**Identification of equine *in vitro* metabolites of seven non-steroidal selective androgen receptor modulators for doping control purposes**

**Charlotte Cutler1,2, Marjaana Viljanto1, Polly Taylor1, Pamela Hincks1, Simon Biddle1 and Peter Van Eenoo2**

1LGC Ltd, Fordham, Cambridgeshire, UK

2University of Ghent, Laboratory of Doping Control, Ghent, Belgium

**SARMs, HORSE, IN VITRO, METABOLISM, LC-HRMS**

**Abstract**

Selective androgen receptor modulators, SARMs, are a large class of compounds developed to provide therapeutic anabolic effects with minimal androgenic side effects. A wide range of these compounds are available to purchase online, and thus provide the potential for abuse in sports. Knowledge of the metabolism of these compounds is essential to aid their detection in doping control samples. *In vitro* models allow a quick, cost-effective response where administration studies are yet to be carried out. In this study, the equine phase I metabolism of the non-steroidal SARMs GSK2881078, LGD-2226, LGD-3303, PF-06260414, ACP-105, RAD-140 and S-23 was investigated using equine liver microsomes. Liquid chromatography coupled to a QExactive Orbitrap mass spectrometer allowed identification of metabolites with high resolution and mass accuracy. Three metabolites were identified for both GSK2881078 and LGD-2226, four for LGD-3303 and RAD-140, five for PF-06260414, twelve for ACP-105 and ten for S-23. The equine metabolism of GSK-2881078, LGD-2226, LGD-3303 and PF-06260414 is reported for the first time. Although the equine metabolism of ACP-105, RAD-140and S-23has previously been reported, the results obtained in this study are compared to any published data.

**1 Introduction**

Selective androgen receptor modulators (SARMs) are a large class of structurally diverse compounds that have been developed primarily for androgen replacement therapy. [1] The main aim of their development has been to design compounds that are orally active and interact selectively with the androgen receptor, in order to provide the therapeutic anabolic benefits of traditional anabolic androgenic steroids, but with fewer of the associated androgenic side effects.[1]

A wide range of SARMs have become available to purchase online via uncontrolled sites as supplement products. While many of these compounds are undergoing (or have undergone) clinical evaluation, to date no SARMs have completed full clinical trials. However, due to their potential for abuse in sports, SARMs have been added to the World Anti-Doping Agency (WADA)[2] prohibited list of banned at all times substances, as well as being prohibited in horseracing by the International Federation of Horseracing Authorities (IFHA).[3] Adverse analytical findings (AAFs) for SARMs have been reported in both human[4-6] and equine sports.[7-9] There is therefore an increasing need for doping control laboratories to expand and update their analytical methods to ensure coverage of these novel compounds.

A substantial amount of work has been carried out using mass spectrometric techniques to characterise SARMs, often under varying ionisation and dissociation conditions, to help laboratories to effectively screen for these compounds,[10-14] as well as the publication of semi-quantitative analytical methods for the detection of multiple parent SARMs.[15,16] Additionally, the stability of a selection of parent SARMs compounds has been assessed, in solvent and fortified in urine from different species.[17] However, with urine being the most widely analysed matrix in doping control, it is also of great importance to identify the metabolic fate of these novel compounds, which could potentially increase the chances of detection of their abuse. While administration studies remain the gold standard for determining accurate metabolic profiles, *in vitro* studies provide a quick, cost-effective and ethically viable approach to studying the metabolism of unapproved compounds. Additionally, metabolite reference materials are largely unavailable, and thus the use of *in vitro* samples as metabolite reference materials is permissible according to the International Laboratory Accreditation Cooperation (ILAC) G7 guidance documents.[18] *In vitro* techniques provide the added benefit of considerably cleaner extracts compared to post-administration samples, making them more reliable for use as metabolite reference materials.

Until recently, far more human metabolism studies had been carried out for SARMs compared to equine metabolism studies.[13,19-22] However, increasing numbers of SARM metabolism studies in the equine have been reported over recent years, with both *in vitro* and *in vivo* methodologies being utilised.[23-31] Species specific metabolite profiles are important for identifying the most appropriate targets for analysis in doping control samples, since the metabolic pathways can differ between the species. To date AAFs have almost exclusively been for the parent compounds in equine sports,[8,9] but identification of target metabolites may increase these findings further.

In this study, the equine phase I metabolism of seven non-steroidal SARMs with differing pharmacophores was investigated *in vitro* using equine liver microsomes and liquid chromatography high resolution mass spectrometry (LC-HRMS). These included, GSK28810788, LGD-2226, LGD-3303, PF-06260414, ACP-105, RAD-140 and S-23.

GSK2881078 ((R)-1-(1-(methylsulfonyl)propan-2-yl)-4-(trifluoromethyl)-1*H*-indole-5-carbonitrile) is an indole-carbonitrile based SARM, that has been shown to be well tolerated in elderly men and women, yielding dose-dependent increases in lean mass.[32,33] Its metabolism has recently been investigated in the human following an oral administration,[34] but no equine metabolism information is currently available.

LGD-2226 (6-[Bis-(2,2,2-trifluoro-ethyl)-amino]-4-trifluoro-methyl-1H-quinolin-2-one) is a tissue selective, bi-cyclic quinolone derivative. LGD-2226 has been shown to have anabolic effects on bone and muscle,[35,36] though clinical trials have been discontinued.[37] LGD-3303 (9-Chloro-2-ethyl-1-methyl-3-(2,2,2-triluoro-ethyl)-3*H*-pyrrolo-(3,2-*f*)quinolin-7(6*H*)-one) is another quinolone based SARM that is a highly selective and potent androgen receptor agonist, shown to stimulate muscle anabolism in rats.[38] To the best of the authors’ knowledge, the metabolism of LGD-2226 and LGD-3303 has not been reported in any species.

PF-06260414 (6-[(4R)-tetrahydro-4-methyl-1,1-dioxido-2H-1,2,6-thiadiazin-2-yl]-1-isochinoline carbonitrile) is an isoquinolone-derived SARM that was evaluated in healthy men and was found to have partial agonist activity compared to testosterone.[39] However, like LGD-2226, clinical trials into this compound have since been discontinued.[37] To the best of the authors’ knowledge, its metabolism has not been reported in any species.

ACP-105 (2-Chloro-4-[(3-*endo*)-3-hydroxy-3-methyl-8-azabicyclo[3.2.1]oct-8-yl]-3-methylbenzonitrile) is a tropanol based, selective and potent SARM, with partial agonist activity relative to testosterone.[40] Its equine metabolism has recently been investigated via *in vitro* experiments[31] and oral administration studies.[29,31]

RAD-140 (2-chloro-4-{[(1*R*,2*S*)-1-[5-(4-cyanophenyl)-1,3,4-oxadiazol-2-yl]-2-hydroxypropyl]amino}-3-methylbenzonitrile), also commonly referred to as Testolone, is a phenyl-oxadiazole based SARM that has been shown to have greater anabolic effects compared to testosterone,[41] giving it high potential for abuse in sport. The metabolism of RAD-140 has been investigated in humans[20] and more recently in the equine, both *in vitro* using homogenised horse liver and following an oral administration.[28]

S-23 ((S)-*N-*(4-cyano-3-trifluoromethyl-phenyl)-3-(3-fluoro,4-chlorophenoxy)-2-hydroxy-2-methyl-propanamide) is an arylpropanamide SARM that has been investigated in animal models for use as a male contraceptive.[42] Other arylpropanamides, such as andarine and ostarine, have been widely studied in terms of their metabolism and mass spectral fragmentation.[11,22-25] More recently the equine phase I and II metabolism of S-23 has been investigated *in vitro* using homogenised horse liver.[28]

Although the equine metabolism of ACP-105,[29,31] RAD-140[28] and S-23[28] has previously been reported, the results obtained in this study are compared to any published data and therefore used as positive controls. Such comparisons allow evaluation of different *in vitro* models and assessment of the *in vitro-in vivo* correlation of different structural classes.

**2 Materials and Methods**

**2.1 Chemicals and reagents**

Trizma base, Trizma HCl, potassium dihydrogen orthophosphate, sodium azide, trichloroacetic acid, ammonium acetate and pancreatin were obtained from Sigma Aldrich (Dorset, UK). Methanol (MeOH), acetonitrile (ACN), hexane, ethyl acetate, chloroform and sodium hydroxide (NaOH) were purchased from Fisher Scientific Ltd. (Loughborough, UK). Tert-butyl methyl ether (TBME) was obtained from Honeywell Research Chemicals (Berkshire, UK). Reagent grade water (RG H2O) was prepared using a Triple Red Duo ultrapure water system (Triple Red Laboratory Technology, UK). Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was obtained from Park Scientific (Northampton, UK) and pooled equine liver microsomes were prepared by Quotient Bioresearch (Cardiff, UK). Pancreatin solution was prepared in acetate buffer (1 M, pH 6.6) and RG H2O.

ACP-105, RAD-140 and S-23 were purchased from Toronto Research Chemicals (Ontario, Canada). LGD-2226 and PF-06260414 were purchased from Sigma Aldrich (Dorset, UK). GSK2881078 was purchased from Cayman Chemicals (Michigan, US) and LGD-3303 was purchased from MedChem Express (Sollentuna, Sweden). All compounds were purchased as powders. Stock solutions were prepared at a concentration of 1 mg/mL in methanol (MeOH) and stored at -20 ºC. Benzyldimethylphenylammonium chloride monohydrate (BDPA) was purchased from Acros Organics (Geel, Belgium).

**2.2 *In vitro* incubations with equine liver microsomes**

Prior to use for *in vitro* incubation, the reference standards were analysed using LC-HRMS instrumentation to ensure their purity. The reference materials were checked for any contaminants or interferences, including potential metabolites that might have led to potential misinterpretation of results. No contaminants were found.

*In vitro* incubations were carried out as has been described previously.[27,30] Briefly, drug (at 11 μg/mL), equine liver microsomes (at 1 mg/mL protein) and NADPH regenerating cofactor solution (0.61 mM) were added in TRIS buffer (50 mM, pH 7.4) for a total incubation volume of 0.34 mL. Samples were incubated at 37 ˚C in a water bath for 180 min alongside negative control samples consisting of no-cofactor control, no protein control and a no drug control, to confirm that any metabolites / analytes of interest detected were genuine metabolites of the incubated compound. Following incubation, aliquots (160 μL) were transferred to Eppendorf tubes and quenched by the addition of 225 μL ice-cold acetonitrile (ACN). Quenched aliquots were centrifuged for 10 min at 8,131 g and the supernatant transferred to a separate tube and evaporated to dryness under nitrogen at ambient temperature. Dried samples were reconstituted in 10 µL of MeOH and 90 µL of RG H2O with BDPA injection marker.

**2.3 LC-HRMS for metabolite identification**

Instrumental analysis was performed using a Thermo Scientific Ultimate 3000 UHPLC system coupled to a Thermo Scientific Q Exactive Orbitrap mass spectrometer, equipped with a heated electrospray (HESI-II) ionisation source. The HESI-II source conditions included sheath gas, of 40 arbitrary units (AU); auxiliary gas of 10 AU; spray voltage of 3.75 kV; heater temperature of 425 °C; ion transfer capillary temperature of 320 °C; and S-lens RF level of 40.0. The mass spectrometer was initially operated in both positive and negative ionisation modes, in full-scan (FS) acquisition mode. FS analysis was subsequently followed by tandem mass spectrometry (MS/MS) for the parent drug, and the metabolites identified from the FS spectra. The FS data was acquired with a resolution of 70,000 and a scan range of 90 – 700 *m/z*, whilst the MS/MS data was acquired with a resolution of 17,500. MS/MS data was acquired with a stepped collision energy (CE) of 15, 35, 50 eV; if minimal fragmentation was observed increased CEs were utilised on a compound by compound basis. Software for data evaluation was Thermo Fisher Xcalibur Version 2.2.

Gradient elution was performed using a Phenomenex HST Luna (100 x 2.1 mm, 3 µm) reversed phased column and mobile phase solutions of 0.1% acetic acid in RG H2O (A), 0.1% acetic acid in MeOH (B) and 20 mM acetate buffer (C). The gradient was initiated at 90% A (0% C) where it was held for 2 min, before ramping to 2% A (75% C) by 3.5 min. The gradient was held until 4.5 min, before altering to 2% A (0% C) by 4.51 min, and then returning to initial gradient conditions 90% A (0% C) by 5 min where it was held for the remainder of the run. Initial flow rate was 385 µL/min; at 3.5 min the flow rate was increased to 410 µL/min, where it was held until 4 min. The flow rate was then increased to 510 µL/min by 4.2 min where it was held until 5 min before returning to the initial flow rate of 385 µL/min by 5.5 min. Column temperature was maintained at 40 °C throughout the run. The injection volume was 5 µL.

**2.4 Identification of metabolites**

Metabolites were identified in the *in vitro* incubates using a two-step process, initially with FS accurate mass analysis and data mining for postulated metabolites, followed by targeted MS/MS analysis on any metabolites observed. FS accurate mass analysis of the *in vitro* sample was carried out in positive and negative ionisation modes. Any peaks identified in the extracted ion chromatograms (EIC) were investigated by interpretation of the acquired spectra and compared with controls. Following tentative identification of the *in vitro* metabolites, MS/MS analysis was used to further investigate and characterise the metabolite structures, and the most appropriate ionisation mode was selected.

**3 Results and Discussion**

**3.1 *In vitro* metabolite identification**

**3.1.1 GSK2881078**

GSK2881078 and its observed metabolites were best detected under positive ionisation. GSK2881078 was detected with a theoretical protonated accurate mass [M+H]+ of *m/z* 331.0723. The dissociation pathways of GSK2881078 using positive ESI-MS/MS has been reported previously, and the fragmentation observed in this study was consistent with what was reported (Table 1).[14] The major fragment ions at theoretical *m/z* 311.0660, 251.0791 and 231.0728 were proposed to be formed via the elimination of hydrogen fluoride (-HF), methanesulfinic acid and the combination of both, respectively (Table 1 and Figure1). Additionally, the ions at theoretical *m/z* 211.0478 and 210.0399 were proposed to represent protonated 4-(trifluoromethyl)-1*H*-indole-5-carbonitrile and the corresponding radical cation. A further elimination of -HF from the *m/z* 211.0478 ion resulted in an ion at theoretical *m/z* 191.0415*.*

Three phase I metabolites (M1a-c) of GSK2881078 were identified *in vitro* (Table 1 and Figure 1) and all were formed via mono-hydroxylation. Metabolite M1a was the most abundant metabolite detected *in vitro* followed by M1b. M1a and M1b were postulated to be positional isomers as the observed spectra were almost identical, with hydroxylation at the indole ring moiety. This was supported by fragment ions at theoretical *m/z* 267.0740, 227.0427, 226.0349 and 207.0364, representing the parent ring fragments with a mass shift of 15.995 amu. Metabolite M1c was postulated to be hydroxylated on the side chain. This was supported by the absence of the parent fragment at theoretical *m/z* 121.0318, and the presence of the ion at theoretical *m/z* 249.0634. The latter is thought to represent the loss of the methanesulfinic acid group, but with a proton rearrangement likely caused by the presence of a hydroxylation on the side chain.

Table 1 – Details of parent GSK2881078 and all identified metabolites in the *in vitro* sample. MS/MS analysis using positive ionisation with stepped CE 15, 35, 50

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Analyte ID**  **(Elemental composition)**  **Biotransoformation** | **Observed precursor ion**  **(*m/z*)**  **(mass error, mmu)** | **tR**  **(min)** | **Observed product ions** | | **FS instrument signal intensity**  **(Relative intensity %)** |
| **m/z**  **(mass error, mmu)** | **Elemental composition** |
| **GSK2881078**  (C14H13N2O2SF3)  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML2e807062.PNG | 331.0720\*  (0.30) | 2.94 | 311.0659 (0.30)  251.0788 (0.30)  231.0727 (0.17)  211.0477 (0.11)  210.0398 (0.15)  191.0415 (0.01)  121.0320 (0.18) | C14H13O2N2SF2  C13H10N2F3  C13H9N2F2  C10H6N2F3  C10H5N2F3  C10H5N2F2  C4H9O2S | 4.78e8 (N/A) |
| **M1a**  (C14H13N2O3SF3)  Mono-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML2e815600.PNG | 347.0669\*  (0.24) | 2.22 | 327.0608 (0.19)  307.0545 (0.25)  267.0738 (0.21)  227.0425 (0.20)  226.0347 (0.14)  207.0364 (0.02)  121.0319 (0.16) | C14H13O3N2SF2  C14H12O3N2SFC13H10ON2F3  C10H6ON2F3  C10H5ON2F3  C10H5ON2F2 C4H9O2S | 3.66e7 (100.0) |
| **M1b**  (C14H13N2O3SF3)  Mono-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML2e815600.PNG | 347.0670\*  (0.21) | 2.42 | 327.0608 (0.19)  307.0548 (0.09)  267.0739 (0.12)  227.0425 (0.16)  226.0348 (0.04)  207.0365 (0.00)  121.0321 (0.28) | C14H13O3N2SF2 C14H12O3N2SF  C13H10ON2F3  C10H6ON2F3 C10H5ON2F3  C10H5ON2F2  C4H9O2S | 1.52e7 (41.5) |
| **M1c**  (C14H13N2O3SF3)  Mono-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML2e825238.PNG | 347.0669\*  (0.24) | 2.60 | 327.0609 (0.07)  267.0728 (0.43)  249.0633 (0.09)  237.0632 (0.16)  229.0572 (0.01)  180.0681 (0.08) | C14H13O3N2SF2  C13H10ON2F3  C13H8N2F3  C12H8N2F3  C13H7N2F2 C12H8N2 | 5.24e6 (14.3) |

\*Base peak ion of observed spectra

As a comparison, four mono-hydroxylated metabolites of GSK2881078 were identified in human urine following an oral administration.[34] Three of the identified metabolites were postulated to be hydroxylated at the indole ring, with the fourth hydroxylated at the side chain. No phase II metabolites were detected, suggesting that further biotransformation was not required for excretion of this compound. While the metabolic pathways in the equine differ, it is possible that GSK2881078 may also be excreted mainly as phase I metabolites in the equine. However, an equine administration study would be required to support this theory.

**3.1.2 LGD-2226**

LGD-2226 and its identified metabolites were best detected using negative ionisation, however positive ionisation was also effective. LGD-2226 was detected with a theoretical deprotonated accurate mass [M-H]- of *m/z* 391.0498. The major fragment ions observed at theoretical *m/z* 308.0389 and 239.0437 were postulated to be formed by the sequential losses of trifluoroethyl (-83.0109 amu) and trifluoromethyl (-68.9955 amu) radicals (Table 2 and Figure 2). This dissociation pattern for LGD-2226 has been previously observed under positive electrospray ionisation.[10]

Three phase I metabolites of LGD-2226 were identified (Table 2 and Figure 2), postulated to be formed by mono- and bis-*N-*dealkylation of the trifluoroethyl moieties (M1 and M2, respectively), as well as via reduction of the keto group (M3). The most abundant metabolite was the mono-*N-*dealkylated M1, detected with a theoretical [M-H]- of *m/z* 309.0468. The base peak ion of the observed MS/MS spectrum was at theoretical *m/z* 226.0359, thought to represent the loss of a trifluoroethyl radical (-83.0109 amu). The second most abundant metabolite detected was the bis-*N-*dealkylated metabolite M2, detected with a theoretical [M-H]- of *m/z* 227.0437. This metabolite was very stable, with the deprotonated molecular ion representing the base peak, with only two very minor fragment ions observed at theoretical *m/z* 199.0488 and 179.0426, postulated to represent sequential losses of -CO and -HF. The keto-reduced metabolite M3 was detected with a theoretical deprotonated accurate mass of [M-H]- of *m/z* 393.0654, representing a mass increase of 2.0156 amu from the deprotonated parent compound. Furthermore, the observed fragment ions at theoretical *m/z* 310.0546 and 241.0594, representing the same losses as observed for the parent compound, support the proposed identity of the metabolite.

Since this was the first reported metabolism study for LGD-2226 in any species, no data comparisons could be made.

Table 2 - Details of parent LGD-2226 and all identified metabolites in the *in vitro* sample. MS/MS analysis using negative ionisation with stepped CE 15, 35, 50

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Analyte ID**  (Elemental composition)  Biotransformation | **Observed precursor ion**  **(*m/z*)**  **(mass error, mmu)** | **tR**  **(min)** | **Observed product ions** | | **FS instrument signal intensity**  **(Relative intensity %)** |
| **m/z**  **(mass error, mmu)** | **Elemental composition** |
| **LGD-2226**  (C14H9N2OF9)  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML2e88cfd0.PNG | 391.0495  (0.76) | 3.74 | 308.0392\* (1.26)  239.0433 (0.66) | C12H6ON2F6  C11H6ON2F3 | 2.27e9 (N/A) |
| **M1**  (C12H8N2OF6)  *N-*dealkylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML2e89ba5d.PNG | 309.0468  (1.13) | 3.26 | 226.0354\* (0.57)  206.0288 (0.16) | C10H5ON2F3 | 1.02e8 (100.0) |
| **M2**  (C10H7N2OF3)  Bis-*N-*dealkylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML2e8b14cc.PNG | 227.0432\*  (0.53) | 2.31 | 199.0482 (0.11)  179.0416 (0.11) | C9H6N2F3  C9H5N2F2 | 2.54e7 (24.9) |
| **M3**  (C14H11N2OF9)  Keto-reduction  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML2e8beddb.PNG | 393.0654  (0.98) | 3.22 | 310.0550\* (1.43)  241.0592 (0.90) | C12H8ON2F6  C11H8ON2F3 | 5.02e6 (4.9) |

\*Base peak ion in observed spectra

**3.1.3 LGD-3303**

LGD-3303 and its identified metabolites were best detected under positive ionisation. LGD-3303 was detected with a theoretical protonated accurate mass [M+H]+ *m/z* 343.0820. LGD-3303 is relatively stable, and even with an increased energy spread (CE of 33, 55, 70 eV) utilised to encourage fragmentation, the molecular ion remained the base peak of the spectrum. The fragment at theoretical *m/z* 259.0633 was postulated to be formed by the loss of the trifluoroethyl group, an additional loss of -CH2 resulted in the ion at theoretical *m/z* 245.0476 (Table 3 and Figure 3). Sequential losses of –HCl, -H2O and -C2H4 were postulated to result in the ions at theoretical *m/z* 307.1053, 289.0947 and 261.0634 respectively.

Four phase I metabolites of LGD-3303 were identified *in vitro* (Table 3 and Figure 3), two of which were postulated to represent mono-hydroxylated LGD-3303 (M1a and 1b) and two to represent di-hydroxylated metabolites (M2a and 2b). The most abundant metabolite detected was M1a. This was postulated to be hydroxylated within the core ring structure due to the ions at theoretical *m/z* 344.0534, and 329.0299, representing losses of the unchanged methyl and ethyl side-chains, respectively, and the ion at theoretical *m/z* 261.0425, representing the loss of the tri-fluoroethyl moiety and methyl group. However, the exact location of the transformation was not determined due to the stability of the core ring structure. The spectrum acquired for M1b, contained ions at theoretical *m/z* 275.0582 and 261.0425, thought to represent the loss of the trifluoroethyl moiety alone, and in combination with the methyl side chain, respectively. Additionally, the ion at theoretical *m/z* 330.0377 likely represented the loss of the unchanged ethyl side chain, thus M1b was also postulated to be hydroxylated within the core ring structure. The MS/MS spectrum of the di-hydroxylated metabolite M2a showed a fragment ion at theoretical *m/z* 344.0534, representing a loss of -CH2OH, and thus suggesting a hydroxylation at the methyl side-chain. The spectrum of M2b contained an ion at theoretical *m/z* 295.0689, thought to represent the loss of -C2H4O and -HCl, and thus suggesting a hydroxylation at the ethyl side-chain. The exact location of the second hydroxylation was not determined for either compound, but was postulated to occur within the core ring structure, as for the mono-hydroxylated metabolites.

Since this was the first reported metabolism study for LGD-3303 in any species, it was not possible to make any data comparisons.

Table 3 - Details of parent LGD-3303 and all identified metabolites in the *in vitro* sample. MS/MS analysis using positive ionisation with stepped CE 33, 55, 70

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Analyte ID**  (Elemental composition)  Biotransformation | **Observed precursor ion**  **(*m/z*)** | **tR**  **(min)** | **Observed product ions** | | **FS instrument signal intensity**  **(Relative intensity %)** |
| **m/z**  **(mass error, mmu)** | **Elemental composition** |
| **LGD-3303**  (C16H14N2OClF3)  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML2e957504.PNG | 343.0815\*  (0.44) | 4.07 | 328.0582 (0.27)  307.1049 (0.32)  293.0894 (0.26)  289.0945 (0.23)  261.0631 (0.30)  259.0641 (0.85)  245.0473 (0.36)  225.1021 (0.17)  223.0863 (0.24)  205.0760 (0.07) | C15H12ON2ClF3  C16H14ON2F3  C15H12ON2F3  C16H12N2F3  C14H8N2F3  C14H12ON2Cl  C13H10ON2Cl  C14H13ON2  C14H11ON2  C14H9N2 | 1.35e9 (N/A) |
| **M1a**  (C16H14N2O2ClF3)  Mono-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML2e968385.PNG | 359.0762\*  (0.67) | 3.42 | 344.0529 (0.44)  329.0294 (0.47)  261.0417 (0.84)  233.0473 (0.32)  211.0863 (0.24) | C15H12O2N2ClF3  C14H9O2N2ClF3  C13H10O2N2Cl  C12H10ON2Cl  C13H11ON2 | 7.04e7 (100.0) |
| **M1b**  (C16H14N2O2ClF3)  Mono-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML2e968385.PNG | 359.0767\*  (0.20) | 3.94 | 330.0386 (0.86)  309.0844 (0.19)  275.0582 (0.05)  261.0428 (0.23)  259.0628 (0.46)  245.0470 (0.64)  225.1021 (0.11)  207.0914 (0.30) | C14H10O2N2ClF3  C15H12O2N2F3  C14H12O2N2Cl  C13H10O2N2Cl  C14H12ON2Cl  C13H10ON2Cl  C14H13ON2  C14H11N2 | 2.77e6 (3.9) |
| **M2a**  (C16H14N2O3ClF3)  Bis-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML2e97ccf3.PNG | 375.0713\*  (0.52) | 2.72 | 344.0531 (0.26)  329.0297 (0.23)  261.0422 (0.17)  233.0475 (0.12)  211.0865 (0.10) | C15H12O2N2ClF3  C14H9O2N2ClF3  C13H10O2N2Cl  C12H10ON2Cl  C13H11ON2 | 1.28e7 (18.2) |
| **M2b**  (C16H14N2O3ClF3)  Bis-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML2e985d6f.PNG | 375.0713  (0.52) | 2.83 | 339.0949 (0.22)  311.0999\* (0.24)  295.0690 (0.08)  283.0687 (0.23)  265.0944 (0.32)  261.0639 (0.52)  238.0839 (0.05)  227.0812 (0.26) | C16H14O3N2F3  C15H14O2N2F3  C14H10O2N2F3  C13H10O2N2F3  C14H12N2F3  C14H8N2F3  C13H11NF3  C13H11O2N2 | 1.03e7 (14.6) |

\*Base peak ion in observed spectra

**3.1.4 PF-06260414**

While both positive and negative ionisation were effective for the analysis of PF-06260414 and all identified metabolites, negative ionisation provided detection with greater signal intensities. PF-06260414 was detected with a theoretical deprotonated accurate mass [M-H]- of *m/z* 301.0764. The observed fragmentation under negative electrospray ionisation followed equivalent dissociation routes as previously reported in positive mode.[14] The base peak ion of the spectrum was observed at theoretical *m/z* 208.0880, suggested to represent the deprotonated 6-(propylideneamino)isoquinoline-1-carbonitrile (Table 4 and Figure 4). A low abundance ion at theoretical *m/z* 237.1145 was expected to be produced by the loss of the sulfonyl residue (-63.9619 amu). Ions observed at theoretical *m/z* 195.0676 and 168.0567 have been proposed to form via the elimination of propene from the *m/z* 237.1145 ion and subsequent loss of HCN, respectively.[14]

Five phase I metabolites of PF-06260414 were identified *in vitro*, postulated to be formed via mono-hydroxylation (M1a and 1b), bis-hydroxylation (M2a and M2b) and thiadiazinan-bis-*N-*dealkylation (M3). The most abundant metabolite was the thiadiazinan-bis-*N-*desalkyl metabolite M3, detected with a theoretical deprotonated accurate mass [M-H]- of *m/z* 247.0295. The postulated structure of M3 is shown in Table 4. Only two product ions were observed for M3 at theoretical *m/z* 168.0567 and 77.9655, representing two halves of the metabolite; deprotonated 6-aminoisoquinoline-1-carbonitrile and sulfonylazanide.

The mono-hydroxylated metabolite M1b was the second most abundant metabolite detected *in vitro.* Both M1a and 1b were detected with a theoretical deprotonated accurate mass [M-H]- of *m/z* 317.0713. The ion observed at theoretical *m/z* 107.9760 detected in the spectra of both M1a and 1b, suggested hydroxylation at the thiadiazynyl ring, at one of the nitrogen adjacent carbons. However, the exact location of hydroxylation was not determined (Table 4 and Figure 4). Both M2a and 2b were detected with very low signal with a theoretical deprotonated mass [M-H]- of *m/z* 333.0662, and MS/MS information could only be obtained for M2a. The ions detected in negative mode with the theoretical *m/z* 164.0023 and 149.9866 suggest di-hydroxylation of the thiadiazynyl ring. (Table 4 and Figure 4).

No data comparisons were possible for the metabolism of PF-06260414, since it has not been investigated previously in any species.

Table 4 - Details of parent PF-06260414 and all identified metabolites in the *in vitro* sample. MS/MS analysis using negative ionisation with stepped CE 15, 35, 50

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Analyte ID**  (Elemental composition)  Biotransformation | **Observed precursor ion**  **(*m/z*)**  **(mass error, mmu)** | **tR**  **(min)** | **Observed product ions** | | **FS instrument signal intensity**  **(Relative intensity %)** |
| **m/z**  **(mass error, mmu)** | **Elemental composition** |
| **PF-06260414**  (C14H14N4O2S)  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML2ea6bc41.PNG | 301.0766  (1.26) | 2.76 | 237.1142 (0.77)  208.0874\* (0.47)  195.0670 (0.44)  181.0763 (0.30)  168.0558 (0.14)  153.0448 (0.03)  91.9798 (0.31)  64.9688 (0.38) | C14H13N4  C13H10N3  C11H7N4  C12H9N2  C10H6N3  C10H5N2  CH2O2NS  HO2S | 2.83e8 (N/A) |
| **M1a**  (C14H14N4O3S)  Mono-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML61304c4a.PNG | 317.0714\* (1.15) | 2.39 | 237.1142 (0.74)  208.0875 (0.54)  168.0557 (0.11)  136.0062 (0.08)  107.9747 (0.27)  79.9559 (0.35) | C14H13N4  C13H10N3  C10H6N3  C3H6O3NS  CH2O3NS  SO3 | 1.44e7 (11.8) |
| **M1b**  (C14H14N4O3S)  Mono-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML61304c4a.PNG | 317.0713  (0.97) | 2.52 | 237.1142 (0.59)  208.0874 (0.48)  168.0557\* (0.09)  148.0063 (0.02)  136.0062 (0.08)  107.9747 (0.26)  95.9747 (0.31)  79.9559 (0.38) | C14H13N4  C13H10N3  C10H6N3  C4H6O3NS  C3H6O3NS  CH2O3NS  H2NO3S  SO3 | 1.03e8 (84.4) |
| **M2a**  (C14H14N4O4S)  Bis-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML325ab36f.PNG | 333.0665\* (1.30) | 2.31 | 182.0706 (0.68)  168.0558 (0.16)  164.0014 (0.22)  149.9857 (0.11)  107.9745 (0.51)  95.9747 (0.26)  80.9638 (0.32)  79.9559 (0.40) | C11H8N3  C10H6N3  C4H6O4NS  C3H4O4NS  CH2O3NS  H2O3NS  HSO3  SO3 | 4.93e6 (4.0) |
| **M2b\*\***  (C14H14N4O4S)  Bis-hydroxylation |  | 2.61 | - | - | 1.60e6 (1.3) |
| **M3**  (C10H8N4O2S)  Thiadiazinan-Bis-*N-*dealkylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML325b1443.PNG | 247.0292 (0.75) | 2.29 | 168.0557 (0.51)  77.9640\* (0.95) | C10H6N3  NO2S | 1.22e8 (100.0) |

\*Base peak ion in observed spectra

\*\*Not detected using MS/MS

**3.1.5 ACP-105**

ACP-105 and its metabolites were best detected under positive ionisation, with ACP-105 detected with a theoretical protonated accurate mass [M+H]+ of *m/z* 291.1257. The observed fragmentation of ACP-105 in this study was consistent with what has previously been reported,[12,29,31] with the major fragment ions formed through the dissociation of the tropanol moiety.

Twelve phase I metabolites of ACP-105 were identified *in vitro* (Table 5), formed via mono-hydroxylation (M1a-1c), bis-hydroxylation (M2a-2d), mono-oxidation (M3a and 3b) and as a combination of mono-hydroxylation and mono-oxidation (M4a-4c). All of the observed metabolites have been reported following the oral administrations of ACP-105 to the equine.[29,31]

The most abundant metabolites detected *in vitro* were three mono-hydroxylated metabolites of ACP-105; detected with the theoretical [M+H]+ of *m/z* 307.1208. Based on the observed MS/MS fragmentation, these were postulated to be hydroxylated at different positions of the tropanol moiety, which was consistent with the previous studies.[29,31,43] These mono-hydroxylated metabolites were also the most abundant analytes detected in equine urine,[29,31] however, they were excreted primarily as glucuronide conjugates.[31] The administration study reported by Broberg *et al*[31] also presented plasma data, and suggested glucuronide conjugated mono-hydroxylated metabolites as recommended targets in plasma alongside a bis-hydroxylated and mono-oxidised metabolite.

Although several phase I metabolites that were not observed *in vitro* were identified in equine urine following oral administrations,[29,31] the most abundant phase I metabolites detected *in vivo* andrepresenting the best targets for doping control,corresponded to the most abundant *in vitro* metabolites in this study. This shows a good correlation between *in vitro* and *in vivo* phase I metabolites for this compound and demonstrates that the information obtained *in vitro* would be invaluable were *in vivo* studies not possible. This information shows the applicability of *in vitro* produced material for use as reference materials.

Table 5 - Details of parent ACP-105 and all identified metabolites in the *in vitro* sample. MS/MS analysis using positive ionisation with stepped CE 15, 35, 50

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Analyte ID**  (Elemental composition)  Biotransformation | **Observed precursor ion**  **(*m/z*)**  **(mass error, mmu)** | **tR**  **(min)** | **Observed product ions** | | **FS instrument signal intensity**  **(Relative intensity %)** |
| **m/z**  **(mass error, mmu)** | **Elemental composition** |
| **ACP-105**  (C16H19ClN2O)  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML42743f9b.PNG | 291.1258\* (0.10) | 3.96 | 273.1147 (0.55)  233.0840 (0.03)  197.1073 (0.03)  193.0527 (0.02)  179.0370 (0.06)  177.0231 (0.07)  167.0369 (0.11)  107.0859 (0.33)  79.0549 (0.65) | C16H18N2Cl  C13H14N2Cl  C13H13N2  C10H10N2Cl  C9H8N2Cl  C9H6N2Cl  C8H8N2Cl  C8H11  C6H7 | 2.14e8 (N/A) |
| **M1a**  (C16H19ClN2O2)  Mono-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML4274cbe3.PNG | 307.1209  (0.13) | 3.07 | 289.1101 (0.08)  231.0682\* (0.16)  195.0915 (0.16)  189.0215 (0.07)  142.0524 (0.11)  79.0549 (0.72) | C16H18ON2Cl  C13H12N2Cl  C13H11N2  C10H6N2Cl  C9H6N2  C6H7 | 3.04e8 (97.1) |
| **M1b**  (C16H19ClN2O2)  Mono-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML42763ff9.PNG | 307.1207\*  (0.08) | 3.35 | 289.1098 (0.45)  271.0994 (0.22)  233.0839 (0.09)  193.0528 (0.06)  179.0371 (0.01)  167.0371 (0.01)  142.0524 (0.11)  105.0702 (0.34)  79.0549 (0.63) | C16H18ON2Cl  C16H16N2Cl  C13H14N2Cl  C10H10N2Cl  C9H8N2Cl  C8H8N2Cl  C9H6N2  C8H9  C6H7 | 3.13e8 (100.0) |
| **M1c**  (C16H19ClN2O2)  Mono-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML427698f1.PNG | 307.1207\*  (0.05) | 3.65 | 289.1102 (0.01)  207.0318 (0.14)  179.0370 (0.09)  167.0371 (0.01)  142.0526 (0.03) | C16H18ON2Cl  C10H8ON2Cl  C9H8N2Cl  C8H8N2Cl  C9H6N2 | 6.76e7 (21.6) |
| **M2a**  (C16H19ClN2O3)  Bis-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML4276fd6e.PNG | 323.1157  (0.01) | 2.67 | 305.1051 (0.06)  247.0629 (0.34)  231.0682\* (0.14)  195.0917 (0.04)  189.0214 (0.02)  142.0526 (0.02) | C16H18O2N2Cl  C13H12ON2Cl  C13H12N2Cl  C13H11N2  C10H6N2Cl  C9H6N2 | 4.63e7 (14.8) |
| **M2b**  (C16H19ClN2O3)  Bis-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML4276fd6e.PNG | 323.1155  (0.16) | 2.85 | 305.1057 (0.61)  287.0942 (0.33)  247.0630\* (0.31)  231.0682 (0.19)  229.0526 (0.15)  194.0839 (0.07)  179.0603 (0.11)  142.0525 (0.01) | C16H18O2N2Cl  C16H16ON2Cl  C13H12ON2Cl  C13H12N2Cl  C13H10N2Cl  C13H10N2  C12H7N2  C9H6N2 | 5.40e7 (17.3) |
| **M2c**  (C16H19ClN2O3)  Bis-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML4276fd6e.PNG | 323.1159  (0.24) | 2.99 | 305.1047 (0.45)  287.0945 (0.03)  247.0632\* (0.10)  231.0685 (0.19)  207.0322 (0.18)  179.0371 (0.03)  142.0529 (0.31) | C16H18O2N2Cl  C16H16ON2Cl  C13H12ON2Cl  C13H12N2Cl  C10H8ON2Cl  C9H8N2Cl  C9H6N2 | 4.35e6 (1.4) |
| **M2d**  (C16H19ClN2O3)  Bis-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML4276fd6e.PNG | 323.1154\*  (0.31) | 3.11 | 305.1046 (0.52)  287.0944 (0.21)  247.0631 (0.14)  231.0682 (0.17)  179.0370 (0.09)  167.0370 (0.08)  142.0526 (0.09) | C16H18O2N2Cl  C16H16ON2Cl  C13H12ON2Cl  C13H12N2Cl  C9H8N2Cl  C8H8N2Cl  C9H6N2 | 5.94e6 (1.9) |
| **M3a**  (C16H17ClN2O)  Mono-oxidation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML5676348d.PNG | 289.1100  (0.23) | 3.67 | 271.0988 (0.83)  207.0319 (0.09)  193.0531 (0.42)  179.0370\* (0.05)  167.0370 (0.02)  142.0528 (0.20)  105.0702 (0.31) | C16H16N2Cl  C10H8ON2Cl  C10H10N2Cl  C9H8N2Cl  C8H8N2Cl  C9H6N2  C8H9 | 3.94e6 (1.3) |
| **M3b**  (C16H19ClN2O3)  Mono-oxidation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML5676348d.PNG | 289.1105\*  (0.29) | 3.94 | 207.0321 (0.15)  177.0214 (0.04) | C10H8ON2Cl  C9H6N2Cl | 8.55e5 (0.3) |
| **M4a**  (C16H17ClN2O)  Mono-hydroxylation + mono-oxidation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML5679c7a8.PNG | 305.1055  (0.40) | 3.05 | 287.0946 (0.07)  245.0839 (0.06)  229.0527\* (0.02)  194.0840 (0.15)  179.0604 (0.02)  142.0527 (0.12) | C16H16ON2Cl  C14H14N2Cl  C13H10N2Cl  C13H10N2  C12H7N2  C9H6N2 | 1.10e7 (3.5) |
| **M4b**  (C16H17ClN2O)  Mono-hydroxylation + mono-oxidation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML5679c7a8.PNG | 305.1052\*  (0.06) | 3.26 | 231.0682 (0.11)  207.0319 (0.05)  179.0371 (0.06)  177.0216 (0.24)  142.0524 (0.18) | C13H12N2Cl  C10H8ON2Cl  C9H8N2Cl  C9H6N2Cl  C9H6N2 | 6.86e6 (2.2) |
| **M4c\*\***  (C16H17ClN2O)  Mono-hydroxylation + mono-oxidation | - | 3.49 | - | - | 1.89e6 (0.6) |

\*Base peak ion in observed spectra

\*\*Not detected using MS/MS

**3.1.6 RAD-140**

Under positive ionisation RAD-140 was detected with a theoretical protonated accurate mass [M+H]+ of *m/z* 394.1065. Under negative ionisation extensive in-source fragmentation was observed resulting in the loss of the acetaldehyde moiety (-CH3COH), as reported previously.[20] In the current study, the best detection of RAD-140 was achieved when using negative ionisation and targeting the in-source fragment with a theoretical [M-H]- of *m/z* 348.0661. The MS/MS fragmentation observed using both positive and negative ionisation was consistent with what has been reported previously.[12,20,28]

Four phase I metabolites of RAD-140 were identified using this *in vitro* system, formed via mono-hydroxylation (M1), amide hydrolysis (M2) and combinations thereof (M3a and 3b) (Table 6). Both positive and negative ionisation modes were required for identification of metabolites of RAD-140, with M3a and 3b only detected using negative ionisation. The most abundant metabolite observed *in vitro* in this study was M1, which was best detected under negative ionisation when targeting the in-source fragment, detected with a theoretical deprotonated accurate mass [M-H]- of *m/z* 364.0601. An equivalent metabolite was reported in human urine,[20] with the in-source fragment also recommended as the analytical target. Interestingly, no mono-hydroxylated metabolites of RAD-140 were reported following incubation with homogenised equine liver or equine oral administration,[28] highlighting a difference between these models. However, it was unclear whether in-source fragmentation was monitored.

All of the other metabolites identified in this study (M2, M3a and 3b) were reported unconjugated in equine urine following oral administration of RAD-140, with M3a and 3b also detected as sulphate conjugates. Only unconjugated and glucuronide conjugated parent RAD-140 was detected in plasma.[28] The fragmentation observed in this study was consistent with what has been reported, suggesting equivalent metabolites. One of the hydroxylated amide hydrolysis metabolites was also detected as a sulfate conjugate in urine, which was detected for the longest time period after administration in all of the horses.[28] Unconjugated parent RAD-140 gave the longest detection time in equine plasma.

Table 6 - Details of parent RAD-140 and all identified metabolites in the *in vitro* sample. MS/MS analysis using both positive and negative ionisation with stepped CE 15, 35, 50

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Analyte ID**  (Elemental composition)  Biotransformation | **Observed precursor ion**  **(*m/z*)**  (Polarity) | **tR**  **(min)** | **Observed product ions** | | **FS instrument signal intensity**  **(Relative intensity %)** |
| **m/z**  **(mass error, mmu)** | **Elemental composition** |
| **RAD-140**  (C20H16N5O2Cl)  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML2e9faeca.PNG | 394.1054 (1.64)  (+ve) | 3.35 | 223.0630 (0.86)  205.0525 (0.73)  172.0502 (0.86)  170.0836\* (0.81)  169.0758 (0.73) | C11H12ON2Cl  C11H10N2Cl  C9H6ON3  C11H10N2  C11H9N2 | 1.31e8 (N/A) |
| **RAD-140 in-source fragment**  (C18H11N5OCl)  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML57531cbe.PNG | 348.0653 (0.68)  (-ve) | 3.35 | 321.0550 (0.68)  175.0059 (0.12)  170.0349\* (0.02)  127.0289 (0.21) | C17H10ON4Cl  C9H4N2Cl  C9H4ON3  C8H3N2 | 6.62e8 (N/A) |
| **M1 (in-source fragment)**  (C18H11N5O2Cl)  Mono-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML5755e402.PNG | 364.0608 (0.63)  (-ve) | 2.95 | 193.0166 (0.22)  170.0349\* (0.50)  127.0289 (0.71) | C9H6ON2Cl  C9H4ON3  C8H3N2 | 5.66e7 (100.0) |
| **M2**  (C8H7ClN2)  Amide hydrolysis  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML576358a5.PNG | 167.0369\* (0.20)  (+ve) | 2.82 | 131.0604 (0.50)  104.0498 (0.23)  77.0392 (0.07) | C8H7N2  C7H6N  C6H5 | 5.54e6 (9.8) |
| **M3a**  (C8H7ClN2O)  Amide hydrolysis and mono-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML57653b94.PNG | 181.0165\* (0.37)  (-ve) | 2.37 | 145.0395 (0.67)  118.0285 (0.81)  90.0333 (1.02) | C8H5ON2  C7H4ON  C6H4N | 3.84e6 (6.8) |
| **M3b**  (C8H7ClN2O)  Amide hydrolysis and mono-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML57653b94.PNG | 181.0165\* (0.37)  (-ve) | 2.77 | 145.0396 (0.61)  118.0284 (0.89)  90.0335 (0.85 | C8H5ON2  C7H4ON  C6H4N | 2.29e6 (4.0) |

\*Base peak ion in observed spectra

**3.1.7 S-23**

As has been reported for other arylpropionamide SARMs,[11,22,24] negative ionisation provided the best detection of S-23 and its *in vitro* metabolites. S-23 was detected with a theoretical deprotonated molecular mass [M-H]- of *m/z* 415.0477. The observed dissociation of S-23 was consistent with structurally similar arylpropionamide SARMs,[11,22,24] and the MS/MS fragments observed reflected those reported previously.[28]

Ten phase I metabolites were identified for S-23 using this *in vitro* system (Table 7), formed via mono-hydroxylation (M1a-1d), bis-hydroxylation (M2), amide hydrolysis (M3 and M6), a combination of mono- and bis-hydroxylation with amide hydrolysis (M4 and M5, respectively) and dephenylation (M7). The most abundant metabolite was M3, corresponding to 4-amino-2-(trifluoromethyl)benzonitrile, which was detected with the theoretical [M-H]- of *m/z* 185.0332. This metabolite is equivalent to an abundant metabolite of ostarine that has been detected in equine urine following its intravenous (IV) administration.[24] Ostarine (S-22) is analogous to S-23, differing only by the substituent groups of the phenoxy ring, and it can therefore be postulated that S-23 would follow very similar routes of metabolism *in vivo*. The mono-hydroxylated version of this metabolite (M4) was the next most abundant detected in this study. Both of these metabolites (M3 and M4) were also produced when S-23 was incubated with homogenised horse liver,[28] with M4 also detected as a sulphate conjugate. In the reported metabolic study of ostarine, the sulphate conjugate of M4 was the major urinary metabolite and allowed the longest urinary detection.[24] Thus, it is likely also the best target for detection of S-23 in equine urine.

Another potential target analyte would be a sulphate conjugate of metabolite M5 (bis-hydroxylated M3), which was detected following incubation of S-23 with homogenised equine liver,[28] and in the metabolic study of ostarine in urine.[24] M6 was also reported following *in vitro* incubation of S-23,[28] but no analogous metabolite was reported for ostarine in equine urine.[24] Dephenylated metabolite M7 was reported in both previous studies; meanwhile, the mono- and bis-hydroxylated metabolites of S-23 (or analogous metabolites for ostarine) were not reported in either of the other studies.[24,28]

It is important to note that the administration of analogous arylpropionamide SARMs has been carried out via an IV dosing route rather than oral. A comparison of metabolites identified for the SARM LGD-4033 following oral[27] and IV[26] administrations highlighted the differences in urinary metabolites that can be observed between the two routes. Particularly, a greater array of metabolites were reported following oral administration. The non-detection of mono-hydroxylated metabolite for the other arylpropionamides following IV administration cannot rule out their production following oral administration, with an administration of S-23 required to confirm the proposed analytical targets.

These results suggest that phase II *in vitro* systems are valuable for certain groups of compounds, particularly arylpropionamides, that have been shown to produce metabolites that are highly sulphate-conjugated in equine urine.[24]

Table 7 - Details of parent S-23 and all identified metabolites in the *in vitro* sample. MS/MS analysis using negative ionisation with stepped CE 15, 35, 50

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Analyte ID**  (Elemental composition)  Biotransformation | **Observed precursor ion**  **(*m/z*)** | **tR**  **(min)** | **Observed product ions** | | **FS instrument signal intensity**  **(Relative intensity %)** |
| **m/z**  **(mass error, mmu)** | **Elemental composition** |
| **S-23**  (C18H13N2O3ClF4)  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML5766b45b.PNG | 415.0483  (1.05) | 4.07 | 269.0541 (0.90)  241.0590 (0.69)  185.0323 (0.21)  144.9850 (0.15)\* | C12H8N2O2F3  C11H8N2OF3  C8H4N2F3  C6H3OClF | 2.56e9 (N/A) |
| **M1a**  (C18H13N2O4ClF4)  Mono-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML576908d1.PNG | 431.0425  (0.34) | 3.44 | 269.0542 (0.93)  241.0589 (0.53)  185.0323 (0.21)  160.9800\* (0.01)  125.0030 (0.35) | C12H8N2O2F3  C11H8N2OF3  C8H4N2F3  C6H3O2ClF  C6H2O2F | 9.60e6 (2.4) |
| **M1b**  (C18H13N2O4ClF4)  Mono-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML576908d1.PNG | 431.0421  (0.12) | 3.63 | 269.0543 (1.08)  241.0594 (1.04)  185.0323 (0.18)  160.9800\* (0.00) | C12H8N2O2F3  C11H8N2OF3  C8H4N2F3  C6H3O2ClF | 3.07e6 (0.8) |
| **M1c**  (C18H13N2O4ClF4)  Mono-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML576908d1.PNG | 431.0421  (0.12) | 3.86 | 269.0543 (1.05)  255.0383 (0.76)  185.0323 (0.18)  160.9799\* (0.08)  144.9850 (0.13)  125.0030 (0.30) | C12H8N2O2F3  C11H6N2O2F3  C8H4N2F3  C6H3O2ClF  C6H3OClF  C6H2O2F | 3.54e7 (9.0) |
| **M1d**  (C18H13N2O4ClF4)  Mono-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML576908d1.PNG | 431.0424  (0.25) | 3.92 | 269.0543 (1.08)  185.0323 (0.22)  160.9800\* (0.01)  125.0031 (0.22) | C12H8N2O2F3  C8H4N2F3  C6H3O2ClF  C6H2O2F | 8.71e7 (22.0) |
| **M2**  (C18H13N2O5ClF4)  Bis-hydroxylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML576a6062.PNG | 447.0378  (0.75) | 3.56 | 269.0538 (0.53)  185.0325 (0.36)  176.9751\* (0.14)  140.9981 (0.17)  113.0031 (0.22) | C12H8N2O2F3  C8H4N2F3  C6H3O3ClF  C6H2O3F  C5H2O2F | 3.31e6 (0.8) |
| **M3**  (C8H5N2F3)  Amide hydrolysis  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML5758af2d.PNG | 185.0323\*  (0.36) | 2.70 | 158.0211 (0.06)  145.0195 (0.13)  115.0288 (0.29) | C7H3NF3  C8H2NF  C7H3N2 | 3.95e8 (100.0) |
| **M4**  (C8H5N2OF3)  Mono-hydroxylation of M3  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML575b9ff1.PNG | 201.0274\*  (0.20) | 2.67 | 181.0209 (0.12)  161.0145 (0.09)  154.0099 (0.03)  134.0034 (0.24) | C8H4N2OF2  C8H3N2OF  C7H2NOF2  C7HNOF | 3.39e8 (85.8) |
| **M5**  (C8H5N2O2F3)  Bis-hydroxylation of M3  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML575de8d3.PNG | 217.0227\*  (0.20) | 2.49 | 197.0160 (0.48)  169.0210 (0.04) | C8H3N2O2F2  C7H3N2OF2 | 6.94e6 (1.8) |
| **M6**  (C10H10ClFO4)  Amide hydrolysis  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML5759a280.PNG | - | 3.69 | 144.9849\* (0.77)  109.0084 (0.52) | C6H3OClF  C6H2OF | 1.60e8 (40.5) |
| **M7**  (C12H10N2O3F3)  Dephenylation  C:\Users\CD619~1.CUT\AppData\Local\Temp\SNAGHTML575a28ee.PNG | 287.0644  (0.04) | 2.79 | 185.0320\* (0.45)  158.0213 (0.47)  145.0194 (0.78)  115.0291 (0.54) | C8H4N2F3  C7H3NF3  C8H2NF  C7H3N2 | 1.24e8 (31.4) |

\*Base peak ion in observed spectra

**3.1.8 *In vitro-in vivo* correlation**

The comparison of the results obtained in this study to those where administration has now taken place, showed a good correlation with observed phase I metabolites. However, the *in vitro* model used in this study is unable to generate phase II metabolites. The equine administrations of ACP-105,[29,31] RAD-140,[28] ostarine[24] and AC-262536,[30] have indicated that phase II conjugates are often the most abundant metabolites excreted in equine urine. However, this is not the case for all the SARMS, such as LGD-4033.[27] The use of liver homogenatesmodels with phase II cofactors has been shown to produce good correlation to the *in vivo* profile.[28] However, the use of enzyme hydrolysis in extraction techniques, particularly when *in vivo* data is not available, would aid detection regardless of the conjugation status.[27,30] As can be seen from the results of this study, and from some previously reported studies,[27,30] the major phase I metabolites identified *in vitro,* very often mirror the most abundant metabolites detected in urine if enzyme hydrolysis or solvolysis was utilised. For example, the major ACP-105 and S-23 metabolites.

**4 Conclusions**

The *in vitro* metabolism of seven non-steroidal SARMs has been presented. To the best of the authors’ knowledge this is the first report of the equine metabolism of GSK2881078, LGD-2226, LGD-3303 and PF-06260414. Three metabolites of GSK2881078 were detected, formed via mono-hydroxylation. Four metabolites of LGD-3303 were detected, formed via mono- and di-hydroxylation. Five metabolites of PF-06260414 were detected, with the most abundant formed via thiadiazinan-bis-*N*-dealkylation. Three metabolites of LGD-2226 were detected with the major metabolite formed by mono-*N-*dealkylation of a tri-fluoromethyl moiety. These results should provide additional targets for analysis of these SARMs in doping control analysis. As no phase II metabolism was investigated, the use of hydrolysis methodologies is advised.

Additionally, twelve phase I metabolites of ACP-105 were identified, all of which have been reported following oral administration.[29,31] The major metabolites observed were mono-hydroxylated metabolites, corresponding to those detected following administration. Four metabolites of RAD-140 were identified, the most abundant of which was a mono-hydroxylated metabolite that was not detected following oral administration.[28] The other metabolites did correspond to the *in vivo* results. Finally, ten phase I metabolites of S-23 were identified. The major metabolite was formed via amide hydrolysis, and this was also the major metabolite identified *in vitro* for S-23 with equine liver homogenates[28] and for ostarine following IV administration,[24] albeit a phase II conjugate. The hydroxylated metabolites of S-23 were not reported in incubations with equine liver homogenates,[28] or analogous metabolites following IV administration of ostarine.[24] However, metabolites can differ following oral administration, and no administration of S-23 via this route has thus far been reported. Administration studies of all the reported SARMs that have only been investigated *in vitro* would be useful to help further refine analytical targets.

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