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Implementation of the prolyl hydroxylase inhibitor Roxadustat (FG-4592) and its main metabolites into routine doping controls

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The utility of hypoxia-inducible factor (HIF) prolyl hydroxylase inhibitors as a therapeutic means of treating patients suffering from anaemia has been demonstrated for various clinical settings. However, besides this intended use, HIF stabilizers can be the subject of misuse in amateur and elite sports due to their erythropoietic properties, as recently proven by several cases of adverse analytical findings in doping control testing. Consequently, to allow for adequate and comprehensive test methods, knowledge of the drug candidates' metabolism and analytical options enabling appropriate detection windows in sports drug testing samples (i.e., blood and urine) is essential to doping control laboratories. In the present study, a novel HIF prolyl hydroxylase inhibitor referred to as Roxadustat (FG-4592) and main plasma- and urine-derived metabolites were investigated in the context of routine doping control analytical approaches. Liquid chromatography-mass spectrometry-based test methods were used to study the target analytes' dissociation pathways following electrospray ionization and collision-induced dissociation. Diagnostic precursor-product ion pairs were selected to enable the implementation of the intact drug Roxadustat and selected metabolites into multi-analyte initial testing procedures for plasma and urine specimens. The assays were validated in accordance to guidelines of the World Anti-Doping Agency (WADA) and results demonstrated the suitability (fitness-for-purpose) of the employed analytical methods with detection limits ranging from 0.05 to 1 ng/mL and 1 to 5 ng/mL for urine and plasma, respectively. Subsequently, elimination study plasma and urine samples collected up to 167 h post-administration were analyzed using the validated methods, which suggested the use of different target analytes for blood and urine analyses with FG-4592 and its glucuronide, respectively, for optimal detection windows. Additionally, a light-induced rearrangement product (photoisomer) of Roxadustat resulted in the formation of an additional compound of identical mass. Copyright © 2017 John Wiley & Sons, Ltd.

Keywords: doping; sport; mass spectrometry; HIF stabilizer; erythropoietin

Introduction

The pharmacological manipulation of hypoxia-inducible factor (HIF) prolyl hydroxylase activities by orally available drug candidates is advancing as a viable alternative to established approaches commonly relying on the intravenous or sub-cutaneous administration of recombinantly produced erythropoietin (EPO).[1–3] The use of HIF prolyl hydroxylase inhibitors (PHIs) has been shown to allow for correcting and maintaining target haemoglobin levels in chronic kidney disease (CKD) patients,[4–6] and although full clinical approval has not been issued for any representative of this emerging class of therapeutics, concerns regarding illicit use, especially in professional and amateur sport, arose approximately a decade ago. Consequently, pilot studies aimed at testing model substances and *in vitro* derived metabolites were initiated[7–9] before 2011 the World Anti-Doping Agency (WADA) first mentioned HIF PHI explicitly in the annually issued Prohibited List.[10] Banned at all times, HIF PHI have since been on the radar of doping control laboratories,[11–13] and anti-doping efforts have lately been substantially facilitated by collaborative studies with the pharmaceutical industry. As demonstrated by the 2015 report of FG-4592 in an athlete's urine sample,[14] the criticality of establishing an appropriate analytical method is vital. Comprehensive knowledge about the drugs' metabolism and elimination is desirable to optimize the best possible detection windows during events and in retain samples for doping controls.

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In this study, routine sports drug testing methods were expanded to include the new HIF PHI drug candidate Roxadustat (FG-4592, Figure 1, **1**) and its main metabolites (as identified in quantitative metabolite profiling studies using radiolabelled Roxadustat) plus a light-induced rearrangement product (Figure 1, **6**). In contrast to earlier approaches,[14] existing assays were assessed for their sensitivity and selectivity to measure the most relevant target analytes inclusive of metabolic products at concentrations commonly observed in blood and urine following therapeutic dosing of Roxadustat. Urine and plasma samples from a Phase 1 clinical trial were used to generate proof-of-concept data allowing estimate of a detection window for the target analytes.[4,15]

Experimental

Chemicals and reagents

The reference substances for FG-4592 (**1**) and corresponding metabolites M1-M4 (**2**–**5**) as well as two stable isotope-labelled analogues (internal standards, ISTDs) and a light-induced rearrangement product (compound **6**) were provided by FibroGen Inc. (San Francisco, CA, USA). Oasis HLB 3 mL/60 mg solid-phase extraction (SPE) cartridges were purchased from Waters (Eschborn, Germany). Bond Elut Nexus 1.5 mL/30 mg SPE cartridges were purchased from Agilent (Santa Clara, CA, USA). All organic solvents (Honeywell, Seelze, Germany or Morris Plains, NJ, USA) used were of analytical grade and deionized water was obtained from a Sartorius (Göttingen, Germany) Arium pro ASTM or a Millipore (Billerica, MA, USA) Advantage A10 system.

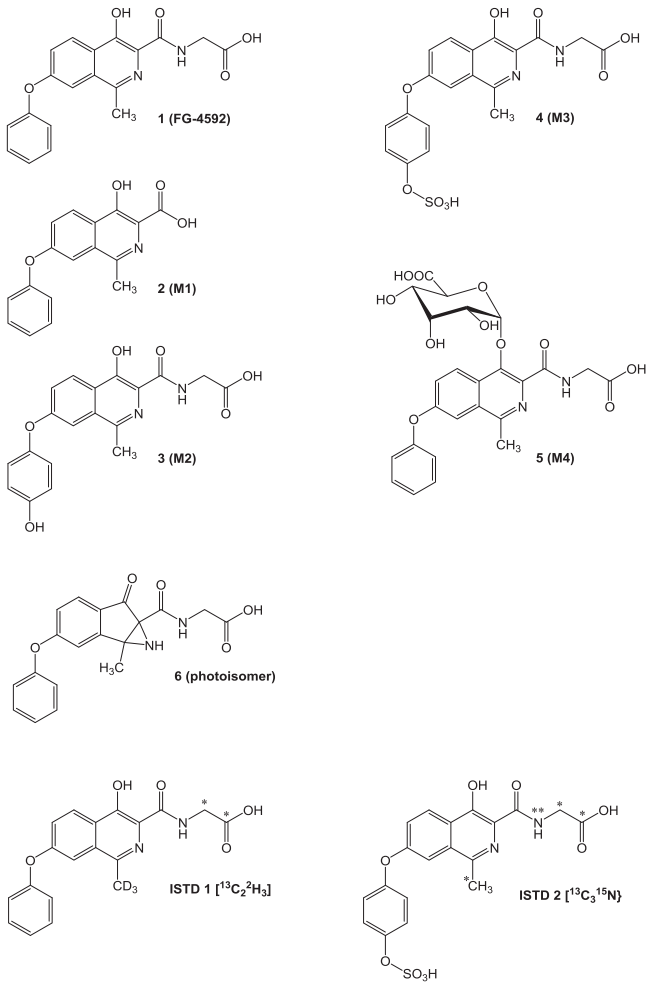


Figure 1. Structures of Roxadustat (**1**, mol wt = 352) and its metabolites M1 (**2**, mol wt = 295), M2 (**3**, mol wt = 368), M3 (**4**, mol wt = 448), M4 (**5**, mol wt = 528), the light-induced rearrangement product (**6**, mol wt = 352), and the two internal standards ISTD 1 (mol wt = 357) and ISTD 2 (mol wt = 452). * indicates positions of ^{13}C and ** indicates positions of ^{15}N stable isotope abelling.

Urine sample preparation for initial testing procedure (ITP, dilute-and-inject)

In accordance to an existing dilute-and-inject initial testing procedure,[16] 90 μL of urine were enriched with 30 ng of the ISTDs by adding 10 μL of a methanolic solution containing 3 μg/mL ISTDs. The sample was briefly mixed by vortex mixing (5 s), centrifuged for 1 min at 600 x *g*, and subjected to liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis.

Urine sample preparation for confirmatory testing procedure

Two mL of urine were spiked with 100 ng of the ISTDs by adding 10 μL of a methanolic solution containing 10 μg/mL of the ISTDs. The sample was gently mixed by vortex mixing for 5 s and then loaded onto an Oasis HLB SPE cartridge preconditioned sequentially with 1 mL of water, 1 mL of methanol, and again with 1 mL of water. The urine was passed through by gravity flow, the SPE was washed with 2 mL of water, and then eluted with 2 volumes of 0.5 mL of methanol. The combined eluates were concentrated to dryness in a stream of nitrogen at 50°C, and the residue was finally reconstituted in 100 μL of acetonitrile/water (1:1, *v/v*).

Plasma sample preparation for initial testing procedure

For the screen, 100 μL of plasma were spiked with 4 ng of the ISTDs by adding 20 μL of an aqueous solution containing 0.2 μg/mL of the ISTDs. The sample was treated by addition of 200 μL of isopropanol and mixed by vortexing for 30 s or sonicating for 5 min. The sample was centrifuged at 558 x *g* for 5 min and the supernatant transferred to a fresh container. The sample was evaporated under an air stream at 40°C then reconstituted in 20 μL of methanol followed by 40 μL of 25 mM aqueous ammonium acetate in 0.1% formic acid.

Plasma sample preparation for confirmatory testing procedure

A 500 μL aliquot of plasma was spiked with 20 ng of the ISTDs by adding 20 μL of an aqueous solution containing 1 μg/mL of the ISTDs. The sample was treated by addition of 1000 μL of isopropanol and mixed by gentle vortexing for 30 s or sonicating for 5 min. The sample was centrifuged at 558 x *g* for 5 min and the supernatant transferred to a fresh container. The sample was concentrated under an air stream at 40°C to a volume below 500 μL. The sample was diluted with 1 mL of 0.05 M aqueous sodium acetate at pH 5 prior to loading onto a Bond Elut Nexus SPE cartridge that had been pre-washed with 1 mL of methanol and 1 mL of water. After loading the cartridge was washed with 1 mL of water and then eluted with 1 mL of methanol. The sample was evaporated under an air stream at 40°C then reconstituted in 20 μL of methanol followed by 40 μL of 25 mM ammonium acetate in 0.1% formic acid.

Liquid chromatography - tandem mass spectrometry (LC–MS/MS)

LC–MS/MS measurements for both the ITP and the confirmatory analyses for urine were performed using an Agilent Technologies (Waldbronn, Germany) 1200SL liquid chromatograph interfaced to a Thermo Fisher Scientific (Dreieich, Germany) Vantage triple quadrupole instrument. Solvents used for LC were 5 mM aqueous ammonium acetate buffer with 1% acetic acid (A) and acetonitrile (B). Gradient elution with online trapping was used, employing an Accucore (Thermo Fisher Scientific, Dreieich, Germany) phenyl-hexyl trapping column (10 × 3 mm, 2.6 μm particle size) and an Accucore C8 analytical column (50 × 3 mm, 2.6 μm particle size). Following injection of 20 μL of sample, loading onto the trapping column was done with 99% A at 800 μL/min for 2 min (flow diverted to waste), before the elution was done within 7 min (at 300 μL/min) by linearly decreasing from 99% A to 20% A. The column was flushed with 100% B for 0.5 min prior to a 2.7 min re-equilibration period at starting conditions. The overall run time was 12.2 min. The mass spectrometer, using the multiple reaction monitoring (MRM) mode, was operated with electrospray ionization and scan-to-scan alternating polarity. The HESI-II source used a spray voltage of 3500 V and −2500 V for positive and negative ionization, respectively, and the temperature was maintained at 450°C. Nitrogen used as collision and auxiliary/sheath gas was provided by a CMC (Eschborn, Germany) nitrogen generator. Collision offset voltages were optimized for each precursor-product ion pair, which are summarized in Table 1.

Further, to probe for the impact of the mass analyzer and to exploit additional analytical options, a high resolution/high accuracy quadrupole-orbitrap-based mass spectrometer (Q Exactive plus HF, Thermo Fisher Scientific, Dreieich, Germany) was interfaced to the same LC set-up reported for the triple quadrupole instrument. The mass spectrometer was operated in full scan (*m/z* 200–1000) and targeted MS/MS mode, employing a precursor ion isolation width of *m/z* 2.0. Resolution (full width at half maximum) was set to 30 000 in full scan and 15 000 in MS/MS experiments, and normalized collision energies in MS/MS were set at 35% (arbitrary units).

Table 1. Precursor-product ion pairs for all target analytes included in the ITP and confirmatory test methods

Urine testing			
Analyte	Precursor/product ion pairs (coll. Offset voltage [V])		
FG-4592	*m/z* 353–222 (50)	*m/z* 353–250 (39)	*m/z* 353–278 (26)
Comp. **6**	*m/z* 353–224 (50)	*m/z* 353–252 (37)	*m/z* 353–234 (40)
M1	*m/z* 296–222 (45)	*m/z* 296–250 (33)	*m/z* 296–278 (25)
M2	*m/z* 369–238 (53)	*m/z* 369–266 (42)	*m/z* 369–294 (30)
M3	*m/z* 449–294 (53)	*m/z* 449–369 (27)	*m/z* 449–374 (33)
M4	*m/z* 529–250 (64)	*m/z* 529–278 (47)	*m/z* 529–353 (24)
ISTD 1	*m/z* 358–225 (50)	*m/z* 358–253 (39)	*m/z* 358–281 (26)
ISTD 2	*m/z* 453–267 (50)	*m/z* 453–373 (29)	*m/z* 453–375 (32)
Plasma testing			
Analyte	Precursor/product ion pairs (coll. Offset voltage [V])		
FG-4592	*m/z* 353–222 (31)	*m/z* 353–250 (25)	*m/z* 353–278 (17)
Comp. **6**	*m/z* 353–178 (45)	*m/z* 353–207 (31)	*m/z* 353–252 (16)
M2	*m/z* 369–238 (32)	*m/z* 369–266 (25)	*m/z* 369–294 (18)
M3	*m/z* 449–266 (32)	*m/z* 449–294 (26)	*m/z* 449–374 (19)
M4	*m/z* 529–222 (54)	*m/z* 529–278 (30)	*m/z* 529–250 (40)
ISTD 1	—	*m/z* 358–253 (25)	*m/z* 358–281 (17)
ISTD 2	—	*m/z* 453–267 (32)	*m/z* 453–373 (16)

For plasma, the samples were analyzed on Waters Corporation (Milford, MA, USA) systems incorporating an Acquity Binary Solvent Manager, Acquity FTN Sample Manager, and Acquity Column Manager interfaced with an Xevo TQ-S triple quadrupole mass spectrometer. The solvents used were 25 mM ammonium formate in 0.1% formic acid (A) and acetonitrile (B). A Restek (Bellefonte, PA, USA) Raptor Biphenyl (100 × 2.1 mm; 2.7 μm) column maintained at 40°C was used for the separation. After an injection of 10 μL, the column was maintained at the starting conditions of 0.45 mL/min and 14% B. From 0.5 min to 6.5 min the percentage of B was raised linearly to 70%. The percentage of B was ramped up to 95% over 0.1 min and held there for 0.9 min, after which time the solvent composition was returned to starting conditions over 0.1 min and held there until the end of the run at 9.0 min. The electrospray probe was maintained at 1500 V with a desolvation temperature of 450°C, a desolvating gas flow of 1000 L/h, a nebulizer pressure of 7.0 bar, and a cone gas flow of 150 L/h. The mass spectrometer source was maintained at 150°C. The MRM parameters for each compound are summarized in Table 1.

Urine samples

For method development purposes, 10 blank urine samples (5 female and 5 male donors) were obtained from healthy volunteers. The urine sample collection and use was approved by the local ethics committee and written informed consent was obtained from the donors. Further to spiking experiments, urine specimens collected from 6 individuals (age 19–45 years) during phase-I clinical trials (provided by FibroGen, San Francisco, CA, USA) were used to generate proof-of-concept data and to allow for estimating detection windows for the target analytes. The urine sampled from each participant prior to and 24, 72, 120, and 167 h following the oral administration of 0.3 mg/kg (low dose) or 4 mg/kg (high dose) of Roxadustat was subjected to the validated routine doping control methods.

Plasma samples

Likewise for validation of the plasma methods, 10 blank plasma samples (5 female and 5 male donors) were obtained from healthy volunteers. In addition, plasma samples from clinical studies were provided by FibroGen (San Francisco, CA, USA) from six individuals administered orally with 0.3 mg/kg (low dose) or 4 mg/kg (high dose) of Roxadustat. Samples were taken prior to administration and 8, 24, 48, 72, and 96 h post-administration. Due to limited sample volumes, the administration study samples were examined only using the confirmation method shown above and using only 150 μL aliquots. Samples were missing for two of three subjects at the 24 h sampling for low dose and were of limited volume for an additional sample at 24 h, low dose and a sample at 48 h, low dose. The volume of isopropanol used for precipitation was only 300 μL, but otherwise the same procedure as described for the confirmation method was used.

Routine doping control assay characteristics

The suitability of the ITP was assessed concerning the parameters specificity (10 blank urine samples, 5 female, 5 male), sensitivity (limit of detection, LOD), linearity (0.1, 0.5, 1.0, 10, 50, 100, 250, 500 ng/mL), ion suppression/enhancement, and intra-day and inter-day imprecision. For the determination of the methods' imprecision, three concentration levels of 10, 100, and

500 ng/mL with 6 and 18 sample replicates for each time-point were analyzed for intra-day and inter-day imprecision, respectively. With regards to the confirmatory test method, intra-day and inter-day imprecision data were generated at 1, 10, and 100 ng/mL, and the SPE recovery was determined at 10 ng/mL for all compounds. Therefore, two sets of 6 samples each were prepared, one spiked before SPE and the second spiked with 10 ng/mL of all analytes into the eluate of the SPE prior to evaporation of the solvent. Matrix effects were determined by comparing average peak intensities of each target analyte obtained from the analyses of reference standards and urine samples (n = 3) spiked at 10 ng/mL. Similar studies were carried out for the plasma methods using similar concentration levels as summarized in Tables 2 and 3.

High resolution/high accuracy (tandem) mass spectrometry

Studies concerning the analytes' collision-induced dissociation (CID) behaviour were conducted using identical chromatographic conditions as described for the routine doping control analytical assay, but instead used an ABSciex (Darmstadt, Germany) high resolution/high accuracy TripleTOF® 5600 mass spectrometer as

Table 2. Summary of assay characteristics for the detection of FG-4592 and related metabolites in human urine

ITP

Analyte	LOD (estimated) QqQ [ng/mL]	LOD (estimated) Q Exactive [ng/mL]	matrix effect [%]	conc. [ng/mL]	Intra-day imprecision (n = 18) CV [%]	Inter-day imprecision (n = 54) CV [%]
FG-4592	1	0.1		10	2.9–9.2	10.9
			−24	100	1.4–4.7	7.8
				500	2.1–5.4	5.2
Comp. **6**	1	0.1		10	7.5–13.0	28.0
			−62	100	3.3–6.7	18.6
				500	1.6–5.9	16.1
M1	1	1		10	4.7–8.1	30.5
			−10	100	4.4–6.3	8.9
				500	1.3–8.1	23.1
M2	1	1		10	4.8–13.4	18.3
			−31	100	0.8–10.0	8.9
				500	1.6–9.9	9.1
M3	1	1		10	7.7–17.2	27.0
			+39	100	4.2–5.5	21.3
				500	3.0–5.9	20.2
M4	1	0.1		10	5.1–10.8	14.3
			−53	100	3.5–5.1	9.5
				500	1.5–5.2	10.6

Confirmatory test

Analyte	LOD (estimated) QqQ [ng/mL]	LOD (estimated) Q Exactive [ng/mL]	matrix effect [%]	conc. [ng/mL]	Intra-day imprecision (n = 18) CV [%]	Inter-day imprecision (n = 54) CV [%]	Recovery [%]
FG-4592	0.1	0.05		1	12.0–14.5	14.3	
			+70	10	4.2–18.8	16.8	36
				100	6.7–13.5	16.1	
Comp. **6**	0.1	0.1		1	8.9–18.8	15.3	
			−69	10	4.9–8.6	6.5	65
				100	6.0–10.0	10.4	
M1	0.1	0.1		1	6.1–12.4	24.8	
			−47	10	4.2–7.4	9.5	61
				100	6.1–11.2	12.8	
M2	0.1	0.1		1	8.8–17.2	14.4	
			−53	10	3.5–15.6	9.5	35
				100	4.3–6.2	15.8	
M3	0.1	0.1		1	15.1–23.0	18.4	
			−83	10	8.6–15.1	12.0	35
				100	5.6–17.2	17.5	
M4	0.1	0.05		1	11.7–12.7	14.5	
			−63	10	5.3–16.7	10.3	101
				100	7.5–20.4	19.9	

Table 3. Summary of assay characteristics for the detection of FG-4592 and related metabolites in human plasma

ITP						
Analyte	LOD [ng/mL]	matrix effect [%]	conc. [ng/mL]	Intra-day imprecision (n = 18) CV [%]	Inter-day imprecision (n = 54) CV [%]	Recovery [%]
FG-4592	1		10	1.1–7.3	8.1	
		−15	100	0.8–6.2	11.5	65
			500	2.3–9.2	6.8	
Comp. **6**	1		10	5.7–25.7	62.2	
		+3	100	3.5–15.9	19.6	77
			500	5.5–9.1	14.9	
M2	1		10	6.1–34.1	19.6	
		−37	100	3.5–9.1	10.5	77
			500	4.1–11.1	9.7	
M3	5		10	3.7–8.7	6.5	
		+22	100	2.5–4.4	3.1	73
			500	1.3–5.7	3.7	
M4	2		10	3.3–5.3	11.6	
		+69	100	2.4–6.6	6.3	71
			500	2.4–5.9	5.1	
Confirmatory test						
Analyte	LOD [ng/mL]	matrix effect [%]	conc. [ng/mL]	Intra-day imprecision (n = 18) CV [%]	Inter-day imprecision (n = 54) CV [%]	Recovery [%]
FG-4592	1		1	2.4–5.6	6.6	
		−80	10	2.9–6.9	5.1	45
			100	2.2–3.9	4.0	
Comp. **6**	1		1	5.7–16.9	46.8	
		−5	10	5.9–16.5	23.2	53
			100	8.7–19.6	15.8	
M2	1		1	6.8–9.1	14.8	
		−50	10	3.8–17.1	12.7	51
			100	2.9–13.2	8.7	
M3	1		1	4.0–9.2	8.9	
		−67	10	1.9–6.5	4.5	44
			100	2.0–3.2	3.4	
M4	1		1	5.9–7.0	16.7	
		+5	10	8.0–21.5	15.9	40
			100	7.9–11.5	12.1	

the analyzer. The mass spectrometer was operated in positive ESI mode and calibrated every five injections to minimize mass errors to <5 ppm. Nitrogen required as curtain and sheath gas as well as collision gas was obtained from a nitrogen generator (CMC, Eschborn, Germany) and product ion scan experiments were conducted by isolating the appropriate protonated species $[M + H]^+$ as precursor ions at unit resolution. CID was accomplished using collision energies [CE] adjusted to allow for retaining 10–20% of the respective isolated precursor ion.

Results and discussion

High resolution/high accuracy tandem mass spectrometry

The protonated molecule $[M + H]^+$ of Roxadustat (**1**, Figure 1) is found at *m/z* 353, which dissociates in a series of diagnostic product ions following collisional activation as depicted in Figure 2a and Scheme 1. In accordance to earlier studies with structural analogues,[8] Roxadustat is suggested to eliminate water (18 Da) and carbon monoxide (28 Da) as supported by accurate mass measurements as well as stable isotope labelling (Table 1). The resulting product ion at *m/z* 307 further releases 29 Da as confirmed by pseudo-MS^3 experiments. This neutral loss of 29 Da is attributed to methanimine as corroborated by the elemental compositions of *m/z* 307 and *m/z* 278, with the latter representing the base peak of the product ion mass spectrum of Roxadustat under the chosen collision-induced dissociation conditions (Figure 2a). An acyl cation-bearing structure is suggested for the product ion at *m/z* 278 (Scheme 1), which was found to subsequently eliminate carbon monoxide in MS^3 studies, forming the product ion at *m/z* 250 (Table 1). Further, similar to compounds of related structure composed of an isoquinoline-3-carboxamide nucleus, a spontaneous and reversible water addition (+18 Da) is observed,[8] which is proposed to support the formation of the product ion at

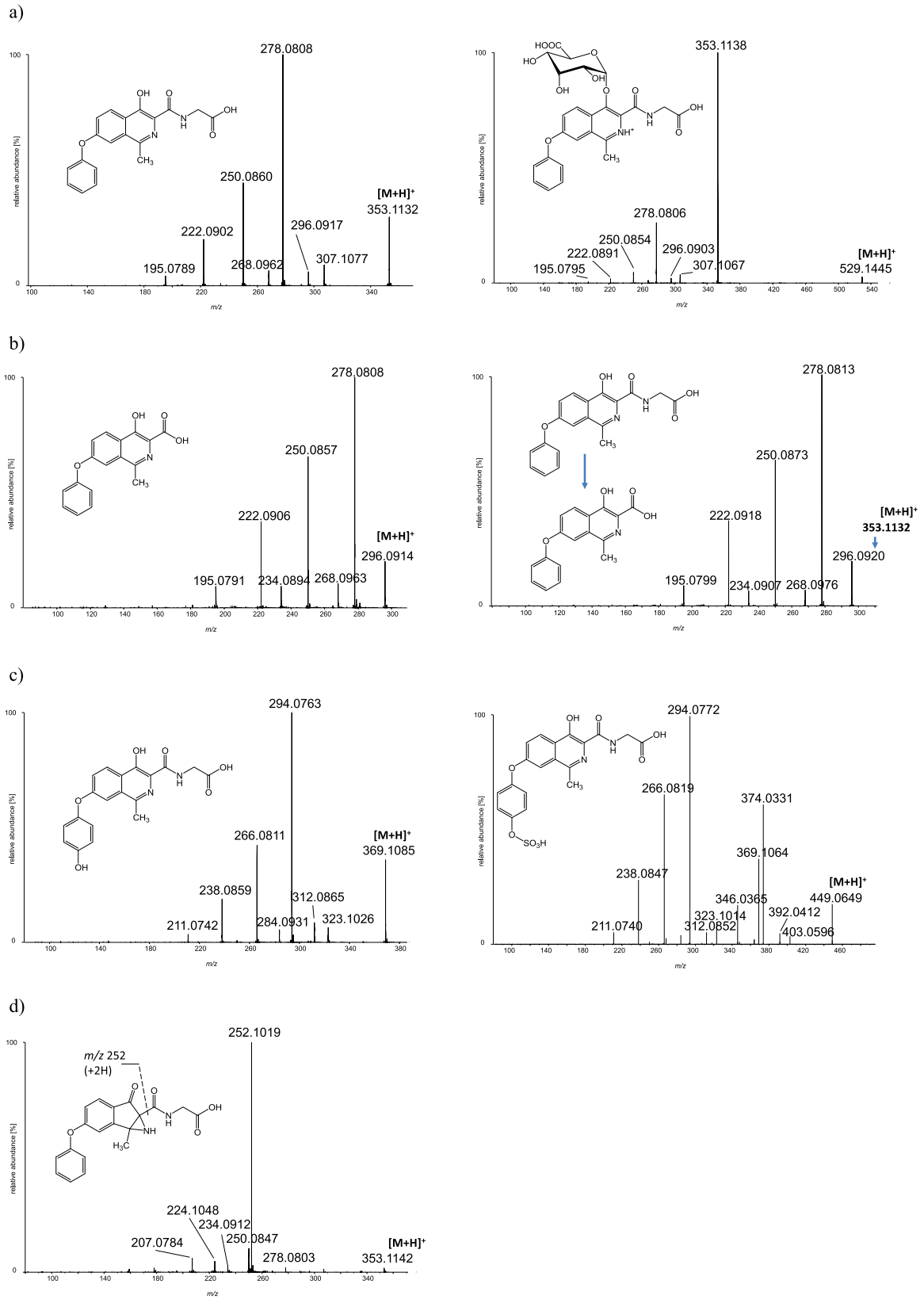
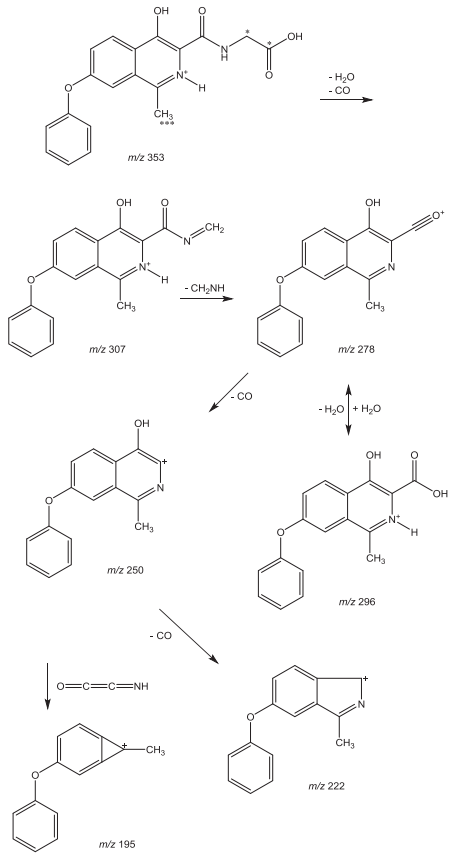


Figure 2. (a) Product ion mass spectra of the protonated molecules $[M + H]^+$ at *m/z* 353 of Roxadustat (left) and at *m/z* 529 of its glucuronidated analogue (M4, right), both recorded at a collision energy of 35 eV; (b) product ion mass spectrum of the protonated molecule $[M + H]^+$ at *m/z* 296 of M1 (left), recorded at a collision energy of 25 eV, and product ion mass spectrum of *m/z* 296 derived from Roxadustat in using MS^3 experiment (right); (c) product ion mass spectra of the protonated molecules $[M + H]^+$ at *m/z* 369 of hydroxylated Roxadustat (M2, left) and at *m/z* 449 of its sulphoconjugated analogue (M3, right), both recorded at a collision energy of 25 eV; (d) product ion mass spectrum of the protonated molecule $[M + H]^+$ at *m/z* 353 of compound **6**, recorded at a collision energy of 25 eV.



Scheme 1. Proposed dissociation pathway of roxadustat under ESI/CID conditions. * indicates labelled positions of the $^{13}C_2$,$^{2}H_3$-labelled analogue.

m/z 296 comprising of a protonated isoquinoline-3-carboxylic acid species (Scheme 1). CID experiments on this product ion at *m/z* 296 as well as on the protonated molecule of 4-hydroxy-1-methyl-7-phenoxyisoquinoline-3-carboxylic acid (**3**, Figure 1), which was synthesized as reference material for metabolism studies, further corroborated the identity of the suggested product ion structure as illustrated in Figure 2b. Here, product ion mass spectra with a convincing presence and abundance of all diagnostic ions are shown. The product ion at *m/z* 250 (Figure 2a) further undergoes two main dissociation routes, leading to *m/z* 222 and 195, respectively as supported by MS^3 data (Table 1). An elimination of carbon monoxide, which necessitates the formation of an isoindole core structure, is proposed to allow for the loss of CO, while the loss of 2-iminoethenone (55 Da) from *m/z* 250 is suggested to yield the product ion at *m/z* 195, tentatively assigned to a 7-methyl-3-phenoxybicyclohepta-1,3,5-triene structure. The proposal of an intact 7-methyl group in the product ion at *m/z* 195 is based on the finding that all three deuterium atoms of the stable isotope-labelled analogue of FG-4592 are retained in the product ion (data not shown).

Routine doping control assay characteristics

Following the identification and characterization of diagnostic precursor/product ion pairs for all target analytes (Table 1), two assays referred to as ITP and confirmatory test method were established. These were based on existing testing strategies to enable the rapid implementation of Roxadustat and relevant metabolites into existing routine doping control analytical protocols for human urine. Both approaches were significantly facilitated using stable isotope-labelled ISTDs, representing the analogues of Roxadustat and its hydroxylated and sulpho-conjugated urinary metabolite M3.

The ITP employs direct urine injection, and the method's characteristics are summarized in Table 2 (upper part). For all target analytes, the assay proved specific as no interfering signals were observed at expected retention times of the compounds when 10 different blank urine samples were analyzed (Figure 3a). Conversely, spiking ten urine samples at 1 ng/mL (the estimated LOD of the triple quadrupole (QqQ)-based mass spectrometer, defined with a signal-to-noise ratio ≥ 3) with all target compounds (**1**–**6**) allowed for the detection of these with at least two diagnostic precursor/product ion pairs (Figure 3a, Table 2). The use of the high resolution/high accuracy mass spectrometry substantially improved the signal-to-noise ratio for Roxadustat, its light-induced rearrangement product (compound **6**), and the glucuronide of Roxadustat (M4), enabling estimated LODs of 0.1 ng/mL (Table 2). No advantage was observed for the analytes M1-M3. Intra-day and inter-day imprecisions varied substantially between the different analytes and concentration levels, and ranged overall between 0.8 and 30.5%. Here, the value of the ISTDs was particularly evident, enabling imprecisions for the main urinary target compounds Roxadustat and M4 (Roxadustat glucuronide) below 15% despite the presence of substantial matrix effects (ion suppression) (Table 2).

To obtain lower LODs, the confirmatory test method is based on SPE using 2 mL of urine followed by an identical LC–MS/MS analytical run as conducted for the ITP. As detailed in Table 2 (lower part), recoveries for all analytes were found between 35 and 101%, and despite considerable matrix effects including ion suppression up to 83%, LODs of 0.1 ng/mL were estimated for all substances using the QqQ-based mass spectrometer. Representative extracted ion chromatograms for blank and spiked specimens are shown in Figure 3b, illustrating the absence of interferences in blank urine as well as the identification of two diagnostic precursor/product ion pairs as required for analyte identification according to WADA guidelines.[17] Superior LODs were obtained by using the quadrupole-orbitrap-based analyzer for Roxadustat and its main metabolite M4 using the same product ions as selected for the QqQ-based analyzer (Table 1), albeit the gain was less than in the ITP procedure. Overall, intra-day and inter-day imprecisions were found below 25% (Table 2).

For plasma, limitations in recovery and the presence of matrix interferences generally yielded higher limits of detection (Table 3). The highest sensitivity was achieved for FG-4592, compound **6**, and M2 at 1 ng/mL in the ITP. The reduced sensitivity in plasma is partially due to reduced recovery. For the ITP, a simple precipitation with isopropanol is performed to remove plasma proteins. The recoveries for all compounds were around 70%. The confirmatory procedure uses a larger volume of plasma and follows a similar precipitation step with a solid phase extraction after organic solvent removal. The recovery of the solid phase extraction step is apparently also around 70%. The matrix effect varied from −37% to +69% in the ITP and from −80% to +13% in the confirmatory test. For the confirmatory test, all metabolites were detectable at 1 ng/mL.

For most of the compounds, the intra-day and inter-day imprecision values were comparable to the levels seen for the urine procedures (Table 3). Nearly all imprecision values were below 20% with the imprecision values being near or below 10% for FG-4592 and M3, compounds for which deuterated internal standards were available. The highest degree of imprecision was seen for the light-

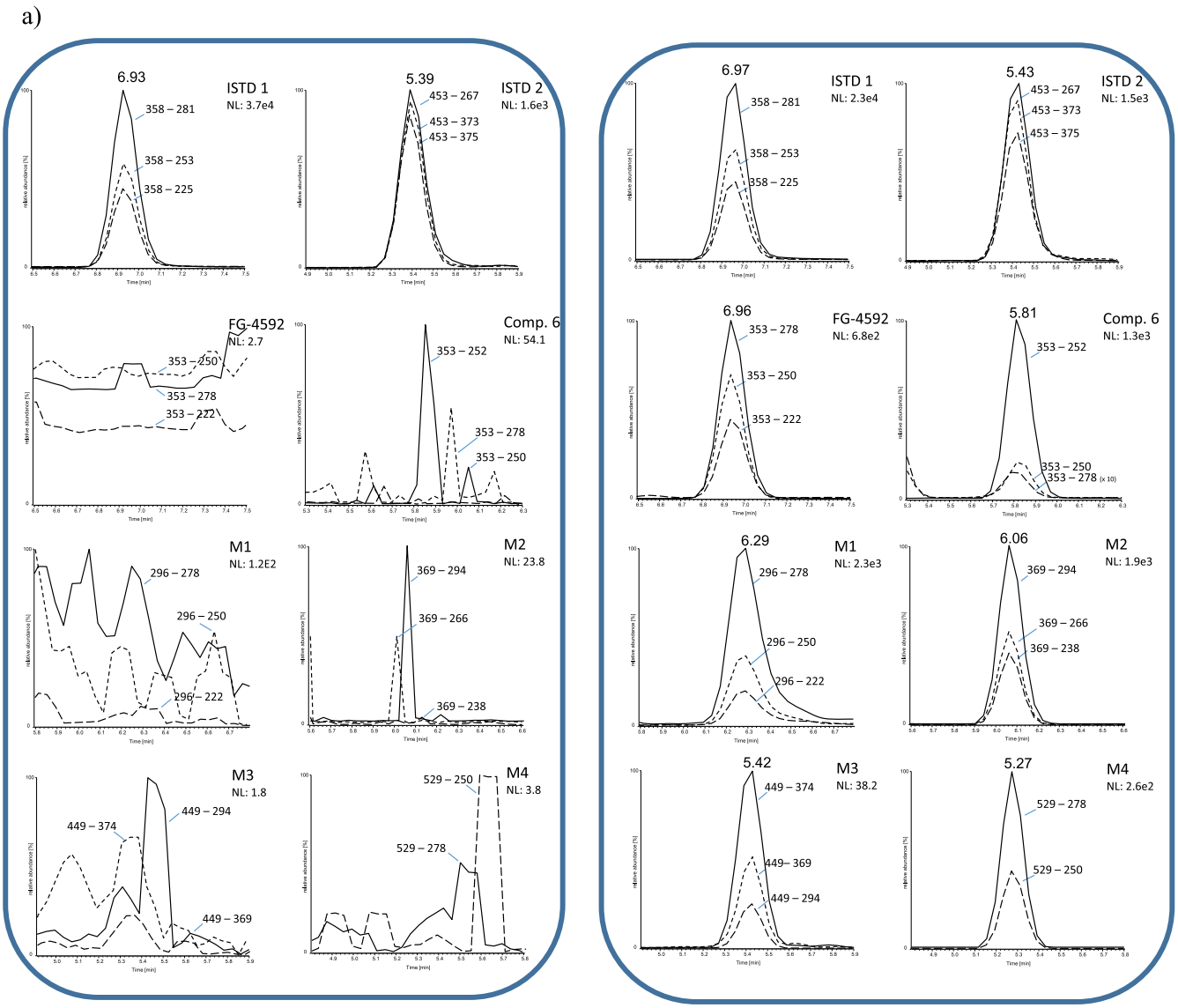


Figure 3. Extracted ion chromatograms of blank urine samples (left) and blank urine spiked at the respective LODs (right) of the initial testing procedure ITP (a, 1 ng/mL) and the confirmatory SPE-based analytical approach (b, 0.1 ng/mL). All extracted ion chromatograms are normalized (NL) to the highest abundance displayed in the respective window.

induced rearrangement product **6**. Assuming photoconversion rates of FG-4592 and its corresponding deuterated internal standard are comparable, the higher imprecision might be accounted for by conversion of FG-4592 to the photoisomer **6** during the extraction process. It should be noted that **6** is an artefact of light exposure, particularly during sample preparation/processing. Therefore, measures should be taken to minimize sample exposure to light. The photoisomer may have been present in the FG-4592 sample that was taken from the athlete due to a consequence of sample handling. Compound **6** is not a metabolic product of FG-4592 and does not form *in vivo*. The rearrangement is a photochemically allowed process by Woodward-Hoffman rules and is thermally reversible.[18–22] Photoisomer **6** was found to be negative in GLP-compliant bacterial reverse mutation (Ames) assays, in the presence or absence of microsomal enzymes (S9 mix, prepared from Arochlor™ induced rat liver) (unpublished observations).

Proof-of-concept testing

Proof-of-concept data were obtained by analyzing 6 sets of 5 urine samples each, collected in the context of phase-I clinical trials following the oral administration of 0.3 mg/kg (low dose, sets 1–3) and 4 mg/kg (high dose, sets 4–6) of Roxadustat. The samples were analyzed using both the ITP and the confirmatory approach, and test results are summarized in Table 4. Overall, FG-4592 and its glucuronic acid conjugate (M4) offered the longest retrospective analyses with detection times up to 167 h. Using a high-dose regimen, M3 was further traceable up to 120 h, while all other analytes (M1, M2 and **6**) did not appear to contribute to extended detection windows concerning the analyses of doping control urine samples. Consequently, targeting FG-4592 and M4 is recommended for routine sports drug testing, with direct urine injection offering a facile and appropriate initial testing procedure.

b)

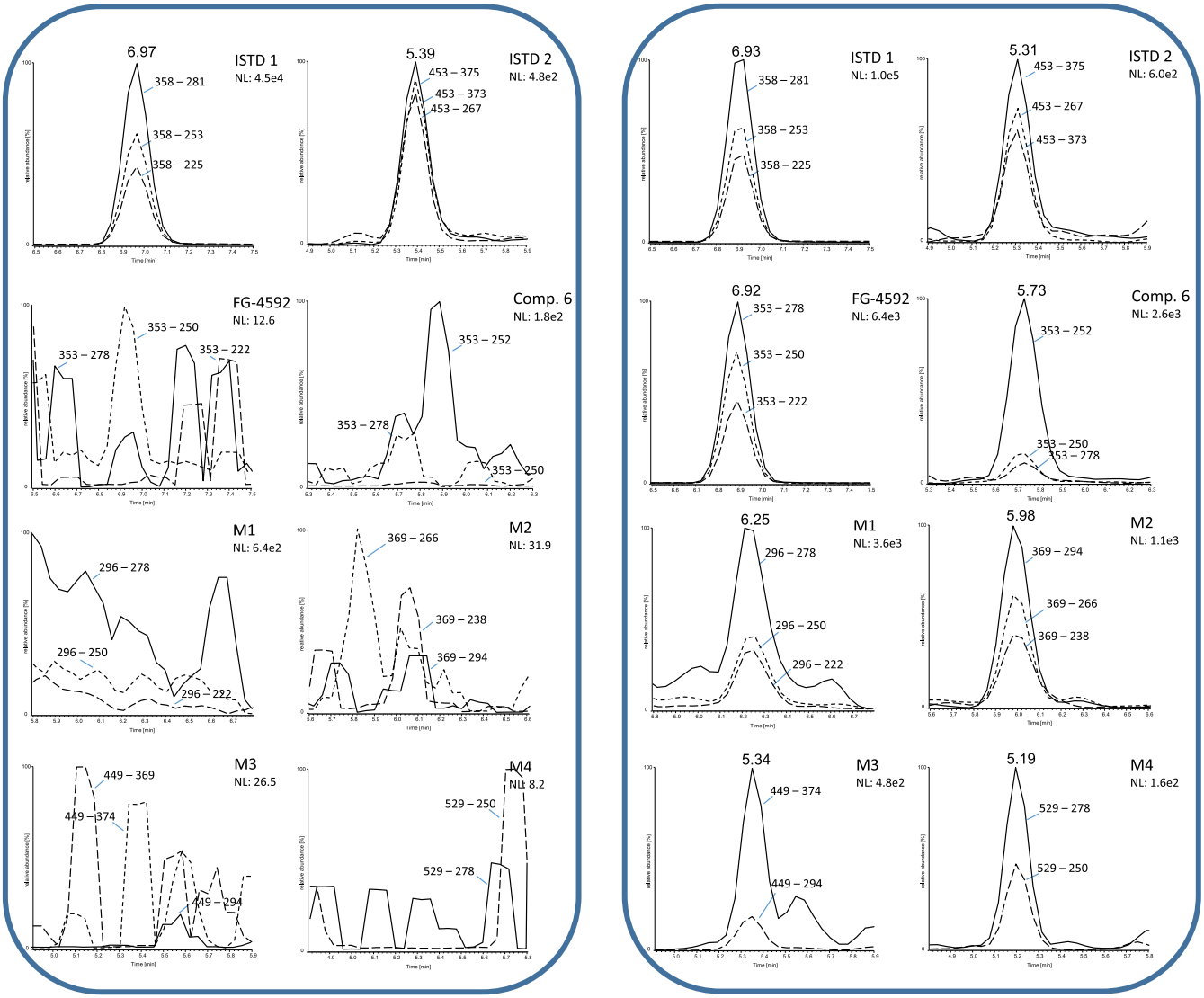


Figure 3. (*continued*)

Table 4. Summary of elimination study urine analyses. Numbers in brackets indicate how many samples of the tested set were successfully analyzed for the respective compound

Urine sample	Identified analyte(s)											
	ITP						confirmatory analysis					
low-dose	FG-4592	Comp. **6**	M1	M2	M3	M4	FG-4592	Comp. **6**	M1	M2	M3	M4
blank	-	-	-	-	-	-	-	-	-	-	-	-
24 h	✓ (3/3)	✓ (3/3)	✓ (3/3)	✓ (3/3)	✓ (3/3)	✓ (3/3)	✓ (3/3)	✓ (3/3)	✓ (3/3)	✓ (3/3)	✓	✓
72 h	✓ (3/3)	-	-	-	✓ (3/3)	✓ (3/3)	✓ (3/3)	-	-	✓ (1/3)	✓ (3/3)	✓(3/3)
120 h	✓(2/3)	-	-	-	-	✓ (3/3)	✓ (3/3)	-	-	-	-	✓ (3/3)
167 h	-	-	-	-	-	✓ (2/3)	✓ (3/3)	-	-	-	-	✓ (2/3)
high-dose												
blank	-	-	-	-	-	-	-	-	-	-	-	-
24 h	✓ (3/3)	✓ (3/3)	✓ (3/3)	✓ (3/3)	✓ (3/3)	✓ (3/3)	✓ (3/3)	✓ (3/3)	✓ (3/3)	✓ (3/3)	✓ (3/3)	✓ (3/3)
72 h	✓ (3/3)	-	-	✓ (1/3)	✓ (3/3)	✓ (3/3)	✓ (3/3)	-	-	✓(1/3)	✓ (3/3)	✓ (3/3)
120 h	✓ (3/3)	-	-	✓ (3/3)	✓ (3/3)	✓ (3/3)	✓ (3/3)	-	-	-	✓(2/3)	✓ (3/3)
167 h	✓ (3/3)	-	-	-	-	✓ (3/3)	✓ (3/3)	-	-	-	-	✓ (2/3)

Table 5. Summary of elimination study plasma analyses. Numbers in brackets indicate how many samples of the tested set were successfully analyzed for the respective compound

Plasma sample	Identified analyte(s)				
	Confirmatory procedure				
low dose	FG-4592	Comp. **6**	M2	M3	M4
blank	-	-	-	-	-
8 h	✓ (3/3)	✓ (3/3)	-	✓ (3/3)	✓ (2/3)
24 h	✓ (1/1)	-	-	-	-
48 h	✓(3/3)	✓ (1/3)	✓ (1/3)	✓ (2/3)	-
72 h	✓(2/3)	✓ (2/3)	-	-	-
96 h	✓(1/3)	-	-	-	-
high dose					
blank	-	-	-	-	-
8 h	✓ (3/3)	✓ (3/3)	✓ (2/3)	✓ (3/3)	-
24 h	✓ (3/3)	✓ (3/3)	✓ (1/3)	✓(3/3)	-
48 h	✓ (3/3)	✓ (3/3)	-	✓ (3/3)	-
72 h	✓ (3/3)	✓ (3/3)	✓ (1/3)	✓ (1/3)	-
96 h	✓ (3/3)	✓ (2/3)	-	✓ (1/3)	-

For plasma, limited sample availability only allowed the confirmatory test. Moreover, the test was modified to utilize only 150 μL aliquots. Despite these limitations, FG-4592 was detectable in nearly all samples up to 96 h at high dose, as was the photoisomer **6** due to uncontrolled/imperfectly controlled exposure to light (Table 5). At the lower dosage level, FG-4592 was detected in all samples through 48 h and in two of three and one of three samples at 72 h and 96 h, respectively. The photoisomer was a less reliable indicator at low dose. Of the excretion metabolites, only M3 was routinely detectable at low and high doses. None of the excreted metabolites provided reliable detection beyond 48 h. For the purposes of doping control, efforts should focus on detecting FG-4592 and its photoisomer with the presence of M3 providing further confidence in cases involving high doses or short time frames between ingestion and blood sampling.

Conclusion

Rapid and specific methods for the detection of FG-4592 and its metabolites in human urine and plasma samples have been established as a means for anti-doping measures in athletics. In urine, the intact drug as well as its glucuronic acid conjugate, which is not yet commercially available, were found to represent the target analytes of choice, offering detection windows of up to 167 h under the chosen drug administration regimen. Monitoring both compounds further compensates for potential hydrolysis effects, transforming the phase-II metabolite back to FG-4592. Although detection of FG-4592 and its metabolites in plasma is less sensitive and has a shorter detection window, the method could prove useful when a urine sample is not available or to provide greater flexibility in proving a case of repeated use. Roxadustat and its photoisomer are the critical congeners for detection in blood. The sulphate M3 is less reliable but can corroborate findings for FG-4592 and its photoisomer in favorable circumstances. For difficult samples, more extensive clean-up may be required as the use of larger sample volumes increases the matrix effect observed for the confirmatory test relative to the ITP. If one decides to focus on FG-4592 and its photoisomer, which is justifiable given the results seen in the administration study, more aggressive washing can be used during solid-phase extraction that would otherwise lead to loss of more polar metabolites, especially M3 and M4.

The incorporation of the photoisomer into screening programs is highly recommended based on the observed variability in its recovery throughout the extraction process. Samples for doping control are not always subjected to the most stringently regulated conditions and variability in degree of exposure to conditions yielding conversion of FG-4592 to its isomer is likely to be high. Excluding the isomer from analysis removes a potential explanation for variability in response observed between the ITP and confirmatory testing.

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