In vivo and *in vitro* metabolism of the designer anabolic steroid furazadrol in thoroughbred racehorses

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Abstract:

Furazadrol ([1',2']isoxazolo[4',5':2,3]-5α-androstan-17β-ol) is a designer anabolic androgenic steroid that is readily available *via* the internet. It contains an isoxazole fused to the steroid A-ring which offers metabolic stability and noteworthy anabolic activity raising concerns over the potential for abuse of this compound in equine sports. The metabolism of furazadrol was studied by *in vivo* and *in vitro* methods for the first time. Urinary furazadrol 17-sulfate and furazadrol 17-glucuronide metabolites were detected *in vivo* after a controlled administration and compared with synthetically-derived reference materials in order to confirm their identities. They were quantified to establish the excretion profile and a suitable limit of detection. Minor metabolites were also detected, including epifurazadrol, hydroxylated furazadrol, and hydroxylated and oxidised furazadrol, present as the sulfate and glucuronide conjugates. Phase II metabolites were subjected to enzymatic hydrolysis by *Escherichia coli* β-glucuronidase and *Pseudomonas aeruginosa* arylsulfatase to further confirm the identity of the corresponding phase I metabolites. The metabolism profile was compared to the products obtained from an *in vitro* phase I metabolism study, with all but two of the minor *in vivo* phase I metabolites observed in the *in vitro* system. These investigations identify the key urinary metabolites of furazadrol following oral administration, which can be incorporated into anti-doping screening and confirmation procedures.

Keywords:

furazadrol; [1',2']isoxazolo[4',5':2,3]-5α-androstan-17β-ol; designer steroid; metabolism; anti-doping; liquid chromatography-mass spectrometry

1 Introduction:

Over past decades numerous instances of the use of androgenic anabolic steroids (AAS) as performance enhancing drugs have been documented resulting in prohibition by the majority of sporting governing bodies[1]. In order to enforce these bans, analytical methods which primarily utilise gas or liquid chromatography coupled to detection by mass spectrometry (GC-MS or LC-MS) have been developed[2][3][4][5]. In the past decade however, numerous designer AAS compounds have made their way onto the market and with them the potential for widespread abuse in sporting competition[6][7]. They are readily available *via* the internet in so-called "dietary supplements", which frequently contain misleading or false content information in order to circumvent attempts to control their use by law-enforcement authorities. Their novel structures present a variety of problems to anti-doping laboratories as these compounds may pass undetected in routine screening[6]. Additionally, these agents present problems for animal welfare as safety and efficacy have generally not been established. Of the vast variety of designer compounds now available, very few have been selected for study in thoroughbred racehorses[8][9][10][11][12][13], despite extensive attention in human sport[6][7][14][15]. As such, development of methods to detect these compounds in equine systems is highly desirable.

Steroid isoxazoles such as [1',2']isoxazolo[4',5':2,3]-5α-androstan-17β-ol (called furazadrol **F** in this work) and its isomer [1',2']isoxazolo[4',3':2,3]-5α-androstan-17β-ol (isofurazadrol **IF**) have been detected as components of so called "dietary supplements"[16][17]. Mixtures of **F** and **IF** were first identified in Orastan-A (Gaspari Nutrition) predominately as the tetrahydropyranyl ether[16] and more recently in Furazadrol (Axis Labs)[17], with incorrect labelling of the contents in both cases. Both **F** and **IF** have been reported to exert anabolic activity in the older steroid literature that was dependent on the mode of administration[18][19] and more recently in both yeast and human HuH7 androgen bioassays[17], with the former isomer reported to display greater activity[18][19]. Related isoxazole containing steroids, including the structurally similar danazol, are banned in competition by the World Anti-Doping Agency (WADA) and the International Federation of Horseracing Authorities (IFHA)[20][21]. To the best of our knowledge, there have been no reported studies of **F** metabolism highlighting the need for work in this area. To this end the equine metabolism of this compound has been investigated through use of an *in vivo* drug administration study as well as comparative *in vitro* techniques, with major metabolites matched against synthetically-derived reference materials. This metabolic profiling study provides anti-doping laboratories with the information required to establish the routine screening for the detection of **F** abuse in horses.

2 Experimental

2.1 Materials

Chemicals, enzymes and solvents including sulfur trioxide pyridine complex (SO_3.py), dihydrotestosterone (17β-hydroxy-5α-androstan-3-one), *Helix pomatia* β-glucuronidase, trypsin and 1,4-dioxane were purchased from Sigma–Aldrich (Castle Hill, Australia) and were used as supplied unless otherwise stated. *N,N*-Dimethylformamide (DMF) and aqueous ammonia solution were obtained from Chem-Supply (Gillman, Australia). Formic acid and magnesium chloride was obtained from Ajax Chemicals (Auburn, Australia). Ammonia gas was purchased from BOC (North Ryde, Australia). Testosterone (17β-hydroxyandrost-4-en-3-one) was purchased from Steraloids (Newport RI, USA). d_3-Testosterone 17-glucuronide and d_3-testosterone 17-sulfate were purchased from the National Measurement Institute (North Ryde, Australia). d_4-Hydrocortisone was purchased from BDG Synthesis (Wellington, New Zealand). Epitestosterone (17α-hydroxyandrost-4-en-3-one) was synthesised from testosterone according to literature methods[22]. MilliQ water was used in all aqueous solutions. Solid-phase extraction (SPE) was performed using Waters (Rydalmere, Australia) Oasis WAX 6cc cartridges (PN 186004647), Oasis WAX 3cc cartridges (PN 186002492) and UCT (Bristol PA, USA) XTRACKT 3cc cartridges (PN XRDAH203) as specified. *Escherichia coli* β-glucuronidase solution was used as supplied from Roche (Castle Hill, Australia). Equine liver S9 fraction was used as supplied from XenoTech (Lenexa KS, USA). *Escherichia coli* glucuronylsynthase was expressed in *E. coli* according to literature methods[23]. *Pseudomonas aeruginosa* arylsulfatase was expressed in *E. coli* according to literature methods[24].

2.2 Furazadrol Reference Materials

A range of reference materials were employed to aid the identification of phase I and phase II metabolites. These were **F**[25]; **IF**[25]; epifurazadrol, **EF**; oxidised furazadrol, **OF**; oxidised isofurazadrol, **OIF**; furazadrol 17-sulfate, **FS**[25]; isofurazadrol 17-sulfate, **IFS**[25]; epifurazadrol 17-sulfate, **EFS**; furazadrol 17-glucuronide, **FG**; isofurazadrol 17-glucuronide, **IFG**; and epifurazadrol 17-glucuronide, **EFG**. The IUPAC names and structures of these compounds are given in the supplementary material, together with experimental details and characterisation data for new compounds **EF**, **OF**, **OIF**, **EFS**, **FG**, **IFG** and **EFG**, and copies of the 400 MHz ^{1}H NMR, 100 MHz ^{13}C NMR, and +EI LRMS or –ESI LRMS spectra where appropriate.

2.3 Instruments

Melting points were determined using a SRS (Sunnyvale CA, USA) Optimelt MPA 100 melting point apparatus and are uncorrected. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded using either a Varian (Santa Clara CA, USA) 400 MHz, Bruker (Alexandria, Australia) Ascend 400 MHz or Bruker Avance 400 MHz spectrometer at 298 K using deuterated chloroform or deuterated methanol solvent. Data is reported in parts per million (ppm), referenced to residual protons or ^{13}C in deuterated methanol (CD_3OD: ^{1}H 3.31 ppm, ^{13}C 49.00 ppm) or chloroform ($CDCl_3$: ^{1}H 7.26 ppm, ^{13}C 77.16 ppm), with multiplicity assigned as follows: br = broad, s = singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quartet,

m = multiplet. Coupling constants *J* are reported in Hertz. Low-resolution mass spectrometry (LRMS) and high-resolution mass spectrometry (HRMS) were performed using positive electron ionisation (+EI) on a Micromass VG Autospec mass spectrometer or negative electrospray ionization (–ESI) on a Micromass ZMD ESI-Quad, or a Waters LCT Premier XE mass spectrometer. Reactions were monitored by analytical thin layer chromatography (TLC) using Merck (Bayswater, Australia) Silica gel 60 TLC plates and were visualised by staining with a solution of potassium permanganate ($KMnO_4$ [3 g], K_2CO_3 [20 g], NaOH [0.25 g], H_2O [305 mL]) or concentrated sulfuric acid-methanol (5:95 v/v), with heating as required.

2.4 Analytical Methods

Positive mode liquid chromatography-high resolution accurate mass (LC-HRAM) spectrometry analysis was undertaken using a Thermo Fisher Scientific (Bremen, Germany) Ultimate 3000 HPLC coupled to an Q Exactive Hybrid Quadrupole Orbitrap or an Exactive Plus Orbitrap mass spectrometer equipped with a Waters SunFire C18 column (100 x 2.1 mm, 3.5 um) eluting with a gradient consisting of the following mobile phases, A: 0.1% formic acid in water, B: 0.1% formic acid in methanol, gradient: 0-1 min A-B (95:5 v/v), 1-15 min A-B (95:5 v/v) to A-B (5:95 v/v), 15-19 min A-B (5:95 v/v), 5 min re-equilibration, flow rate 0.4 mL min^{-1}. Unconjugated steroids and steroid glucuronides were monitored for the proton adduct ($[M+H]^+$) using HESI in positive full scan mode at a resolution of 70,000 (FWHM).

Negative mode LC-HRAM spectrometry analysis was undertaken using a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer equipped with a Phenomenex (Torrance CA, USA) Gemini C18 column (50 x 2 mm, 5 um), eluting with a gradient consisting of the following mobile phases, A: aqueous ammonium acetate (0.01 M, pH 9.0), B: 0.1% acetic acid in acetonitrile, gradient: 0-2 min A-B (99:1 v/v), 2-8.5 min A-B (99:1 v/v) to A-B (20:80 v/v), 2.7 min re-equilibration, flow rate 0.5 mL min^{-1}. Steroid glucuronide and sulfate conjugates were monitored for the anion ($[M-H]^-$) using HESI in negative full-scan or targeted MS/MS mode at a resolution of 70,000 (FWHM).

2.5 *In vivo* equine metabolism study

2.5.1 Animal Administration

Animal administration was approved by the Racing NSW Animal Care and Ethics Committee. A sample of **F-IF** (10:1 w/w, 200 mg) was administered orally as a suspension in water by way of a nasal-gastric tube to a thoroughbred gelding (20 years old, 580 kg) and samples of urine were collected at 0, 2, 4, 6 and 24 h post-administration and daily thereafter up to 7 days post administration. Urine samples were collected by spontaneous voiding with carrot reward and were immediately frozen and stored at -20 °C until required for analysis.

2.5.2 Sample Preparation without fractionation

An aliquot of urine (2 mL) was fortified with d_3-testosterone 17-sulfate (100 ng mL^{-1}) internal standard and treated with sodium phosphate buffer (100 mM, pH 7.4, 1 mL) and centrifuged (2000 rpm, 5 min) to pellet

solids. The supernatant was then loaded onto an Oasis WAX SPE cartridge (3 cc) that was pre-conditioned with methanol (1 mL) and water (2 mL), and then washed with aqueous sodium hydroxide solution (0.1 M, 2 mL), sodium phosphate buffer (0.1 M, pH 7.4, 2 mL) and water (2 mL). The urinary steroid metabolites were then eluted with a solution of methanol-ethyl acetate-diethylamine (25:25:1 v/v/v, 2 mL). Concentration under a stream of nitrogen at 60 °C afforded a residue which was reconstituted in methanol-water (5:95 v/v) and transferred to a sealed vial for subsequent analysis by LC-MS as per section 2.4.

2.5.3 Sample preparation with fractionation

Sample preparation was conducted according to Section 2.5.2 using modified elution conditions. The urinary steroid metabolites were fractionated by elution with methanol (2 mL), a solution of methanol-ethyl acetate-formic acid (25:25:1 v/v/v, 2 mL) and a solution of methanol-ethyl acetate-diethylamine (25:25:1 v/v/v, 2 mL) which afforded the unconjugated, glucuronide and sulfate fractions respectively[26].

2.5.4 Enzymatic hydrolysis of the glucuronide fraction by *E. coli* β-glucuronidase

The glucuronide fraction from section 2.5.3 above was reconstituted in sodium phosphate buffer (50 mM, pH 7.4, 2 mL) and a solution of *E. coli* β-glucuronidase (140 units mL^{-1}, 50 µL) was added. The solution was incubated at 37 °C for 18 h before being subjected to purification by SPE without fractionation as per section 2.5.2 above.

2.5.5 Enzymatic hydrolysis of the sulfate fraction by *P. aeruginosa* arylsulfatase

The sulfate fraction from section 2.5.3 above was reconstituted in tris(hydroxymethyl)amino-methane buffer (0.1 M, pH 9.0, 2 mL) and a solution of *P. aeruginosa* arylsulfatase (70 mg/mL, 50 µL) was added[24]. The solution was incubated at 37 °C for 18 h before being subjected to purification by SPE without fractionation as per 2.5.2 above. Concentration afforded a residue which was reconstituted in methanol-water (5:95 v/v) and transferred to a sealed vial for subsequent positive mode LC-MS analysis as per section 2.4.

2.5.6 Quantification of furazadrol 17-glucuronide (FG) in equine urine

Duplicate aliquots of blank equine urine (2 mL) were fortified with d_3-testosterone 17-glucuronide (100 ng mL^{-1}) internal standard and spiked with **FG** to generate a zero solution and calibrators at concentrations of 1, 5, 10, 50, 100, 500, 1000 and 1500 ng mL^{-1}, respectively. These were then subjected to SPE as per section 2.5.2 above and subsequent positive mode LC-MS analysis as per section 2.4. Data analysis was performed using Xcalibur software and Microsoft Excel. The limit of detection (LOD) was estimated from separate sets (n=3) of equine urine spikes at concentrations of 0, 0.05, 0.1, 0.2, 0.5, 1 and 2 ng mL^{-1} achieving consistent responses with signal-to-noise (*S/N*) greater than 3.

2.5.7 Quantification of furazadrol 17-sulfate (FS) in equine urine

Duplicate aliquots of blank equine urine (2 mL) were fortified with d_3-testosterone 17-sulfate (100 ng mL^{-1}) internal standard and spiked with **FS** to generate a zero solution and calibrators at concentrations of 1, 5, 10, 50, 100, 500, 1000, 1500 and 2000 ng mL^{-1}, respectively. These were then subjected to SPE as per

section 2.5.2 above and subsequent negative mode LC-MS analysis as per section 2.4. Data analysis was performed using Xcalibur software and Microsoft Excel. The LOD was estimated as described in section 2.5.6.

2.6 *In vitro* phase I metabolism using equine liver S9 fraction

The *in vitro* phase I metabolism study was conducted according to literature protocols[10] with only minor modifications. A stock solution containing **F**-**IF** (10:1 w/w, 860 µg) in sodium phosphate buffer (50 mM, pH 7.4, 20.0 mL) was prepared and an aliquot of this solution (136.5 µM, 250 µL) was transferred to a new reaction vial. The solution was treated in order with a solution of nicotinamide adenine dinucleotide-nicotinamide adenine dinucleotide phosphate-glucose-6-phosphate-magnesium chloride (15 mM/15 mM/75 mM/45 mM, 10x final concentration, 50 µL), additional sodium phosphate buffer (87 µL), equine liver S9 fraction solution (20 mg/mL, 25 µL) and glucose-6-phosphate dehydrogenase solution (11.4 units mL^{-1}**,** 88 µL). The final solution (500 µL) was then incubated in an open tube with agitation for 3 h at 37 °C. The reaction was quenched with acetonitrile (1 mL), centrifuged (2000 rpm, 5 min) to pellet solids and the supernatant was decanted. Concentration of the supernatant under a stream of nitrogen at 60 °C afforded a residue which was reconstituted in methanol-water (5:95 v/v) and transferred to a sealed vial for subsequent positive mode LC-MS analysis as per section 2.4. Control experiments excluding cofactors, equine liver S9 fraction or steroid respectively were performed alongside the above reaction with addition of sodium phosphate buffer to maintain a constant final reaction volume.

2.7 Translation to routine screening

An aliquot of urine (3 mL) was adjusted to pH 5.0-5.5 using aqueous hydrochloric acid solution (3 M) and a solution of internal standard containing d_4-hydrocortisone (1.5 µg mL^{-1}) in methanol (155 µL) was added followed by a solution of *H. pomatia* β-glucuronidase (16.2 µL/mL, 155 µL), trypsin (25 mg/mL, 37.5 µL) and sodium phosphate buffer (0.1 M, pH 5.15, 4 mL). The solution was incubated at 37 °C for 17 h before loading onto a UCT XTRACKT SPE cartridge (3 cc) pre-conditioned with methanol (1 mL) and sodium phosphate buffer (1.5 mM, pH 7.0, 1 mL). The sample was washed with aqueous acetic acid solution (0.1 M, 3 mL), eluted with a solution of ethyl acetate-*n*-hexane (3:2 v/v) and dried under stream of nitrogen at 60 °C. The residue was reconstituted in aqueous formic acid (0.1 M, 50 µL) and formic acid in methanol (0.1 M, 50 µL) and transferred to a sealed vial for subsequent positive mode LC-MS analysis as per in section 2.4. The LOD was estimated as described in section 2.5.6.

3 Results and Discussion

3.1 Synthesis of furazadrol (F) reference materials

Furazadrol was prepared according to the reported methods with only minor modifications[19][25]. Isoxazole formation in ethanol solvent afforded a 10:1 mixture of **F** and **IF** that was not readily separable by column chromatography or purified by recrystallisation. Given that the materials sourced on-line are

reported to contain both isomeric steroids in varying ratios, the mixture was deemed suitable to conduct *in vivo* and *in vitro* metabolism studies. Isoxazole formation in pyridine solvent afforded **IF** as the sole product[16][19][25]. This material was identical to the minor isomer described above.

In addition, a number of predicted phase I metabolites of **F** were selected based on the equine metabolism of structurally similar steroidal agents, and these were synthesised in an effort to unambiguously identify the metabolites derived from *in vivo* and *in vitro* metabolism studies[27]. Access to the **EF** proved more challenging than expected as direct Mitsunobu inversion under a variety of conditions failed to afford the desired isomer in appreciable yield[22]. Instead, Birch reduction of epitestosterone gave 17α-hydroxy-5α-androstan-3-one which underwent formylation and heterocycle formation in ethanol to give **EF** and epiisofurazadrol (**EIF**) as a 5:1 mixture (**Scheme 1**). Oxidation of **F** with pyridinium chlorochromate (PCC) afforded the desired **OF** (**Scheme 1**) in 79% yield with **OIF** (97%) prepared in a similar manner.

Scheme 1

Synthesis of the phase II sulfate and glucuronide metabolites in high purity could be achieved using recently reported methods (**Scheme 2**)[23][25]. Sulfation of **F** gave **FS** with >98% conversion[25]. Glucuronylation of **F** afforded **FG** with a modest 38% conversion. These methods also allowed rapid and efficient access to **IFS** (>98% conversion)[25], **EFS** (>98% conversion), **IFG** (69% conversion) and **EFG** (29% conversion).

Scheme 2

3.2 *In vivo* equine metabolism

There have been no reported studies detailing the metabolism of furazadrol in equine systems. To address this, an *in vivo* equine administration study was undertaken in which a synthetically derived sample of furazadrol (200 mg, 10:1 mixture of **F**:**IF**) was administered orally to a thoroughbred gelding and urine samples were collected up to 7 days post-administration. Sample preparation employed solid phase extraction without fractionation (Section 2.5.2) using Waters Oasis WAX cartridges to provide an extract containing free, glucuronide conjugated and sulfate conjugated steroids that were subjected to positive and negative mode LC-MS analysis. Data were examined using mass filters for predicted metabolites formed from up to three metabolic transformations including oxidation, reduction and hydroxylation, with or without subsequent sulfation or glucuronylation. Metabolite peaks were identified where exact masses were observed within 10 ppm of the predicted mass and by comparison with blank urine. Metabolites identified directly by LC-MS were matched against reference materials where available (**Table 1**).

Table 1. *In vivo* equine metabolism of furazadrol

Metabolite	**Precursor ion and MS/MS fragments** (% of base peak), [collision energy][A]	**RT** (min)[A]	**Precursor ion**	**Theoretical *m/z***
FS	394.1682 (15%), 96.9589 (100%), [60	6.23[B]	$[M-H]^-$	394.1683

	eV]			
IFS	394.1682 (30%), 96.9588 (100%), [60 eV]	6.10[B,C]	$[M-H]^-$	394.1683
hydroxylated furazadrol sulfate (**S1**)	410.1663 (10%), 364.1579 (20%), 96.9588 (100%), [60 eV]	5.11	$[M-H]^-$	410.1632
oxidised and hydroxylated furazadrol sulfate (**S2**)	408.1476 (20%), 96.9589 (100%), [60 eV]	4.31	$[M-H]^-$	408.1475
oxidised and hydroxylated furazadrol sulfate (**S3**)	408.1478 (10%), 365.0479 (30%), 96.9589 (80%), 79.9561 (100%), [60 eV]	5.80	$[M-H]^-$	408.1475
FG	492.2578 (100%), 316.2262 (80%), 141.0179 (20%), 113.0232 (30%), 84.0446 (70%), [40 eV]	13.70[D]	$[M+H]^+$	492.2592
IFG	492.2574 (25%), 316.2262 (90%), 288.2312 (35%), 189.1268 (40%), 113.0232 (45%), 85.0286 (100%), [50 eV]	13.25[D,E]	$[M+H]^+$	492.2592
EFG	492.2582 (15%), 316.2263 (100%), 163.1477 (20%), 113.0232 (35%), 84.0446 (70%), [40 eV]	14.07[F]	$[M+H]^+$	492.2592
oxidised and hydroxylated furazadrol glucuronide (**G1**)	506.2369 (10%), 330.2055 (100%), 294.1845 (20%), 141.0179 (45%), [40 eV]	12.07	$[M+H]^+$	506.2385

[A]From targeted MS/MS data acquisition on the Q Exactive instrument using conditions specified for positive mode or negative mode analysis (section 2.4). [B]Matched against mixed **FS** and **IFS** reference material. [C]Matched against **IFS** reference material. [D]Matched against mixed **FG** and **IFG** reference material. [E]Matched against **IFG** reference material. [F]Matched against mixed **EFG** and epiisofurazadrol 17-glucuronide reference material.

Negative mode analysis showed that furazadrol was primarily excreted without phase I transformation as the sulfate conjugate (**Table 1**). Metabolites corresponding to **FS** (**Figure 1**) and **IFS** were identified and matched against synthetic reference materials. As the administered drug was a 10:1 mixture of **F** and **IF**, metabolites derived from both isomers were observed in the *in vivo* samples, with **IF** giving rise to a number of minor metabolites. Since there appeared to be little discernible difference in the metabolism of each isomer in the mixture, metabolism is described for the major isomer only. Hydroxylated furazadrol sulfate (**S1**), and two oxidised and hydroxylated furazadrol sulfate metabolites (**S2**, **S3**) were also observed as minor metabolites. No **EFS** was detected by comparison with the synthetically-derived reference material. Although glucuronide metabolites were detected by negative mode analysis, the sensitivity was significantly lower than that observed under positive mode analysis[28].

Figure 1.

Positive mode analysis showed that furazadrol was also primarily excreted without phase I transformation as the glucuronide conjugate (**Table 1**). Metabolites corresponding to **FG** (**Figure 2**) and **IFG** were identified and matched against reference materials. Minor **EFG** was also observed and matched against the reference material. Epimerisation of the C17 alcohol, through a two-step oxidation and reduction sequence is typically observed as a significant pathway for a range of steroids including boldenone[27][29]. Additionally, a minor oxidised and hydroxylated furazadrol glucuronide metabolite (**G1**) was also detected. No unconjugated furazadrol metabolites were detected by positive mode analysis in the *in vivo* samples. Comparison of urine samples against synthetically derived reference materials failed to identify the presence of **F**, **IF**, **EF**, **OF** or **OIF** as significant equine urinary metabolites.

Figure 2.

3.3 Enzyme hydrolysis of *in vivo* equine metabolites

Although the major phase II furazadrol metabolites were matched against synthetic reference materials, further characterisation of the corresponding phase I metabolites was attempted by LC-MS. Hydrolysis of both the glucuronide and sulfate metabolites would provide additional evidence of metabolite structure and also afford unconjugated metabolites suitable for confirmatory analysis. Aliquots of the 4 h urine were subjected to SPE with fractionation (section 2.5.3) to afford glucuronide and sulfate fractions suitable for enzyme hydrolysis.

Hydrolysis of the glucuronide fraction with *E. coli* β-glucuronidase at 37 °C overnight (section 2.5.4) followed by SPE without fractionation afforded the glucuronidase hydrolysed extract. Within this extract, hydrolysis of **FG** (major), **IFG** and **EFG** in the urine gave rise to **F** (major), **IF** and **EF** respectively and these were matched against reference materials by positive mode LC-MS analysis (section 2.4). Additionally, hydrolysis of the minor unidentified oxidised and hydroxylated furazadrol glucuronide (**G1**) gave rise to a peak with a mass corresponding to an unconjugated oxidised and hydroxylated furazadrol metabolite (**M9**,

see section 3.5). Residual glucuronide metabolites were not observed by positive mode LC-MS analysis of the enzyme hydrolysed extract.

Detection of the sulfate metabolites required negative mode LC-MS analysis, with the only major peaks observed the parent sulfate anion $[M-H]^-$ and hydrogen sulfate $[HSO_4]^-$. Two major peaks are insufficient for confirmatory analysis by AORC criteria providing additional impetus for hydrolysis to the corresponding unconjugated metabolites[30]. Hydrolysis of the sulfate metabolites was carried out with *P. aeruginosa* arylsulfatase, a purified enzyme with steroid sulfate hydrolysis activity comparable to commercially available crude enzyme preparations but without glucuronidase, oxidase or reductase activity[24]. Treatment of the sulfate extract at 37 °C overnight (section 2.5.5) followed by SPE without fractionation afforded the sulfatase hydrolysed extract. Hydrolysis of **FS** (major) and **IFS** in the urine gave rise to **F** (major) and **IF** respectively and these were matched against reference materials by positive mode LC-MS analysis (section 2.4). Additionally, hydrolysis of the minor hydroxylated (**S1**), and oxidised and hydroxylated furazadrol sulfate metabolites (**S2**, **S3**) gave rise to peaks corresponding to one hydroxylated furazadrol (**M3**, see section 3.5), and one oxidised and hydroxylated furazadrol metabolite respectively. A second oxidised and hydroxylated furazadrol metabolite was not observed. Residual sulfate metabolites were not observed by negative mode LC-MS analysis of the enzyme hydrolysed extract (section 2.4).

3.4 Quantification of *in vivo* equine metabolites in equine urine

Having identified **FG** and **FS** as the major equine metabolites following oral administration attention turned to establishing the quantification of these metabolites. Although the detection of exogenous steroids or their metabolites is sufficient grounds for prosecution in the racing industry, the investigation of excretion profiles provides information relevant to the development of screening strategies for illicit substances. Calibrators were generated from separately spiking blank urine with **FG** and **FS** reference materials which were subjected to sample preparation (section 2.5.2) and LC-MS analysis (section 2.4) to generate a calibration plot. The plots were linear over the range 1-1500 ng mL^{-1} for **FG** (R^2 0.999) and 1-2000 ng mL^{-1} for **FS** (R^2 0.993). For **FS** detected in negative mode, peak excretion of 1642 ng mL^{-1} was observed at 4 h, before decreasing to 43 ng mL^{-1} at 24 h (mean ± 20%, n = 3) above the LLOQ (1.0 ng mL^{-1}) and LOD (0.5 ng mL^{-1}) for this analyte. For **FG** detected in positive mode, peak excretion of 143 ng mL^{-1} was observed at 4 h, before decreasing to 6.0 ng mL^{-1} at 24 h (mean ± 20%, n = 3) above the LLOQ (1.0 ng mL^{-1}) and LOD (0.2 ng mL^{-1}) for this analyte. No metabolites were detected beyond the 24 h sample. The excretion profile for the major urinary metabolites is presented below (**Figure 3**).

Figure 3

3.5 *In vitro* equine metabolism with liver S9 fraction

Whilst *in vivo* metabolism studies provide the most complete picture of steroid metabolism, this approach may not always be possible due to ethical or financial constraints. In such situations *in vitro* metabolism studies provide an alternate means of metabolic profiling. Metabolism platforms such as equine liver

hepatocytes, microsomes or S9 fraction offer convenient systems to study the *in vitro* metabolism of steroid compounds. A key question associated with the use of *in vitro* metabolic platforms is how closely such systems replicate the metabolism observed *in vivo*. To address this question a brief phase I *in vitro* metabolism study of furazadrol was conducted using equine liver S9 fraction to compare the metabolic profile generated.

The *in vitro* metabolism of furazadrol using equine liver S9 fraction afforded a range of metabolites (**Table 2**). These included **EF**, **EIF**, **OF** and **OIF** that were matched to reference materials. The study also gave rise to a number of additional unidentified metabolites including eight hydroxylated furazadrol isomers (**M1**-**M8**), one oxidised and hydroxylated furazadrol isomer (**M9**) and two dihydroxylated furazadrol isomers (**M10**, **M11**).

Table 2. *In vitro* equine metabolism of furazadrol

Metabolite	**Precursor ion and MS/MS fragments** (% of base peak), [collision energy][A]	**RT** (min)[A]	**Precursor ion**	**Theoretical *m/z***
F	316.2270 (25%), 199.1479 (10%), 159.1165 (15%), 145.1011 (25%), 119.0855 (30%), 105.0700 (45%), 84.0447 (100%), [60 eV]	14.25[B,C,D]	$[M+H]^+$	316.2271
IF	316.2269 (95%), 288.2314 (25%), 187.1479 (30%), 159.1165 (35%), 145.1011 (65%), 105.0700 (100%), [60 eV]	13.90[B,C,D,E]	$[M+H]^+$	316.2271
EF	316.2266 (30%), 199.1479 (15%), 171.1166 (10%), 157.1010 (30%), 131.0854 (20%), 119.0855 (20%), 105.0700 (35%), 93.0701 (30%), 84.0447 (100%), [60 eV]	14.41[D,F]	$[M+H]^+$	316.2271
OF	314.2108 (25%), 199.1480 (15%), 173.1322 (20%), 159.1166 (30%), 145.1011 (45%), 108.0810 (55%), 84.0447 (100%), [60 eV]	13.81[G]	$[M+H]^+$	314.2115
OIF	314.2109 (85%), 286.2164 (45%), 199.1478 (20%), 185.1322 (35%), 145.1011 (55%), 119.0856 (65%), 97.0651 (100%), [60 eV]	13.52[G,H]	$[M+H]^+$	314.2115
hydroxylated furazadrol (**M1**)	332.2214 (10%), 169.1010 (10%), 145.1010 (25%), 105.0700 (40%), 84.0448 (100%), [60 eV]	11.48	$[M+H]^+$	332.2220

hydroxylated furazadrol (**M2**)	332.2216 (20%), 171.1168 (10%), 145.1010 (25%), 105.0701 (40%), 84.0447 (100%), [60 eV]	11.65	$[M+H]^+$	332.2220
hydroxylated furazadrol (**M3**)	332.2217 (30%), 304.2267 (15%), 185.1324 (10%), 145.1011 (30%), 108.0809 (95%), 96.0810 (100%), [60 eV]	12.08[C]	$[M+H]^+$	332.2220
hydroxylated furazadrol (**M4**)	332.2216 (20%), 197.1322 (10%), 171.1166 (15%), 145.1010 (30%), 119.0856 (30%), 105.0700 (50%), 84.0447 (100%), [60 eV]	12.45	$[M+H]^+$	332.2220
hydroxylated furazadrol (**M5**)	332.2218 (40%), 304.2267 (10%), 185.1322 (15%), 157.1010 (25%), 119.0857 (30%), 91.0545 (100%), [60 eV]	12.64	$[M+H]^+$	332.2220
hydroxylated furazadrol (**M6**)	332.2218 (25%), 171.1167 (15%), 145.1011 (30%), 105.0701 (55%), 84.0447 (100%), [60 eV]	12.95	$[M+H]^+$	332.2220
hydroxylated furazadrol (**M7**)	332.2218 (20%), 169.1011 (15%), 145.1011 (35%), 105.0701 (45%), 84.0447 (100%), [60 eV]	13.26	$[M+H]^+$	332.2220
hydroxylated furazadrol (**M8**)	332.2214 (15%), 185.1324 (30%), 131.0856 (35%), 105.0701 (45%), 84.0447 (100%), [60 eV]	13.33	$[M+H]^+$	332.2220
oxidised and hydroxylated furazadrol (**M9**)	330.2059 (20%), 240.2684 (30%), 110.0602 (10%), 91.0545 (100%), [50 eV]	12.33[D]	$[M+H]^+$	330.2064
dihydroxylated furazadrol (**M10**)	348.2166 (10%), 169.1011 (15%), 131.0855 (25%), 105.0701 (30%), 84.0447 (100%), [60 eV]	11.06	$[M+H]^+$	348.2169
dihydroxylated furazadrol (**M11**)	348.2167 (100%), 330.2062 (25%), 302.2110 (20%), 260.2001 (10%), 159.1168 (15%), 136.0617 (30%), 110.0601 (40%), [60 eV]	11.63	$[M+H]^+$	348.2169

[A]From targeted MS/MS data acquisition on the Q Exactive instrument using conditions specified for positive mode analysis (section 2.4). [B]Matched with mixed **F** and **IF** reference material. [C]Identified in *in vivo* samples after *P. aeruginosa* arylsulfatase hydrolysis. [D]Identified in *in vivo* samples after *E. coli* β-glucuronidase hydrolysis. [E]Matched with **IF** reference material. [F]Matched with mixed **EF** and **EIF** reference material. [G]Matched with mixed **OF** and **OIF** reference material. [H]Matched with **OIF** reference material.

Comparison of the *in vitro* metabolic profile with that obtained *in vivo* after enzyme hydrolysis (section 3.3) showed that a number of the metabolites were common. These included **EF**, and one unidentified oxidised and hydroxylated furazadrol (**G1** → **M9**) observed following hydrolysis of the glucuronide fraction, and one unidentified hydroxylated furazadrol (**S1** → **M3**) following hydrolysis of the sulfate fraction. However, a second oxidised and hydroxylated furazadrol metabolite observed following hydrolysis of the sulfate fraction (**S2** or **S3**) was not detected *in vitro*. Despite this reasonable comparison, it was not possible to distinguish based on relative abundance or other criteria which of the many *in vitro* metabolites formed would likely arise *in vivo*. Thus the ability of this phase I *in vitro* study to identify key *in vivo* metabolites appears to be limited and highlights a major challenge in using *in vitro* methods for metabolic profiling. These differences in metabolic profile could arise from a range of factors including different enzyme activities or cofactor regeneration rates within the two systems, or even the absence of phase II metabolism *in vitro*. These factors were not explored in this work but provide interesting avenues for future investigation.

3.6 Translation to routine screening

The ability of locally implemented protocols to detect furazadrol administration was assessed by subjecting the *in vivo* urine samples to routine screening (section 2.7). Duplicates of each of the *in vivo* samples were manually adjusted to pH 5.0-5.5 and subjected to enzyme hydrolysis using *H. pomatia* β-glucuronidase. Subsequent purification by solid-phase extraction afforded the urinary steroid metabolites in a combined neutral and acidic fraction. Positive mode LC-MS analysis of this fraction detected **F** (major), **IF** and **EF** and these were matched against reference materials. Additionally, an oxidised and hydroxylated furazadrol metabolite (**M9**) was detected. Both **F** and **IF** provide suitable targets for confirmatory analysis, with LOD estimated at 0.2 ng mL^{-1}. The short detection period for furazadrol shown by this work (section 3.4) illustrates the benefits of out-of-competition testing strategies to complement race day sample collection for effective surveillance of anabolic steroid misuse.

4 Conclusions

Designer steroids such as furazadrol pose a significant threat to the integrity of sport if left unchecked. The metabolism of furazadrol was studied by *in vivo* and *in vitro* methods for the first time. Furazadrol 17-sulfate (**FS**) and furazadrol 17-glucuronide (**FG**) metabolites were detected *in vivo* up to one day following controlled oral administration. Minor metabolites including epimerisation (oxidation and reduction),

hydroxylation, and oxidation and hydroxylation, together with sulfate or glucuronide conjugation were also observed (**Figure 4**). These phase II metabolites were subjected to enzymatic hydrolysis by *E. coli* β-glucuronidase and *P. aeruginosa* arylsulfatase to provide further evidence of phase I metabolite identity. The hydrolysed *in vivo* metabolites were compared to those obtained from an *in vitro* study, with reasonable qualitative agreement between systems. These investigations allowed the identification of the key metabolites that can be incorporated into anti-doping screening and confirmation protocols.

Figure 4

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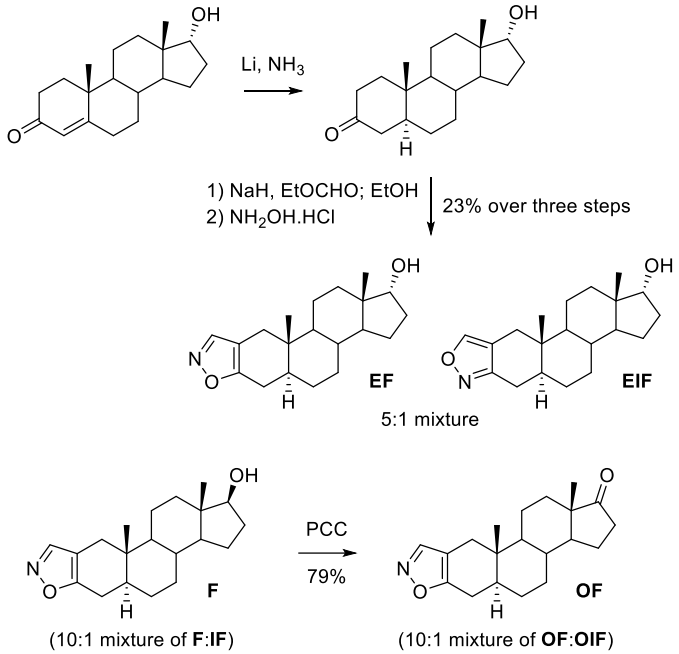
Scheme 1. Synthesis of epifurazadrol (EF) and oxidised furazadrol (OF)



Scheme 2. Synthesis of furazadrol 17-sulfate (FS) and furazadrol 17-glucuronide (FG).

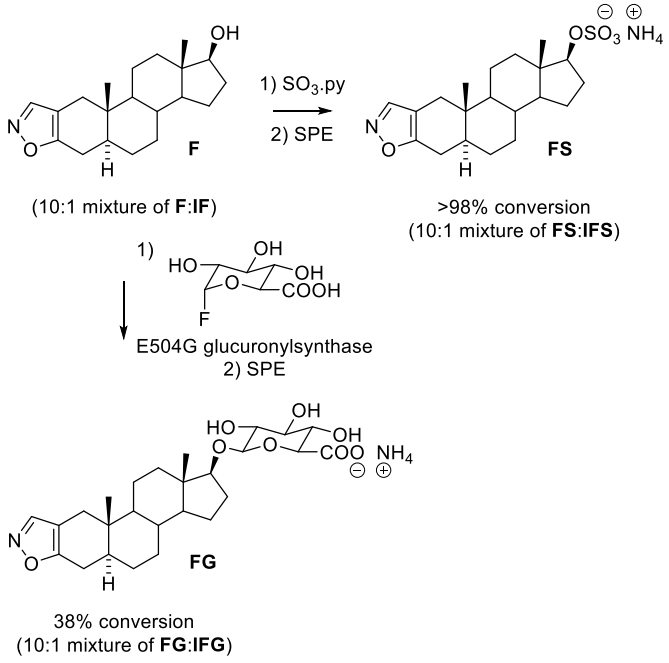


Figure 1. a) Extracted ion chromatogram (*m/z* 394.1683) showing furazadrol 17-sulfate (FS major, 6.23 min) and isofurazadrol 17-sulfate (IFS, 6.10 min) in the 4 h urine; Targeted MS/MS spectrum (60 eV) of b) FS from equine urine and c) FS reference material.

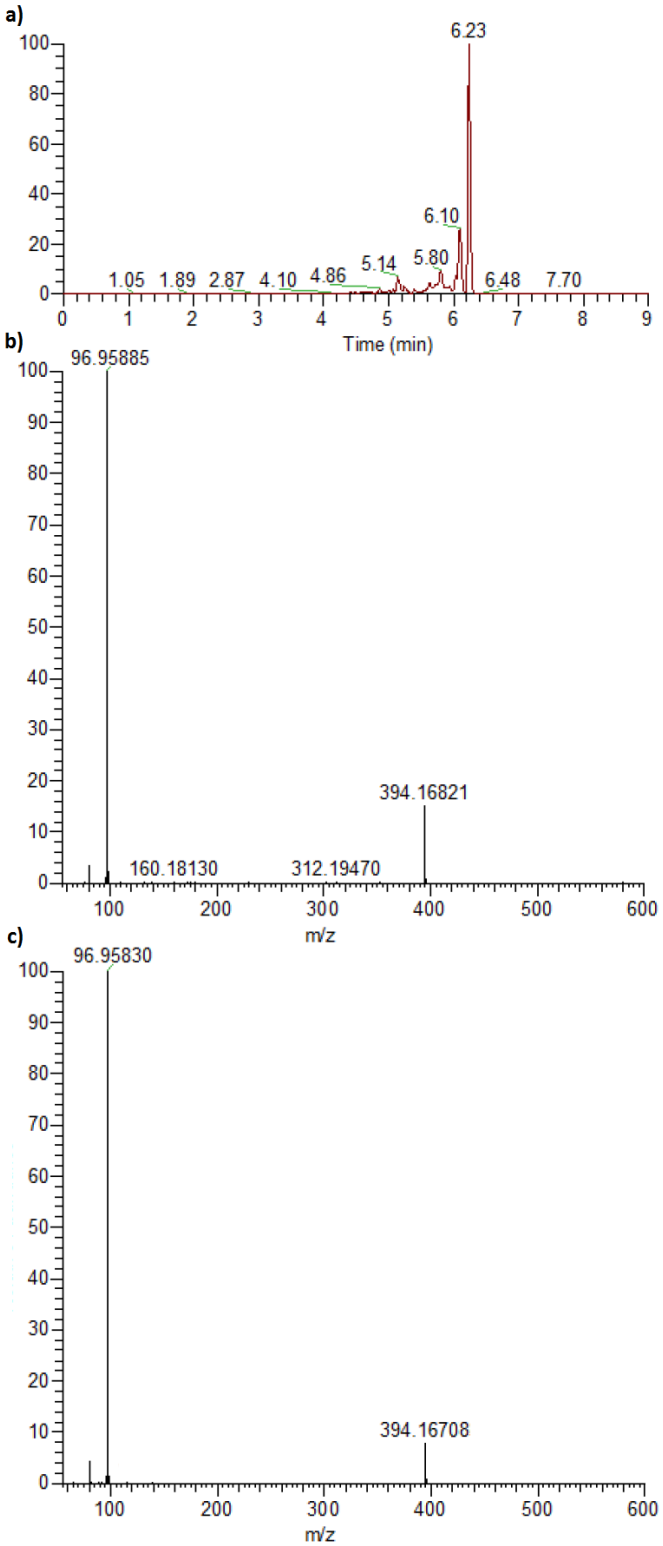
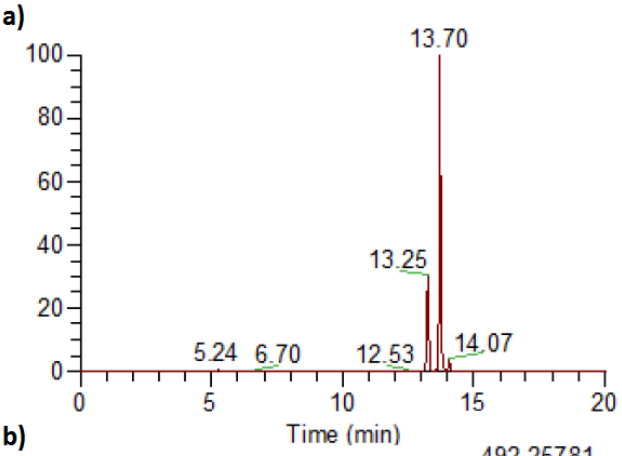
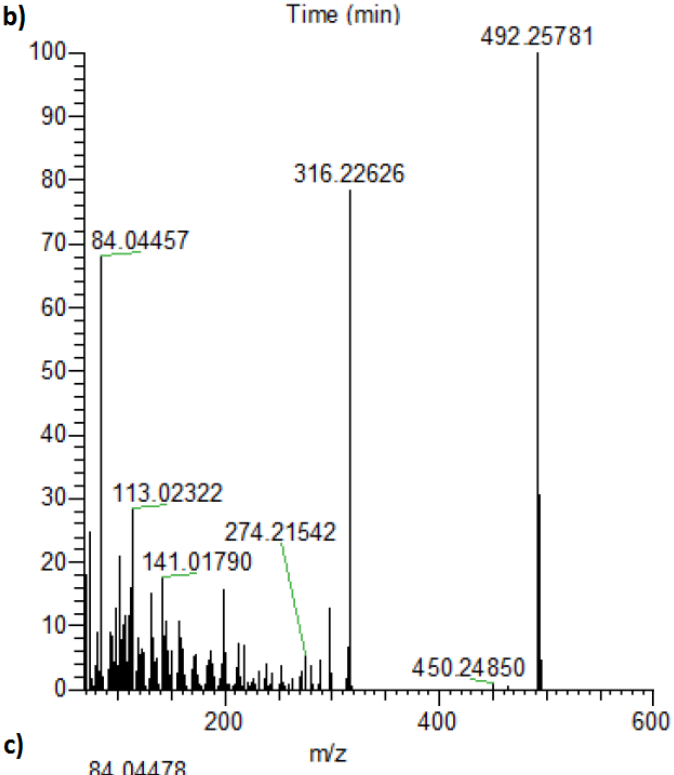


Figure 2. a) Extracted ion chromatogram (*m/z* 492.2592) showing furazadrol 17-glucuronide (FG major, 13.70 min), isofurazadrol 17-glucuronide (IFG, 13.25 min) and epifurazadrol 17-glucuronide (EFG, 14.07 min) in the 4 h urine; Targeted MS/MS spectrum (40 eV) of b) FG from equine urine and c) FG reference material.

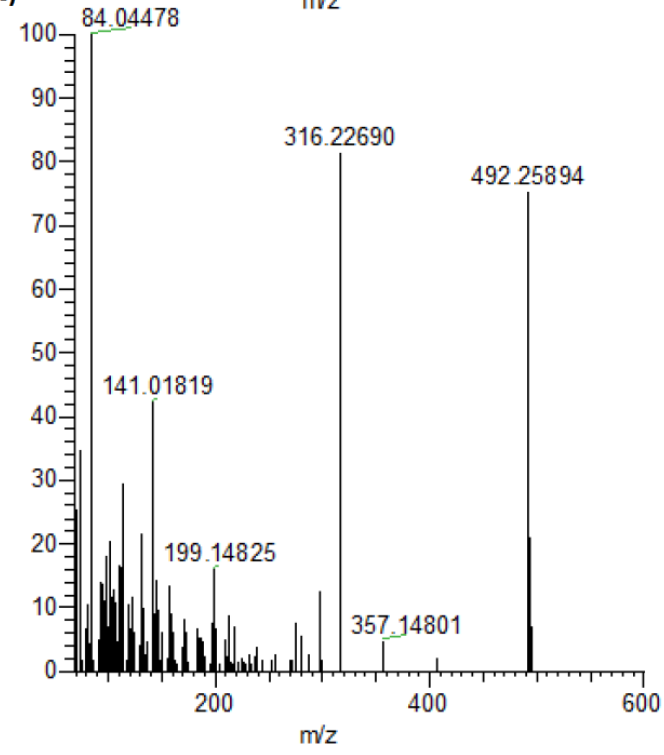
a)


13.70
13.25
5.24
6.70
12.53
14.07
Time (min)

b)

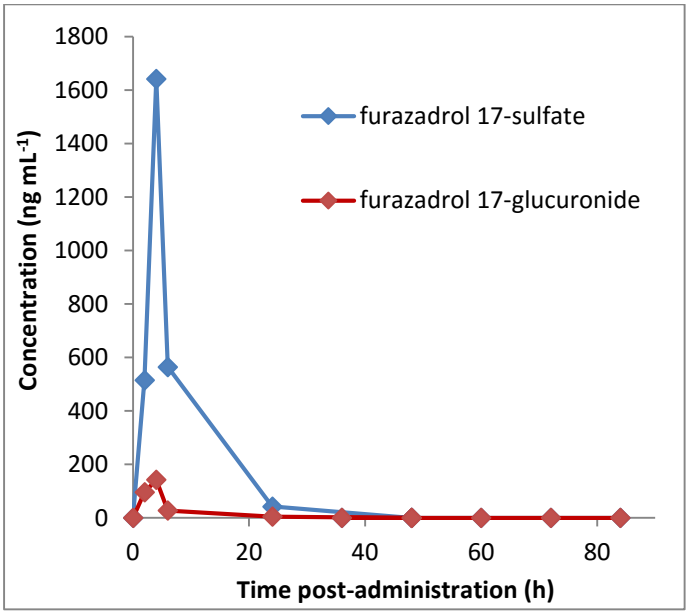

492.25781
316.22626
84.04457
113.02322
274.21542
141.01790
450.24850
m/z

c)


84.04478
316.22690
492.25894
141.01819
199.14825
357.14801
m/z

461

462 **Figure 3. Excretion of major furazadrol metabolites in equine urine**



463

464

Figure 4. Proposed phase I and II metabolism of furazadrol in the horse. [A]Matched to reference material; [B]Structure undefined; [C]Following enzyme hydrolysis with *E. coli* β-glucuronidase; [D]Following enzyme hydrolysis with *P. aeruginosa* arylsulfatase. G, glucuronylation; S, sulfation; O, oxidation; R, reduction; H, hydroxylation.