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# Implementation of the HIF activator IOX-2 in routine doping controls – Pilot study data

Christian Görgens<sup>1</sup> | Sven Guddat<sup>1</sup> | Christina Bosse<sup>1</sup> | Andre Knoop<sup>1</sup> | Hans Geyer<sup>1,2</sup> | Mario Thevis<sup>1,2</sup>

<sup>1</sup>Center for Preventive Doping Research/Institute of Biochemistry, German Sport University Cologne, Cologne, Germany

<sup>2</sup>European Monitoring Center for Emerging Doping Agents (EuMoCEDA), Cologne/Bonn, Germany

## Correspondence

Christian Görgens, Center for Preventive Doping Research/Institute of Biochemistry, German Sport University Cologne, Am Sportpark Müngersdorf 6, 50933, Cologne, Germany.  
Email: c.goergens@biochem.dshs-koeln.de

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## Abstract

Early in 2020, racehorse doping cases revolved around the hypoxia-inducible factor (HIF) activator IOX-2. While the composition of IOX-2 has also been known and monitored in human doping controls for several years, the testing capability of routine sports drug testing methods was revisited for this newly surfaced doping agent. IOX-2 and the analytically well-established HIF activator roxadustat (FG-4592) share identical precursor/product ion pairs, enabling their co-detection in existing initial testing procedures in routine doping controls for the intact unconjugated analytes. In addition, hydroxylated IOX-2 and the corresponding glucuronic acid conjugates were identified as major metabolites in a microdose elimination study, contributing to enhanced initial testing and confirmation procedures.

## KEYWORDS

HIF activator, human urine, IOX-2, roxadustat, sports drug testing

## 1 | INTRODUCTION

Hypoxia-inducible factor (HIF) activators are prohibited in sports both in- and out-of-competition.<sup>1</sup> A variety of drug candidates has been closely monitored in sports drug testing since 2012,<sup>2</sup> and whilst clinical approval was completed in 2019 so far only for one substance (roxadustat (FG-4592), Figure 1A), various adverse analytical findings (AAFs) have been reported since 2015 for different HIF activators. One of the patented drug candidates referred to as IOX-2 was identified in the context of preventive doping research in 2012 and detected early in 2020 in a racehorse doping control sample<sup>3</sup> (Figure 1B), and the testing capability of human routine sports drug testing methods was revisited for this newly surfaced doping agent. By association (and elemental composition), the co-detection of IOX-2 and roxadustat as intact and unconjugated analyte using identical precursor/product ion pairs was utilized (Figures 2 and 3) and since 2015, no adverse analytical findings have been recorded.<sup>4</sup> However, to date no metabolic biotransformation reactions have been assessed.

The aim of this pilot study was the implementation of IOX-2 into an existing initial test method to enable detection at the lowest possible additional workload for the laboratory. Furthermore, a microdose elimination study was performed to identify the compound's major metabolites and to allow a first estimation of urinary excretion profiles and detection times.

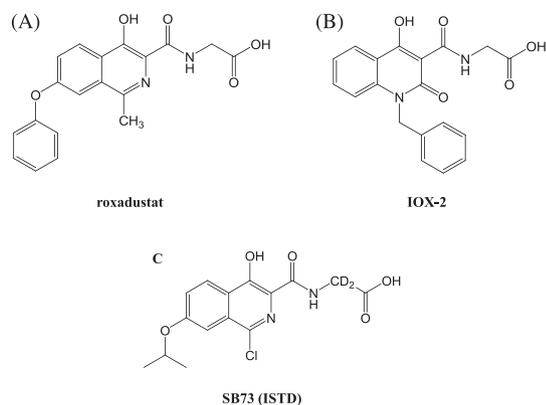
## 2 | EXPERIMENTAL

### 2.1 | Reference material and internal standard (ISTD)

IOX-2 and roxadustat reference materials were obtained from Sigma Aldrich (Deisendorf, Germany). As internal standards (ISTDs), isoxsuprine-D<sub>5</sub> (ISTD of initial testing procedure (ITP)) and SB73 (1-chloro-7-hydroxy-6-isopropoxy-isochinoline-3-yl-glycineamide-D<sub>2</sub>, ISTD of confirmation procedure (CP), Figure 1C), both obtained from in-house syntheses were used.

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**FIGURE 1** Structure formulae of roxadustat (A), IOX-2 (B), and SB73 (C)

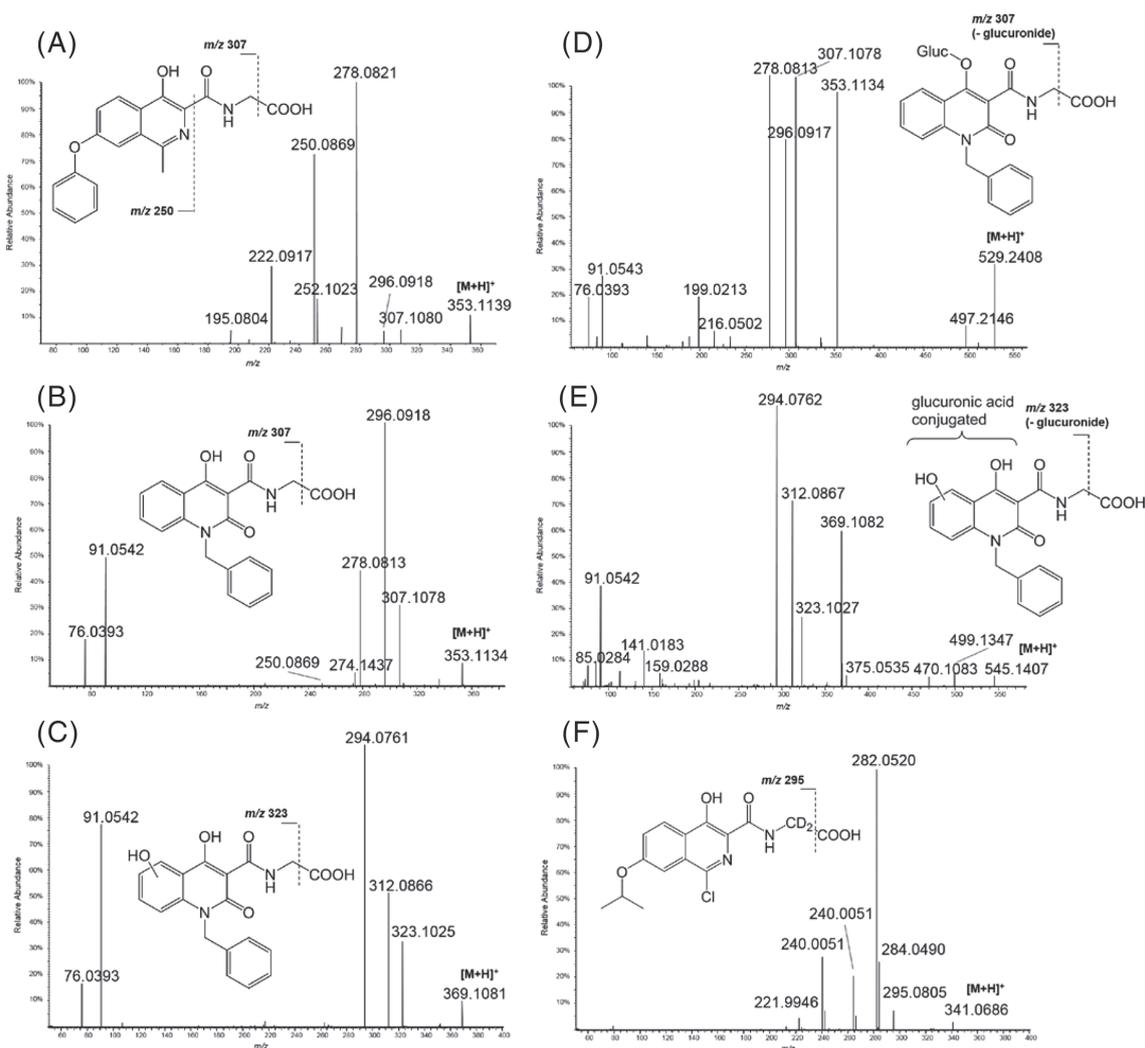
## 2.2 | Sample preparation and instrumentation

Analytical parameters of IOX-2 using established routine doping control methods<sup>5</sup> were determined from reference substance

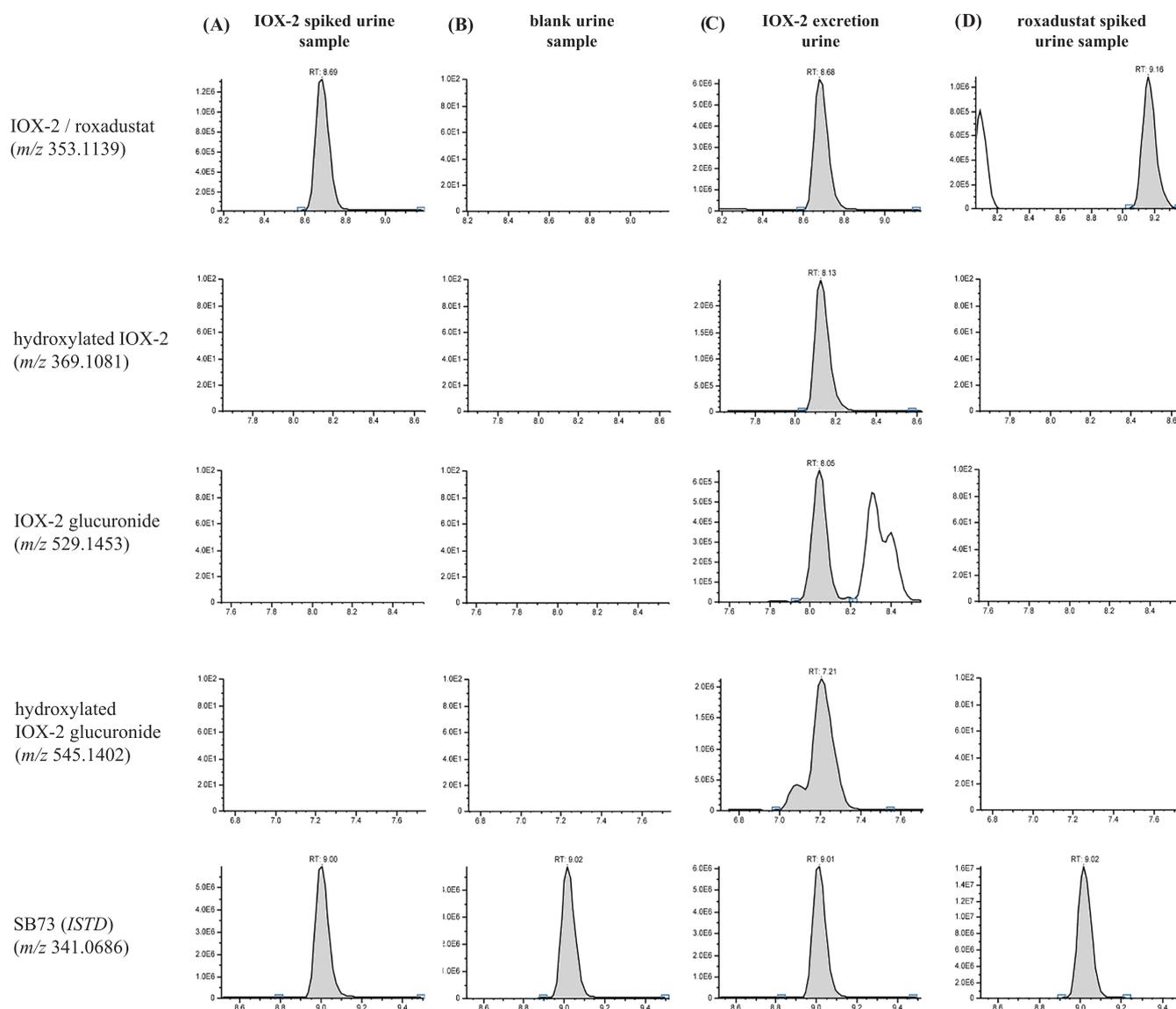
analyses. Urine sample analysis was conducted by means of fortifying an aliquot of 95  $\mu\text{L}$  of urine with 5  $\mu\text{L}$  of an internal standard working solution, and 10  $\mu\text{L}$  was subsequently injected into the liquid chromatographic-mass spectrometric instrument.

For chromatographic separation, a Vanquish UHPLC System (Thermo Scientific, Bremen, Germany) equipped with a Nucleodur C18 Pyramide analytical column ( $2 \times 50$  mm, 1.8  $\mu\text{m}$  particle size; Macherey-Nagel, Düren, Germany) and a guard column ( $2 \times 4$  mm) of the same material was used. The mobile phases were composed of A: 0.1% formic acid and B: acetonitrile. The LC gradient (total run time: 11 min) was set as follows: starting conditions 100% A, 1–5 min: 100–60% A, 5–8 min 60–10% A, 8–11 min 100% A (re-equilibration). The analytical flow rate was 200  $\mu\text{L}/\text{min}$  and for re-equilibration, a flow of 350  $\mu\text{L}/\text{min}$  was employed. The column temperature was set at 30°C.

Mass spectrometric experiments were carried out using an Exploris 480 orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) operating in full scan (scan range:  $m/z$  100–800; orbitrap resolution: 60,000 FWHM) and data independent



**FIGURE 2** Product ion mass spectra of protonated molecules of roxadustat ( $m/z$  353, A), IOX-2 ( $m/z$  353, B), hydroxylated IOX-2 ( $m/z$  369, C), IOX-2 glucuronide ( $m/z$  529, D), hydroxylated IOX-2 glucuronide ( $m/z$  545, E), and SB73 (ISTD,  $m/z$  341, F) using a NCE of 30%



**FIGURE 3** Extracted ion chromatograms of protonated molecules of roxadustat/IOX-2 ( $m/z$  353.1139), hydroxylated IOX-2 ( $m/z$  369.1081), IOX-2 glucuronide ( $m/z$  529.1453), hydroxylated IOX-2 glucuronide ( $m/z$  545.1402), and SB73 (ISTD,  $m/z$  341.0686) of spiked urine specimens (A and D; conc.: 8 ng/mL), a blank urine sample (B) and an IOX-2 excretion urine (C)

acquisition (DIA) (Q1 isolation window:  $m/z$  100; stepped normalized collision energy (NCE): 30 and 55%; orbitrap resolution: 60000 FWHM) mode for initial testing procedure (ITP). In the case of a confirmatory analysis, identical chromatographic conditions were chosen, but with parallel reaction monitoring (PRM) mode (Q1 resolution:  $m/z$  1.0; NCE: 30%; orbitrap resolution: 60000 FWHM) for the mass spectrometric detection and unambiguous identification of the compound.

### 2.3 | Microdose elimination study

With written consent, a microdose elimination study was performed with one healthy male volunteer (64 years, 81 kg, 170 cm), who was

orally administered 1 mg of IOX-2. Urine samples were collected before and up to 91 h post-administration, and specimens were subjected to routine doping control analytical approaches (dilute-and-inject) as well as dedicated product ion scan experiments on *in silico* predicted phase I and phase II metabolites.

### 2.4 | Method validation

The method for the semi-quantitative determination of IOX-2 was comprehensively characterized with regard to the following parameters according to World Anti-Doping Agency (WADA) guidelines.<sup>6</sup> **Selectivity:** Ten different blank urine samples obtained from healthy volunteers were tested for the presence of interfering

signals using the initial testing procedure (ITP) as well as the confirmation procedure (CP).

**Carryover:** The carryover was assessed by analyzing one sample with a concentration set at 400% of the MRPL (8.0 ng/mL) followed by the injection of one blank sample.

**Limit of detection (LOD):** The LOD is the lowest concentration of an analyte that can be detected in 95% of representative samples (ie 95% detection rate). Here, in each case six urine samples were fortified with 0.02–2.00 ng/mL of IOX-2 and analyzed using the ITP.

**Robustness:** The robustness of the approach was determined by analyzing six different urine specimens spiked at 50% of the minimum required performance level (MRPL) of the compound (1.0 ng/mL). The coefficients of variation (%CV) were calculated on the basis of the ISTD-normalized peak areas as well as relative retention times (rRT). Robustness was estimated using the CP.

**Limit of identification (LOI):** The LOI is the lowest concentration of an analyte that meets the WADA Technical document TD IDCR<sup>7</sup> criteria in 95% of representative samples (ie 95% identification rate or 5% false-negative rate). Here, in each case six urine samples were fortified with 0.02–2.00 ng/mL of IOX-2 and analyzed using the CP.

**Linearity:** Calibration curves in the range 0.25–125 ng/mL (0.25, 1.0, 25, 50, 75, 100, 125 ng/mL) were constructed using the ISTD-normalized peak areas and linearity was determined by regression analysis.

## 3 | RESULTS

### 3.1 | Implementation of IOX-2 into routine doping control

Roxadustat (Figure 1A) and IOX-2 (Figure 1B) exhibit the identical sum formulae of  $C_{19}H_{16}N_2O_5$ , and the fact that the structurally related pharmacophores both comprise a glycineamide side chain suggested similar collision-induced dissociation behaviors as reported in 2017<sup>8</sup> and corroborated in Figure 2. The monitoring of diagnostic product ions such as  $m/z$  307, 296, and 278 enabled the detection of intact IOX-2 alongside intact roxadustat in LC-FullIMS-DIA-HRMS

routine analyses, with both analytes separated in retention time by approximately 0.5 min (Figure 3). In addition to the unmodified intact substance of IOX-2, its hydroxylated analog and the corresponding glucuronic acid conjugates were also detected in post-administration urine samples (Figure 3), supporting both initial testing and confirmation procedures in routine doping controls.

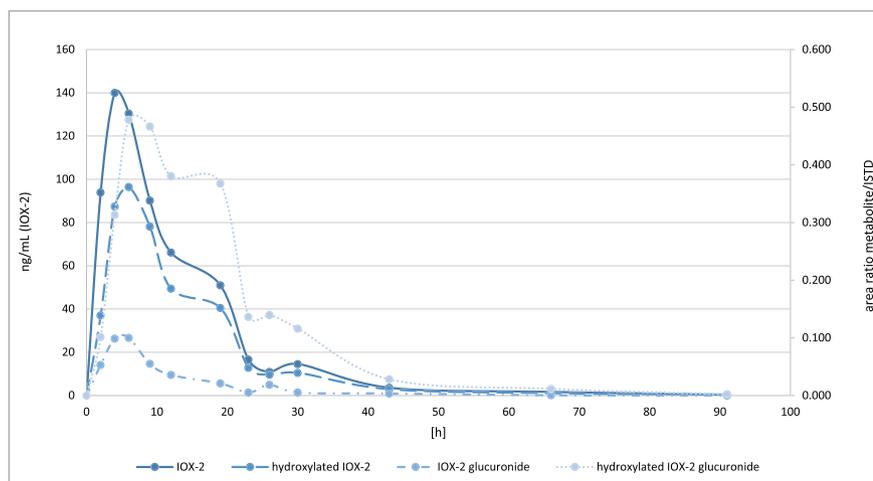
### 3.2 | IOX-2 excretion profile and detection window

As demonstrated in Figure 4, the urinary excretion profiles of the intact drug, as well as the identified major metabolites follow a similar excretion profile with peak concentrations between 4 and 6 hours after application of the substance. All metabolites including the unmodified intact compound were detectable up to 91 h following ingestion of 1 mg of the substance. The maximum concentration of IOX-2 was found to be approximately 140 ng/mL in a urine sample collected 4 h after administration. The last urine sample collected 91 h post administration provided an IOX-2 concentration of approximately 0.4 ng/mL, while hydroxylated IOX-2 and the corresponding phase II metabolites were also detectable.

In a phase 3 clinical trial for the treatment of anemia in Japanese erythropoiesis-stimulating agent-naïve chronic kidney disease patients on dialysis, patients were treated with 50–70 mg of roxadustat 3 times weekly.<sup>9</sup> Consequently, the effective doses in clinical trials of related HIF activators are substantially higher than the performed microdose elimination study. Therefore, a significant extension of the detection window in a potential doping scenario seems possible.

### 3.3 | Retrospective monitoring of urinary IOX-2 metabolites

Due to the fact that routine doping controls of roxadustat and IOX-2 have been performed using LC-FullIMS-DIA-HRMS, retrospective evaluation of IOX-2 metabolites was possible. Despite the



**FIGURE 4** Urinary excretion profiles (specific gravity corrected) of IOX-2, hydroxylated IOX-2, IOX-2 glucuronide, and hydroxylated IOX-2 glucuronide after 1 mg oral administration of IOX-2

**TABLE 1** Method validation results

	Confirmation ions	Selectivity	Carryover [%]	Robustness			LOI [ng/mL]	Linearity
				rRT [%CV]	Area ratio [%CV]	LOD [ng/mL]		
IOX-2	m/z 296.0918 m/z 278.0813 m/z 307.1078	✓	0.2	0.1	13.3	0.6	0.4	0.25–125 ng/mL (R <sup>2</sup> = 0.9971)
Hydroxylated IOX-2	m/z 312.0866 m/z 294.0761 m/z 323.1026	✓	0.0 <sup>b</sup>	0.1 <sup>a</sup>	n.d.	n.d.	n.d.	n.d.
IOX-2 glucuronide	m/z 307.1078 m/z 296.0917 m/z 278.0813	✓	0.4 <sup>b</sup>	0.6 <sup>a</sup>	n.d.	n.d.	n.d.	n.d.
Hydroxylated IOX-2 glucuronide	m/z 312.0867 m/z 294.0761 m/z 323.1026	✓	0.0 <sup>b</sup>	0.1 <sup>a</sup>	n.d.	n.d.	n.d.	n.d.

<sup>a</sup>Estimated by comparison of 12 urine specimens from microdose elimination study.

<sup>b</sup>Determined by comparison of signal areas in a blank urine directly injected after a urine sample containing IOX-2 metabolites (4 h post administration of IOX-2). n.d., not determined due to unavailability of reference standards.

co-detection of IOX-2 and roxadustat in routine doping control samples, another 2000 randomly chosen routine doping control samples from in- and out-of-competition as well as of different sports and gender were retrospectively evaluated for the presence of hydroxylated IOX-2, IOX-2 glucuronide, and hydroxylated IOX-2 glucuronide. In none of the cases examined, were IOX-2 or any of the described metabolites found.

### 3.4 | Method validation

The method employed for a semi-quantitative determination of IOX-2 after direct injection of native urine specimens treated with ISTD was comprehensively characterized in accordance with WADA criteria. The results of the method validation are summarized in Table 1. According to the fact that the aforementioned IOX-2 metabolites are not commercially available, most of the described method validation parameters (robustness, LOD, LOI, and linearity) were estimated with IOX-2 itself, while selectivity and carryover was done for all the investigated metabolites. The assay is characterized by a high selectivity with almost zero biological noise in the blank urine specimens for IOX-2 and all investigated IOX-2 metabolites. The approach was found to be linear from 0.25 to 125 ng/mL (R<sup>2</sup> > 0.99) with an LOD (ITP) of 0.6 ng/mL and an LOI (CP) of 0.4 ng/mL. Furthermore, the assay demonstrates adequate robustness (CV% rRT: 0.1; CV% area ratio: 13.3) and carryover (< 1%).

## 4 | CONCLUSIONS

IOX-2 can adequately be covered in routine doping controls by targeting the intact drug and its hydroxylated metabolite, and the

capability of the combined detection of IOX-2 and roxadustat was demonstrated. The performed pilot microdose elimination study indicates a similar excretion profile of the intact IOX-2 and the identified major urinary metabolites with detection windows longer than 91 h post oral administration of 1 mg of the substance. Although the compound has a high (mis)use potential in sports, retrospective monitoring for IOX-2 and its metabolites indicates that the drug is not likely to have been widely abused over the time period of assessment. However, further studies are indicated in order to complement the pattern of urinary metabolites and their utility in terms of enhanced retrospectivity.

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### ORCID

Christian Görgens  <https://orcid.org/0000-0001-6061-0407>

Andre Knoop  <https://orcid.org/0000-0003-0040-8754>

Mario Thevis  <https://orcid.org/0000-0002-1535-6451>

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