Correspondence case report

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Detection of the selective androgen receptor modulator andarine (S-4) in a routine equine blood doping control sample

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Introduction

Andarine (S-4) is the arylpropionamide-derived compound [*S*-3-(4-acetylamino-phenoxy)-2-hydroxy-2-methyl-*N*-(4-nitro-3-trifluoromethyl-phenyl)-propionamide] first described by Yin *et al.*[1] to be a selective androgen receptor modulator (SARM). Androgen receptor mediated transcription was reported to be 93% that of dihydrotestosterone, the most potent endogenous steroid.[1] This work built on the discovery of nonsteroidal androgens by Dalton *et al.*[2] following investigations of electrophilic ligands binding to the androgen receptor. Andarine (S-4) was shown by *in vitro* and *in vivo* studies to be an effective anabolic agonist, without side-effects often associated with anabolic-androgenic steroids,[1] relating principally to substrate affinity for 5α-reductase to produce DHT in the prostate.[3,4]

Unfortunately the benefits that SARMs such as andarine (S-4) may provide to clinical medicine have the potential for misuse in sports where athletes and/or their handlers may seek to gain unfair advantage with the assumption that these compounds are undetectable by anti-doping laboratories. The pharmacological activity and oral bioavailability of andarine (S-4) at relevant doses in rats (≤10 mg/kg)[5] highlights the potential threat to the integrity of animal and human sports. SARMs are therefore prohibited for use by the International Federation of Horseracing Authorities[6] and the World Anti-Doping Agency (WADA).[7]

Analytical methods utilizing the sensitivity and specificity of liquid chromatography-high resolution accurate mass (LC-HRAM) spectrometry to detect andarine (S-4) have been reported since 2006.[8,9] A black-market product was subsequently found by the Cologne anti-doping laboratory to contain andarine (S-4) in 2009.[10] For the benefit of urine analysis in equine[11–13] and human[13,14] anti-doping, *in vitro* and *in vivo* metabolic studies of andarine (S-4) have been performed. This assisted the reporting of two adverse analytical findings in human doping control urine samples analysed by the Lausanne and Los Angeles anti-doping laboratories in 2011 and 2013, respectively.[15,16] Here we describe, to the best of the authors' knowledge, the first detection of andarine (S-4) in a routine equine blood sample.

Materials and methods

Reference materials, chemicals, and reagents

Andarine (S-4) [*S*-3-(4-acetylamino-phenoxy)-2-hydroxy-2-methyl-*N*-(4-nitro-3-trifluoromethyl-phenyl)-propionamide] was purchased from the Institute of Biochemistry at the German Sports University (Cologne, Germany). d_3-Caffeine was purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada). Mefruside was purchased from Bayer AG (Pymble, NSW, Australia). All general laboratory reagents and solvents used were of HPLC grade and were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA). Water was obtained using a Millipore filtration system (Bedford, MA, USA).

Sample preparation

An equine blood doping control sample consisting of 6x6 mL lithium heparin tubes (BD Vacutainer®LH 102 IU ref 367885) sealed in a plastic integrity pack divided into 2x3 tubes representing the A- and B-portions was submitted to the Australian Racing Forensic Laboratory (ARFL) by an Australian principal racing authority. Following sample receipt, inspection and chain of custody documentation, 3 tubes (A-portion) were centrifuged at 3000 rpm for 10 min to obtain plasma for routine analysis. Blood tubes were stored at 4 °C until analyzed.

Screening analysis

The plasma sample (2 mL) was fortified with mefruside and d_3-caffeine (2 μg/mL, 50 μL) as surrogates before addition of trichloroacetic acid (10% *v/v*, 400 μL) with mixing and pH adjustment to 3.0–3.5. A quality control sample fortified with andarine (S-4; 1 μg/mL, 20 μL) was concurrently analyzed. The acid-neutral fraction containing arylpropionamide SARM compounds was isolated by solid-phase extraction (SPE) using a mixed-mode C8/strong cation exchange XTRACKT® column (200 mg, 3 cc, UCT, Bristol, PA, USA) with the aid of a UCT positive pressure manifold. The cartridge was conditioned with methanol (2 mL) and water (2 mL) before loading supernatant plasma and washing with acetic acid (0.1 M, 2 mL). The cartridge was dried using N_2 gas under positive pressure before elution with ethyl acetate/hexane (3:2 *v/v*, 3 mL). The extract was evaporated to dryness under nitrogen at 60 °C before being reconstituted in 0.1% formic acid/water (50 μL) and 0.1% formic acid/methanol (50 μL) for LC-HRAM spectrometry analysis.

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LC-HRAM analysis

LC-HRAM spectrometry analysis was undertaken using an Ultimate 3000 HPLC coupled to an Exactive Plus benchtop orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a SunFire® C18 column (100 x 2.1 mm, 3.5 um; Waters, Milford, MA, USA) operating at 40 °C. Injection volume was 10 μL. The mobile phase consisted of A: 0.1% formic acid/water and B: 0.1% formic acid/methanol. Gradient elution was performed with a flow rate of 0.4 mL/min according to the following programme: 5% B for 1 min, increased to 95% B at 15.85 min, before returning to 5% B at 16.35 min and held until 20.85 min. The presence of andarine (S-4) was monitored using deprotonated and protonated heated electrospray ionization (HESI) in full-scan mode at a resolution of 70 000 (FWHM) and deconvolution of all-ion fragmentation using the *m/z* 440.1020–440.1108 and *m/z* 442.1176–442.1264 intervals, respectively, between 11.80 min and 12.80 min. The total scan range was *m/z* 50 to *m/z* 850. Mass calibration was performed prior to analysis using Pierce® ESI negative (P/N 88324) and positive (P/N 88323) calibration solutions (Thermo Fisher Scientific, Bremen, Germany) but no lock mass was used. Source temperature, spray voltage, sheath gas (high purity N_2) and auxiliary gas (ultra-high purity N_2) were set at 350 °C, +/−4000 V, 63.74 and 10.30 arbitrary units, respectively. Instrument control and processing were performed using Xcalibur software (version 2.2 SP1).

Confirmation analysis

A separate plasma aliquot (2 mL) from a previously unopened blood tube was used for confirmation. Isolation of the acidic-neutral fraction by SPE was performed as described above for the screening analysis. LC-HRAM spectrometry was undertaken using an Ultimate 3000 HPLC coupled to a QExactive benchtop orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). LC separation was performed as described for the screening analysis. The presence of andarine (S-4) was confirmed using deprotonated and protonated HESI with polarity switching in a single analysis. Targeted MS^2 experiments were performed using parameters in Table 1, together with calibration and source conditions described for the screening analysis. The following sequence order was followed; solvent blank (0.1% formic acid/water (50 μL) and 0.1% formic acid/methanol (50 μL)), equine plasma blank, reagent (MilliQ water extracted concurrently with the sample) blank, test sample, solvent blank, equine plasma blank, equine plasma QC andarine (S-4) spike 1 ng/mL, certified andarine (S-4) standard, mefruside standard, and d_3-caffeine standard.

Table 1. QExactive targeted MS^2 parameters used for confirmation of andarine (S-4)

Compound	Polarity	Precursor ion (*m/z*)	NCE	Resolution (FWHM)	AGC target	Isolation window (*m/z*)
Andarine (S-4)	Negative	440.1064	35	70,000	1x10e^6	0.5
	Positive	442.1221	35	17,500	2x10e^5	4.0
Mefruside (IS)	Negative	381.0340	25	70,000	1x10e^6	0.5
Caffeine-d_3 (IS)	Positive	198.1065	25	17,500	2x10e^5	4.0

NCE = Normalized collision energy (arbitrary units)
AGC = Automatic Gain Control
(IS) = Internal standard

Results and discussion

Screening

On review of LC-HRAM data from the Exactive Plus instrument a peak was found at a retention time (RT) of 12.29 min (relative retention (RR) 1.23 to mefruside) for both *m/z* 440.1020-440.1108 and *m/z* 442.1176–442.1264 intervals. Specificity of these *m/z* intervals has been demonstrated with no response in *bona fide* blank equine plasma used by the ARFL nor any of the more than 4000 routine equine plasma samples analyzed since the inclusion of the parent substance andarine (S-4) as a target. Additional confidence was attributed to the screening abnormality for andarine (S-4) in the sample by having dual deprotonated and protonated ion responses.

Confirmation

Targeted MS^2 experiments enabled confirmation of andarine (S-4) in the equine plasma sample with comparison to the reference standard using the identification criteria for LC-MS techniques prescribed by the *Association of Official Racing Chemists* (Table 2).[17]

Accurate mass assignments for deprotonated and protonated spectra are summarized in Tables 3 and 4, respectively, with good agreement to the characterization of andarine (S-4) reported by Thevis *et al.*[8–10] The deprotonated product ion spectrum (Figure 1) shows elimination of *N*-(4-hydroxyphenyl)-acetamide (−151 Da) yielding *m/z* 289.0447, followed by carbon monoxide to give *m/z* 261.0497. 4-Nitro-3-trifluouromethyl-aniline provides *m/z* 205.0229, while the base ion *m/z* 150.0556 represents the

Table 2. Confirmation criteria for andarine (S-4) in the equine plasma sample

Criterion	Sample	Standard	% difference	Requirement*
Retention time (RT)	12.33	12.34	0.08	<2%
$[M-H]^-$	% rel. abundance			
150.0556	100	100	0	
205.0229	16.50	16.01	0.49	±20% (abs.)
261.0497	19.30	20.07	0.77	±20% (abs.)
289.0447	5.16	5.59	0.43	±20% (abs.)
$[M+H]^+$	% rel. abundance			
208.0968	100	100	0	
190.0862	70.96	71.87	0.91	±40% (rel.)
108.0446	60.90	61.15	0.25	±40% (rel.)
400.1114	42.04	43.25	1.21	±20% (abs.)

* Association of Official Racing Chemists[17]

Table 3. Deprotonated $[M-H]^-$ product ion spectral results for andarine (S-4) in the equine plasma sample from precursor ion *m/z* 440.10640

Product ion (*m/z*) experimental	Product ion (*m/z*) theoretical	Mass error (Δ ppm)	Elemental composition
107.0369	107.0366	−2.8	C_6H_5ON
150.0556	150.0550	−4.0	$C_8H_8O_2N$
205.0229	205.0219	−4.9	$C_7H_4O_2N_2F_3$
261.0497	261.0482	−5.7	$C_{10}H_8O_3N_2F_3$
289.0447	289.0431	−5.5	$C_{11}H_8O_4N_2F_3$

Table 4. Protonated $[M+H]^+$ product ion spectral results for andarine (S-4) in the equine plasma sample from precursor ion *m/z* 442.12205

Product ion (*m/z*) experimental	Product ion (*m/z*) theoretical	Mass error (Δ ppm)	Elemental composition
108.0446	108.0444	−1.9	C_6H_6ON
148.0757	148.0757	0	$C_9H_{10}ON$
166.0863	166.0863	0	$C_9H_{12}O_2N$
190.0862	190.0863	0.5	$C_{11}H_{12}O_2N$
208.0968	208.0968	0	$C_{11}H_{14}O_3N$
400.1114	400.1115	0.2	$C_{17}H_{17}O_5N_3F_3$

p-substituted phenol residue. Prominent features of the protonated product ion spectrum (Figure 2) include the elimination of ketene (C_2H_2O; −42 Da) and 4-nitro-5-trifluoromethylphenyl-formamide ($C_8H_5N_2O_3F_3$; −234 Da). Elemental composition of protonated product ions provided in Table 4 was verified using Mass Frontier 7.0 SR1 software (Thermo Fisher Scientific, Bremen, Germany).

The concentration of andarine (S-4) in the sample was estimated between 0.2 ng/mL and 0.3 ng/mL by comparison to the 1 ng/mL QC equine plasma spike. The sensitivity for MS^2 results to be obtained at this low level together with dual polarity functionality further highlights the benefits of the QExactive instrument to perform this type of forensic analysis. Referee analysis of the sample (B-portion) subsequently conducted by a second accredited Australian racing laboratory confirmed the presence of andarine (S-4).

The illicit administration of SARMs to racehorses may be in response to rules prohibiting the use of anabolic steroids at all times and greater surveillance of conventional doping agents by laboratories. This case illustrates the need for accredited racing laboratories to implement testing methods for substances that become readily available on the

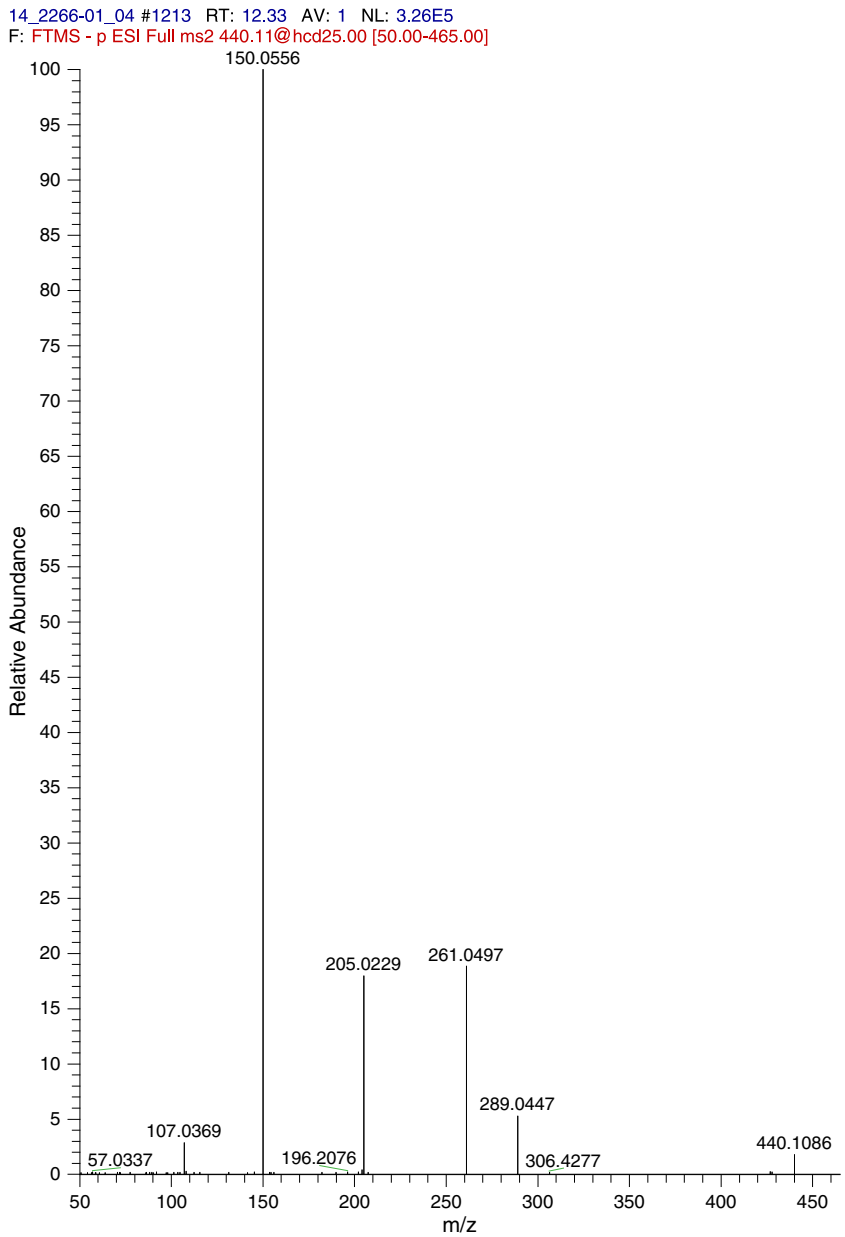

Figure 1. Confirmatory deprotonated $[M-H]^-$ product ion spectra for andarine (S-4) in equine plasma sample.

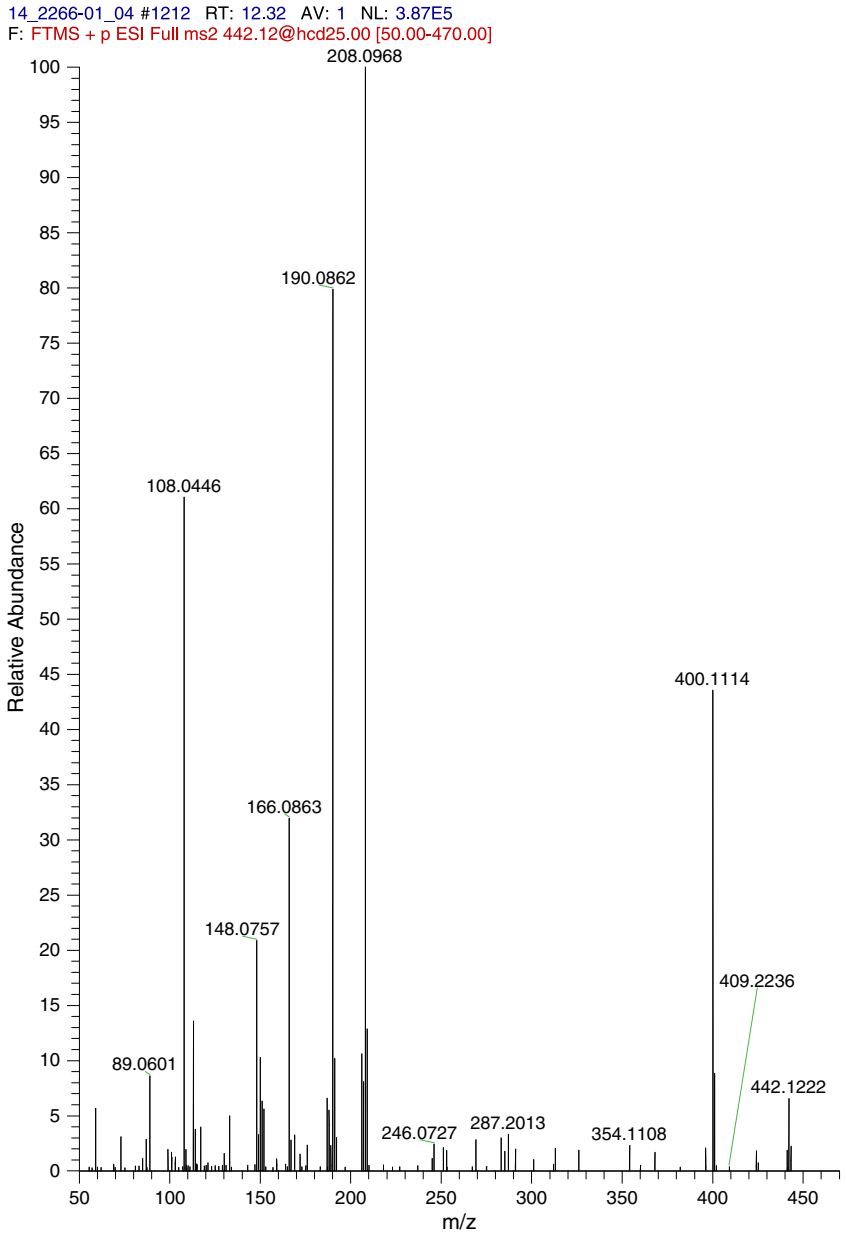

Figure 2. Confirmatory protonated $[M+H]^+$ product ion spectra for andarine (S-4) in equine plasma sample.

black market in order to deter and prosecute licensed persons who are tempted to administer non-approved drugs without knowledge of adverse health effects to an animal.

Conclusion

This case report presents the first analytical finding of andarine (S-4) in an equine blood sample submitted for routine doping control. The use of LC-HRAM spectrometry analysis for screening and confirmatory purposes demonstrated the capability for anti-doping laboratories to combat the misuse of this compound and other SARMs in horseracing.

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