workshops on Doping Analysis, a new gene doping detection approach for multiplex detection of seven potential human gene doping targets in one reaction and analysis was presented. A gene doping panel prototype was developed, suitable for the simultaneous detection of transgenic DNA encoding for *EPO*, *FST*, *GH-1*, *IGF-1*, *MSTN* (-Propeptide), *VEGF-A* and *VEGF-D*. The detection approach is based on a 20-plex Polymerase Chain Reaction (PCR)-amplification step followed by a single-base extension (SBE)-procedure targeting transgenic exon-exon junctions. The SBE-products are then analyzed via Matrix-Assisted Laser Desorption / Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). Further data on the characterization of the panel and its applicability in human sports drug testing programs as well as for a reference material (RM) developed and tested in parallel are presented. In plasmid-spiked human whole blood samples, an estimated $LOD_{95}$ of 1,500 cp/mL or 30 copies (cp) per reaction of the panel and 500 cp/mL or 10 cp per reaction of the RM was determined. The panel also proved to be suitable for the detection of viral vectors by testing equine plasma samples derived from an animal, which received rAAV-delivered transgenic *EPO*. The specificity and applicability of the panel and the RM was further confirmed by the analysis of native human doping control samples.

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Kim M[1,2], Kim E[3], Yi J[1,2], Kim KH[1], Jeong H[3], Hahn J[2], Sung C[1]

# Development of an all-in-one gene doping detection paper chip based on a multiplexed recombinase polymerase amplification and CRISPR/ Cas12a sensing system

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Chemical and Life Engineering Department, Chonnam National University, Yeosu, Korea[3]

## Abstract

Gene doping detection methods has been developed using several technologies such as Polymerase Chain Reaction (PCR), Next Generation Sequencing (NGS), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system. However, in the field of doping control, ensuring the accuracy, and efficiency of the detection methods as well as implementation in international anti-doping laboratories, remains a significant challenge. Thus, we are developing the High-throughput Multiplexing Gene Doping Analysis (HiMGDA) paper chip that integrates multiplexed Recombinase Polymerase Amplification (RPA) and CRISPR-based sensing systems for the detection of exogenous human Erythrypoietin (*hEPO*), Growth Hormone (*hGH*), and Insulin-like Growth Factor ( *hIGF*)-I genes. The best candidate as a miniaturized assay platform is a microfluidic paper-based analytical device (µPAD) that enables a low-cost and multiplex assay, no need for external pumps, and minimum reagent consumption. First, the µPAD was designed for the CRISPR/Cas12a reaction which is composed of a sample loading zone and a fluorescence detection zone, with the reaction occurring between these two zones. Preliminary experimental results showed that fluorescence signal was detected when exogenous *hEPO* gene is loaded, confirming the potential for the practical application of the paper chip. This study will focus on designing a µPAD where RPA occurs and developing a paper chip that enables one-step detection from blood samples.

*The topic of this poster will be published elsewhere.*

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Marchand A, Roulland I, Ericsson M

# Sensitive EPO gene doping detection from dried blood spots for antidoping application

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## Abstract

**Introduction:** In the last 2-3 years, black market products have appeared and were confirmed to contain genetic products coding for human erythropoietin (EPO). These products are prohibited by the World Anti-Doping Agency (WADA) as they could be used to increase EPO hormone level and hence, the red blood cells. In previous work (Marchand *et al.*), we demonstrated the potential of 20 µL-dried blood spots (DBS) to detect presence of EPO transgene in human blood, but the sensitivity was much lower than from 1 mL fresh blood. As use of DBS as collection matrix for antidoping is starting to expend, we developed a new protocol to reach a sensitivity of 1500 copies of EPO transgene/mL (30 copies in one 20 µL-DBS) as requested in WADA Guidelines on gene doping detection.

**Methods:** Three DBS devices were evaluated: polymeric TASSO-20 (TASSO Inc.) and Mitra VAMS (Neoteryx) and cellulose paper Protein Saver 903 (Whatman). The certified reference material RM-EPO (from National Measurement Institute (NMI), Australia) was spiked in venous blood at different number of copies/mL (from 1500 to 5000 c/mL) before spotting DBS. DNA was extracted from a whole spot with Purelink genomic DNA buffer (ThermoFisher) and eluted in 25 µL, real-time PCR amplification cycles were increased from 40 to 45 and the baseline threshold of fluorescence was lowered 5-fold. Post-amplification, Cq value or NA (no amplification) were obtained and fragment size analysis was used to confirm the specificity of amplification and identification of RM-EPO.

**Results:** Polymeric devices gave better sensitivity than cellulose and TASSO-20 were used to fully validate the method using 10 different blood from volunteers. The commercial Taqman EPO assay (Hs01071097_m1, ThermoFisher) was selected as main assay for screening. Sensitivity of 1500 copies/mL was reached for 50% of the spiked samples, and reproducible and robust detection was obtained at 5000 copies/mL (100% of samples) even if only one of the replicate showed amplification, occasionally. For potential confirmation, PCR was performed using NMI confirmation primer assay and RM-EPO was detected with similar sensitivity.

**Conclusion:** These results confirm that polymeric DBS can be used to test for gene doping with acceptable sensitivity and that WADA should authorize this matrix in addition to fresh blood in the Guidelines for Gene Doping detection.

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Marchand A, Roulland I, Ericsson M. Improvement of EPO Transgene Detection From Polymeric Dried Blood Spots for Antidoping Application. *Drug Test Anal.* 2026 Feb;18(2):230-238. doi: 10.1002/dta.70008.

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

# Comparability between different analytical workflows for the analysis of steroid esters

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## Abstract

Dried blood spot was recently accepted as alternative matrix by the World Anti-Doping Agency only for qualitative analyses. There is a variety of commercially available dried blood spot devices that can be used to collect capillary blood. They can be divided in cellulose- and polymer-based collection devices. The materials used for the different microsampling devices might differ in weight, thickness, speed of absorption and presence (or absence) of some chemical compounds. All the above characteristics might influence the uniformity of the absorption and dissemination of the sample as well as the extraction of the analytes from the collection device. During method development, the characteristics of each type of collection device must be considered to know if they can affect the analytical results.

Here we report the extraction recoveries of 25 steroid esters obtained by comparing different collection devices and extraction protocols.

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Jing J, Chen P, Xu X

# Application of automatic dried blood spot analysis combined with metabolomics in the detection of testosterone ester drugs

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## Abstract

**Objectives:** This study aims to optimize the extraction and purification process of fully automated DBS to improve the sensitivity of DBS detection technology, enabling efficient detection of testosterone esters. Additionally, by integrating metabolomics analysis, the study seeks to explore common biomarkers after the use of different testosterone esters, further enhancing the detection strategies for testosterone prohibited substances and providing insights for the further application of DBS samples in doping detection.

**Methods:** The DBS samples from subjects using testosterone propionate and testosterone undecanoate were analyzed using the optimized fully automated DBS analysis system. Data was collected through a non-target data acquisition mode, followed by metabolomics data analysis.

**Results:** Using the optimized DBS analysis system, a sensitive automated screening method for 30 steroid esters was developed, achieving LODs of 0.05 ng/mL for nearly half the compounds, along with a confirmatory method for 26 steroid esters. Metabolomics analysis of the automated detection of DBS samples revealed significant metabolic changes before and after the administration of testosterone propionate and testosterone undecanoate, especially for testosterone propionate, as shown by PCA, PLS-DA, and volcano plot analyses. A large number of differential metabolites were identified based on m/z ratios, p-values, and fold change (FC) values. After merging the results from different time points, 10 common differential metabolites were found for testosterone propionate and 14 for testosterone undecanoate, both including cortisol and its metabolite cortol. The differential metabolites of both drugs were mainly enriched in the biosynthesis of steroid hormones.

**Conclusions:** The innovative modification of the fully automated DBS processing system, including the introduction of a dedicated desorption pump and compensating pump to optimize the flow path, enhanced its sensitivity and expanded the range of detectable substances, thereby promoting its application in doping detection. Additionally, the first integration of the fully automated DBS analysis system with metabolomics fills a significant research gap in the field. Non-targeted metabolomics revealed common biomarkers, offering new indicators for testosterone detection, though further validation is still required.

Salama S, Kraiem S, Saleh A, Saad K, Abushareeda W, Al-Jaber MY, Beotra A, Al Maadheed M

# Method development for the detection of steroid esters in various DBS devices. Evaluation of the derivatization procedures used

Anti Doping Lab Qatar, Doha, Qatar

## Abstract

Steroid esters, commonly used for their ability to extend the biological activity of anabolic steroids, have therapeutic applications but are often misused in competitive sports to enhance performance. Steroid esters are unstable in whole blood therefore, their detection poses significant challenges due to the rapid enzymatic breakdown of these compounds in blood, complicating efforts to identify their abuse in doping control. Therefore, dried blood spot (DBS) samples are used to overcome the limitations posed by esterase activity in liquid blood.

This work explores the potential of dried blood spot (DBS) sampling—using devices such as volumetric absorptive micro sampling VAMS, TASSO, and CARDS. Moreover, DBS sampling offers several advantages, including non-invasive collection, easy storage, and resilience against tampering, making it an efficient alternative for anti-doping purposes.

The study developed and optimized analytical method employing ultra-high-performance liquid chromatography coupled with quadrupole-Orbitrap mass spectrometry (UHPLC-Q-Orbitrap-MS) for the simultaneous detection of 11 Testosterone esters and nandrolone phenylpropionate in DBS samples.

Four chemical derivatization reagents—hydroxyl oxime, methyl-oxime, and the hydrazine-based Girard's Reagents P (GRP) and T (GRT)—were studied and developed for the detection and improvement of the reliability of the method. A comparative evaluation of these derivatization reagents was conducted to determine their effectiveness in improving sensitivity and selectivity across different samples.

The assay demonstrated excellent selectivity, low limits of detection (LOD) ranging from 0.1 to 0.5 ng/mL, reliable precision (intra- and inter-assay) and negligible carry-over less than 0.1%. These results confirm the effectiveness of DBS sampling in simplifying sample preparation procedure for the extraction and the detection of exogenous testosterone administration in doping control. The findings also provide valuable insight into the effectiveness of different derivatization strategies, contributing to the development of more robust anti-doping methodologies.

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Miyamoto A, Ota M, Sato M, Okano M

# Direct analysis of testosterone propionate and methyltestosterone in dried blood spots after transdermal applications in Asian males

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## Abstract

In Japanese market, the cream-based formulation containing testosterone propionate (T propionate) and methyltestosterone (MeT) is available as an over-the-counter drug for hair growth without a prescription. In the past, there have been cases where athletes have claimed to have used this formulation during the investigation of AAF cases, in which urine tests showed the presence of MeT metabolites.

In recent years, dried blood spots (DBS), which are minimally invasive and require little storage space, have been used as test samples in doping tests. Among them, steroid esters are stable in dried blood, and therefore testosterone esters can be directly analyzed, making them the target substances in doping tests.

This study was designed to investigate whether transdermal uptake of this formulation can be detected by expanding the target steroid in the existing method for steroid esters. The study involved some male subjects who applied the cream drug five times. Analysis was performed using a liquid chromatography-tandem mass spectrometry. Simultaneously, MeT and T propionate were detected in DBS samples collected after transdermal administration, which may provide greater accuracy in estimating the drug given than detecting MeT metabolites in urine alone or additional IRMS analysis.

The full paper has been published as:

Miyamoto A, Okano M, Ota M, Sato M. Analysis of Methyltestosterone and Testosterone Propionate in Dried Blood Spots After Transdermal Application. *Drug Test Anal.* 2025 Oct;17(10):2045-2053. doi: 10.1002/dta.3914.

Pecher D, Gmeiner G

# Analysis of testosterone esters in urine samples – evidence of the detectability of testosterone esters in trace amounts

Doping Control Laboratory, Seibersdorf Labor GmbH, Seibersdorf, Austria

## Abstract

The application of exogenous testosterone (T) is still one of the most abused doping practices. Because T is also formed endogenously its exogenous application is usually detected in urine using a combination of gas chromatography and isotope ratio mass spectrometry (GC-IRMS). This approach has limits because preparations currently available on the black market do not necessarily differ significantly in their carbon isotope ratio from endogenously produced T. Testosterone is usually administered as pro-drug formulations in the form of esters with various chain lengths. The T-esters are not produced endogenously and their detection in the athlete's sample automatically results in an Adverse Analytical Finding (AAF). The presence of testosterone esters is currently usually tested in serum, blood (stabilized with NaF), or dried blood spots samples. It is assumed that T-esters are not excreted in the urine in detectable amounts due to their apolar properties and the activity of the esterases. However, to the best of our knowledge, urine samples are not yet analyzed for the presence of T esters in trace amounts.

Therefore, the aim of the presented study was to develop an ultra-sensitive HPLC-MS/MS method suitable for the detection of T-esters in urine samples in low to sub pg/mL amounts and its application for the analysis of samples from individuals with suspected application of T-esters. Sample preparation is based on liquid-liquid extraction (LLE), which was optimized by comparing the extraction recoveries using different extraction solvents at various pH. Extraction with methyl t-butyl ether at pH 6.8 was chosen as optimal. Obtained extracts were evaporated to dryness and T esters derivatized by reconstitution in Girard P solution. The reaction with Girard P allows the creation of products with improved ionization properties resulting in significantly increased sensitivity of the analysis. The chromatographic part of the method is based on an on-line SPE approach combining a Phenyl-Hexyl extraction column (for removal of the excess of derivation agent and matrix interferences) and InfinityLab Poroshell 120 EC-C18 (2.1x100 mm, 1.9 µm) analytical column. This analytical approach included the detection of the presence of 12 T-esters and 1 Trenbolone ester in urine samples with limits of detection in the range 0.05-1 pg/mL. The developed HPLC-MS/MS was applied to 14 doping urine samples with a high T/E ratio (ranging between 5.4-113), where T abuse was previously confirmed by GC-IRMS. Out of the 14 analyzed samples, the Presumptive Adverse Analytical Finding (PAAF) for one or more T-esters was detected in 5 samples. The detected T-esters included T-Propionate and T-Enanthate. An aliquot of one sample with T-Propionate PAAF was prepared and analyzed by a confirmation procedure based on LC-HRMS and LC-MS/MS. The presence of T-Propionate was confirmed. The estimated concentration was in the low pg/mL range.

This is the first evidence that the T-esters were detected in trace amounts in the urine samples.

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Ekström L, Pohanka A, Johansson M

# Sensitivity to detect 100 mg transdermal testosterone in 2016 vs 2024 at Doping Control Laboratory Stockholm

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## Abstract

In a previous administration study conducted 2016 we noted that ABP monitoring compared to population T/E increased the chances to identify suspected cases, and similar results have continuously been reported. However, the IRMS results in our previous study showed poor sensitivity; only 2 of 9 subjects were identified as atypical findings despite elevated T/E. Since then, the IRMS method has been improved. Moreover, serum T has been implemented as a biomarker in ABP, increasing the chances to identify samples for IRMS confirmation. Here we have performed an administration study to healthy males and compared the results with 2016 study results to assess the potential improvement in detection rate. Study population included 5 healthy males. 100 mg testosterone gel was applied on shoulders and abdomen. Urine samples were collected prior, and 1, 10, 24, 36, 48 hours post dose. Moreover, capillary serum was collected with Tasso+SST devices at baseline, 1-, 24- and 48-hours post dose. Urinary steroid profile (GC-MS/MS), serum T (LC-HRMS) and IRMS analyses were performed at Karolinska Doping Control Laboratory, Stockholm.

The urinary T/E and 5αAdiol/E were similar after 100 mg T gel administration in 2016 and 2024, both at baseline (median baseline 1.2 vs 0.9), after 10-12 hours (2.45 vs 2.40), 24 hours (1.68 vs 1.91), and 48 hours (1.34 vs 1.70), respectively. The venous and capillary serum concentrations post administration showed similar concentrations (26 nmol/L vs 24 nmol/L) and (16 nmol/L vs 18 nmol/L) 24- and 48-hours post dose, respectively. It is known that capillary serum and venous serum shows similar results (unpublished results).Two samples from each participant were analyzed with IRMS. All the samples analyzed with IRMS were positive according to WADA technical document using PD as ERC (100 % sensitivity). This is an improvement from the 2016 study where most of the samples analyzed were inconclusive (40% sensitivity). The most common criteria fulfilled were elevated T $\Delta\delta$ >3 ‰ with accompanied 5αAdiol $\Delta\delta$ >3 ‰ (criterium in TD2022IRMS). Notably, even though the T/E was not elevated above 4 or increased substantially (probably not flagged in a passport), IRMS displayed positive results. This discrepancy between urinary T and IRMS result have also been noted after 14 days transdermal T application in men.

The increased sensitivity may depend on the T drug used here was more $^{13}$C-depleted: the T in the Testogel sachets used in this study had a $\delta^{13}$C-value of -31.7 ‰, versus -29.6 ‰ in Mullen et al. The increased chances to detect 100 mg T might also be due to improvements done in sample preparation steps. Conclusively we have shown that the chances to detect micro-doping with T has increased by analyzing serum T and using a more sensitive IRMS method.

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# Investigations into the metabolism and elimination of flmodafinil and fladrafinil in urine and blood (DBS)

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

## Abstract

Flmodafinil (CRL-40,940; 2-[bis(4-fluorophenyl)methylsulfinyl]acetamide) and fladrafinil (CRL-40,41; 2-bis(4-fluorophenyl)methylsulfinyl]-N-hydroxyacetamide) are derivatives of modafinil. The wake-promoting substance modafinil was originally developed for the treatment of narcolepsy and other sleep disorders. Other indications are hyperactivity disorder (ADHD) and Alzheimer's disease. However, the stimulating effects on the central nervous system have contributed to an increasing popularity outside the medical field, particularly for the enhancement of cognitive performance. In recent years, there has been a notable increase in interest within the sporting community regarding substances such as modafinil, due to their purported potential to enhance both physical and mental performance. This has led to concerns about their misuse as doping agents.

The urine sample preparation was conducted as follows: Chromabond® HR-XCW, 45 µm, 1 mL, was conditioned with 1 mL of methanol and 1 mL of $H_2O$. To the urine samples (1 mL), internal standard ($D_5$-furosemide) was added. After loading with urine, the samples were washed with 1 mL $H_2O$/FA (95:5; v/v). Subsequently, the analytes were eluted with 2 mL of MeOH/FA (95:5; v/v). Following evaporation to dryness, the residues were reconstituted in 100 µL $H_2O$/ACN (95:5; v/v).

The dried blood spot (DBS) sample preparation was conducted as follows: The spots were punched out, extracted with 1 mL MeOH/$H_2O$ (60:40; v/v) using an ultrasonic bath for 15 minutes. The solvent was evaporated at 52°C under a nitrogen stream. The dry residue was reconstituted in 100 µL of $H_2O$/ACN (95:5; v/v). Subsequently, the samples were centrifuged, and the supernatant was transferred into a vial for LC-MS analysis.

The LC-HRMS analysis of the target analytes was conducted using a Vanquish UHPLC chromatograph coupled via a HESI source (2.6 kV in negative mode) to a ThermoScientific Exploris™ 480 mass spectrometer. The chromatographic separation was achieved using an EC HPLC NUCLEOSHELL Biphenyl analytical column (100 x 2 mm; 2.7 µm particle size). The mobile phase was 0.1% FA (aq.) (solvent A) and 0.1% FA in ACN (solvent B). Gradient: 100% A at injection, decrease to 0% A over 5 min, the column was then flushed at 0% A for 2 min and subsequently re-equilibrated at 100% A for 3 min. The flow rate was set to 0.3 mL/min, the injection volume was 5 µL.

After ingestion of flmodafinil, the metabolites flmodafinil acid and flmodafinil sulfone were detected. Since fladrafinil acts as a prodrug for flmodafinil, these metabolites were likewise detected after ingestion of fladrafinil. The $MS^2$-product ion mass spectra showed dissociation patterns attributable to the elimination of sulfanol (HSOH) for flmodafinil and fladrafinil, and sulfoxylic acid ($H_2SO_2$) for flmodafinil sulfone. Furthermore, product ions originating from the aliphatic side chain and, in case of flmodafinil acid, also of the fluorinated biphenyl system were observed.

The method was validated for flmodafinil and fladrafinil in urine and DBS. Regarding the elimination profiles of the target analytes, it was found that the maximal values and the detection windows were quite similar after administration of flmodafinil or fladrafinil. When fladrafinil is administered, a slight offset can be seen for the period until the maximal concentration of flmodafinil is reached, related to the conversion of the prodrug fladrafinil to flmodafinil. Determined analyte quantities were considerably lower in blood than in the urine.

Flmodafinil and fladrafinil, with their relationship to modafinil and adrafinil, are subjects of class “S6 stimulants” of the WADA Prohibited List and are therefore only prohibited in-competition. In consideration of the relatively long detection windows of both the intact drugs and their metabolites, careful result interpretation is indicated in case of an AAF.

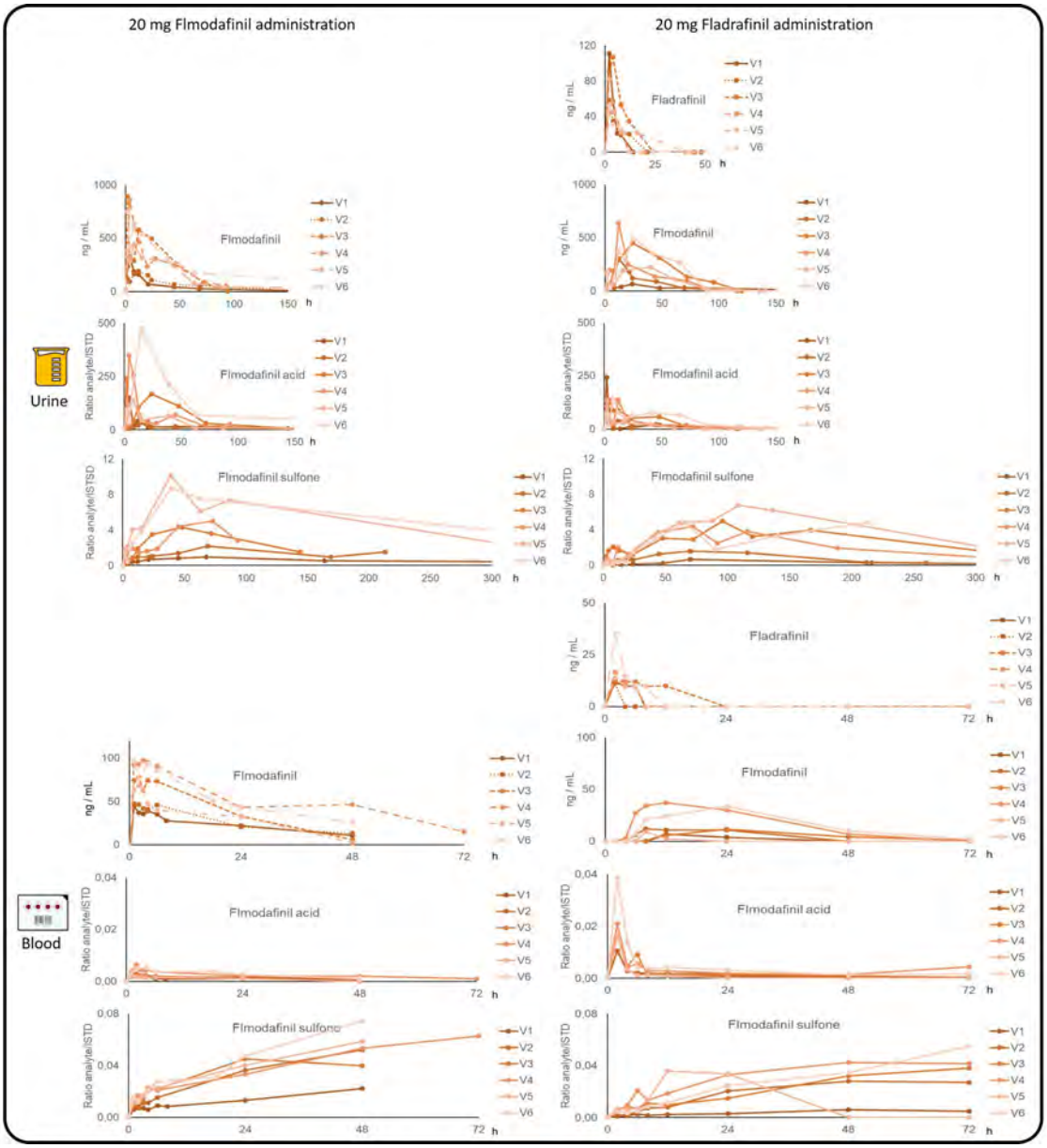


**Figure 1.** Excretion profiles of flmodafinil, fladrafinil, and metabolites in urine and blood (DBS)

The full manuscript has been submitted to *Drug Testing and Analysis.*

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# Sensitive and comprehensive method for small peptides detection by LC-MS/MS

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## Abstract

Small peptides are of increasing concern in doping control because of their potential to stimulate or induce biological processes that enhance athletic performance. Since their inclusion on the WADA prohibited list, continuous advancements have been made in analytical techniques to detect their misuse. This study focuses on the development and validation of a highly sensitive and comprehensive LC–MS/MS method for the detection of forty-five small peptides in human urine, including the recently added peptides Macimoreline and Capromorelin. The analytical procedure involved the preparation of 2 mL urine samples fortified with internal standards, followed by buffering with sodium acetate (pH 5.2) and extraction using Oasis WCX cartridges. After washing, analytes were eluted, dried under vacuum, reconstituted and analyzed by LC–MS/MS. Instrumental analysis was performed using a Thermo TSQ Quantiva triple-quadrupole mass spectrometer coupled with a Dionex Ultimate 3000 UHPLC system operating in positive scheduled MRM mode with a gradient program having a total run time of twenty minutes. The method was validated as per ISO/IEC 17025, WADA ISL (2021), and WADA TD2022MRPL guidelines, assessing parameters such as specificity, limit of detection (LOD), robustness, and carryover. The developed method demonstrated excellent specificity in ten different drug free urine samples of varying pH, specific gravity (SG), and gender. Robustness testing confirmed consistent retention times and signal responses across multiple batches analyzed on different days by different analysts. The LODs for all targeted small peptides ranged from 0.05 ng/mL to 1 ng/mL, fulfilling the acceptance criteria for identification in accordance with WADA TD2023IDCR for signal-to-noise ratio greater than 3.

The optimized solid-phase extraction, refined elution conditions, and carefully optimized mobile-phase conditioned significantly enhanced recovery and minimized matrix effects, contributing to improved detection capability. Currently, the method is routinely implemented in laboratory operations for the detection of small peptides, where successful identification of analytes even below half of the minimum required performance limit (MRPL) has been achieved. This validated LC–MS/MS method offers high sensitivity, selectivity, and robustness for the comprehensive detection of small peptides, including newly listed doping agents such as Macimoreline and Capromorelin. Its routine application strengthened analytical capacity in our laboratory and enhances the ability to detect peptide misuse at trace levels, thereby contributing to fair play and integrity in competitive sports.

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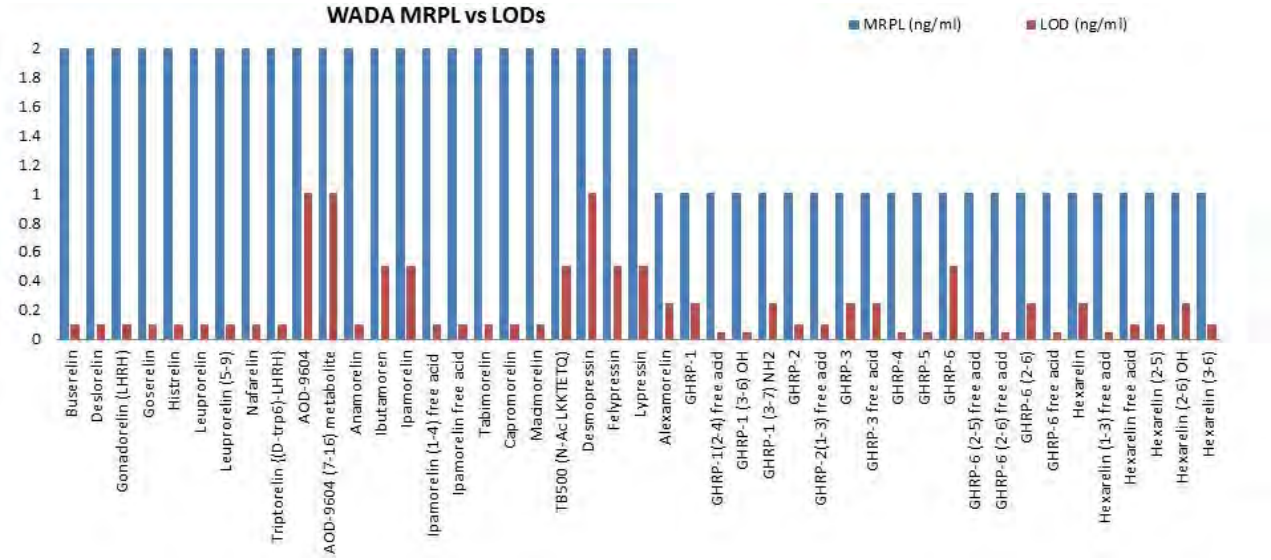


**Figure 1.** Chart showing small peptides MRPL vs LODs

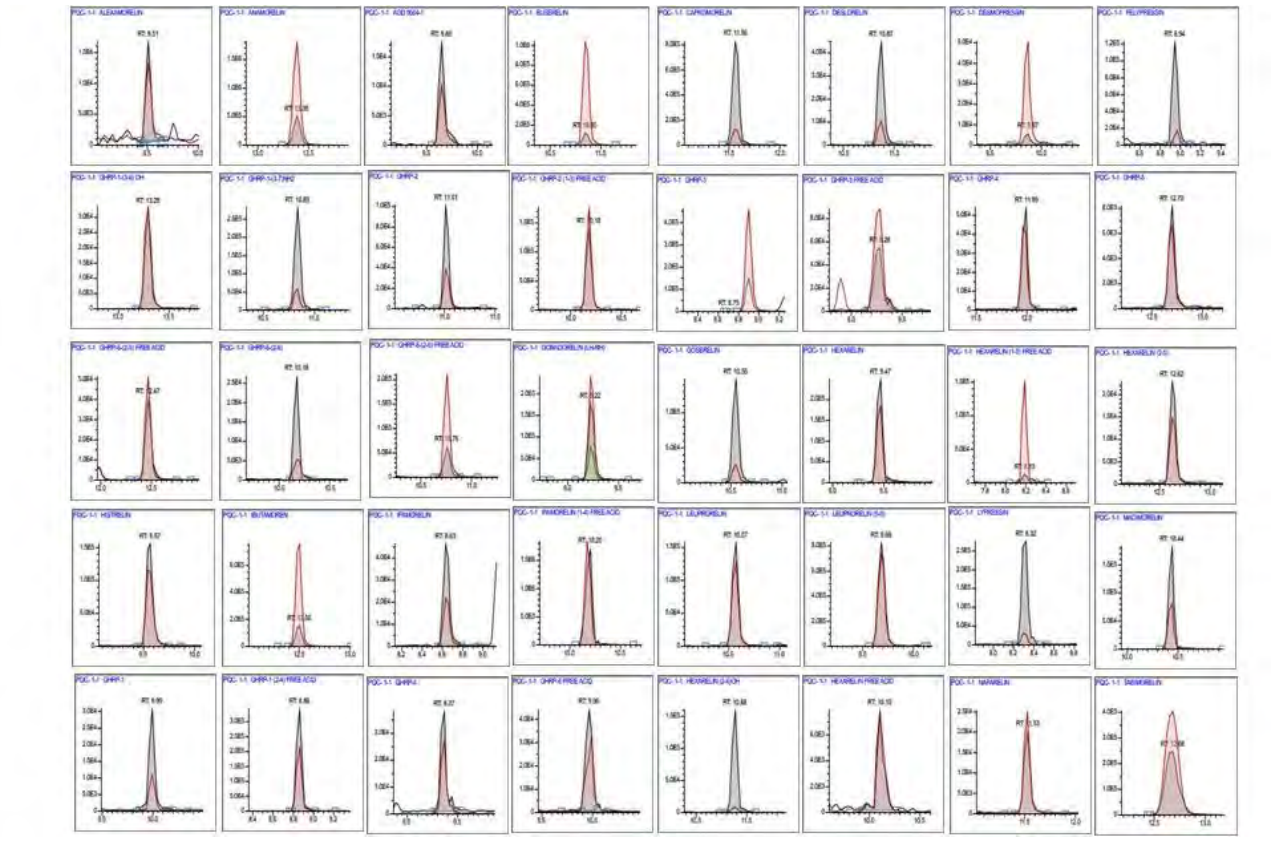

**Figure 2.** PQC at half MRPL chromatograms