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SARM-S4 and metabolites detection in sports drug testing: A case report☆

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ABSTRACT

Recently, pharmaceutical industry developed a new class of therapeutics called Selective Androgen Receptor Modulator (SARM) to substitute the synthetic anabolic drugs used in medical treatments. Since the beginning of the anti-doping testing in sports in the 1970s, steroids have been the most frequently detected drugs mainly used for their anabolic properties. The major advantage of SARMs is the reduced androgenic activities which are the main source of side effects following anabolic agents' administration.

In 2010, the Swiss laboratory for doping analyses reported the first case of SARMs abuse during in-competition testing. The analytical steps leading to this finding are described in this paper. Screening and confirmation results were obtained based on liquid chromatography tandem mass spectrometry (LC–MS/MS) analyses. Additional information regarding the SARM S-4 metabolism was investigated by ultra high-pressure liquid chromatography coupled to quadrupole time-of-flight mass spectrometer (UHPLC–QTOF-MS).

1. Introduction

Arylpropionamide-derived substances are analogs of androgen receptor (AR) agonists like bicalutamide. Since the discovery of these compounds has been reported by Dalton et al. in 1998 [1], these nonsteroidal androgens have been developed and investigated by many authors, especially for their anabolic therapeutics properties [2,3]. Because of their high affinity binding capacity to the androgen receptor, various synthetic drugs have been named Selective Androgen Receptor Modulator or SARMs. At this time four categories of nonsteroidal androgen receptor agonists have been identified as arylpropionamides, bicyclic hydantoins, quinolines and tetrahydroquinoline analogues [4] but none of them are actually available as approved pharmaceutical compound.

The main advantages of SARMs compared to classical steroid replacement therapies have been shown through recent animal models studies which demonstrated that SARMs have a high tissue selectivity in muscle and bone whereas the steroid side effects linked to the androgens tissues are significantly reduced [5,6]. This is mainly due to the AR activation by the SARMs which are not substrates for 5α-reductase and aromatases, thus excluding the amplified androgenic and estrogenic functions in tissues like prostate and seminal vesicles, observed after administration of testosterone and other anabolic androgenic steroids [7–9].

The high anabolic potency of SARMs coupled to a limited or even an absence of androgenic effects are properties which should attract athletes looking for power and strength improvement without undergoing deleterious physiological side effects. Consequently, the SARMs have been prohibited by the World Anti-Doping Agency (WADA) since January 2008 [10] and are included in the S1 class of anabolic agents.

A recent review paper described analytical methods to put forth a doping with SARMs in urine matrix with LC–MS/MS or GC–MS depending on the structure and physicochemical properties of the analyzed product [11]. Also, each of the four above-cited SARMs categories has been investigated regarding the detection of parent compounds and/or metabolites either in vitro or in vivo [12–15]. Considering these published papers, WADA accredited laboratories are currently able to screen for SARMs and their metabolites in doping control urine samples.

In 2008, Thevis et al. identified a significant amount of Andarine (also called SARM-S4 or S-3-(4-acetylamino-phenoxy)-2-hydroxy-2-methyl-N-(4-nitro-3-trifluoromethyl-phenyl)-propionamide) in a freely available internet product [16]. This finding demonstrated the easy accessibility of SARMs products. Thus, athletes could obtain these forbidden substances and use them to improve their physical skills straightforwardly.

Since the introduction of SARMs on the WADA prohibited list, no adverse analytical finding (AAF) was declared by any of the world accredited laboratory. We report here the first AAF in regards to the presence of Andarine and its metabolites identified

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by LC–MS/MS and UHPLC–QTOF-MS in an in-competition female athlete urine sample.

2. Materials and methods

2.1. Chemicals and reagents

All solvents and reagents were of analytical grade purity. Potassium carbonate anhydrous was obtained from Acros Organics (Geel, Belgium). Ethyl acetate was purchased from Panreac (Barcelona, Spain). Sodium hydrogen carbonate and potassium dihydrogen phosphate were supplied by Merck (Darmstadt, Germany). Ultrapure water was produced by a Milli-Q Gradient A10 water purification system with a Q-Gard® 2 and a Quantum™ EX Ultrapure organex cartridge purchased by Millipore Corp. (Billerica, MA, USA). ULC–MS quality acetonitrile was obtained from Biosolve (Chemie Brunschwig, Basel, Switzerland). Formic acid was purchased from Fluka (Buchs, Switzerland). β-Glucuronidase from *Escherichia coli* in a 50% glycerol solution (pH 6.5, 140 U/mL at 37 °C) was supplied by Roche Diagnostics GmbH (Manheim, Germany).

Standard SARM S-4 was provided by the Institute of Biochemistry, Center for preventive Doping Research of the German Sport University in Cologne (Germany).

2.2. Screening LC–MS/MS analysis

Urine samples were thawed at 37 °C and centrifuged at 1350 × *g* for 5 min before sample treatment. Internal standard mefruside was added to 2 mL of urine (125 ng/mL). The first aliquot (2 mL) was saturated with solid potassium carbonate and further extracted at basic pH with 4 mL ethyl acetate by shaking for 10 min. The second aliquot (2 mL) was saturated with solid potassium dihydrogen phosphate and further extracted at acidic pH with 4 mL ethyl acetate by shaking for 10 min. After centrifugation at 1350 × *g* for 5 min, the organic layers were collected. Then, the organic fractions were pooled and evaporated. The residue was dissolved in 125 μL of ammonium formiate buffer 10 mM pH 4/acetonitrile (80/20). Finally, the solution was transferred into a vial prior to LC–MS/MS analysis.

The LC–MS/MS system consisted of a Rheos 2200 CPS-LC system pump (Flux Instrument, Basel, Switzerland) and a HTS Pal autosampler (CTC analytics AG, Zwingen, Switzerland) coupled to a triple stage quadrupole mass spectrometer TSQ Quantum Discovery Max (ThermoFinnigan, San Jose, CA, USA), equipped with an atmospheric pressure ionisation interface Ion MAX™ operated in ESI mode. The separation was performed on a Sunfire™ C8 column (100 mm × 2.1 mm, 3.5 μm) from Waters (Milford, MA, USA). A low dispersion in-line filter (0.2 μm frits) from Agilent (Palo Alto, CA, USA) was installed between the injector and the column. Elution solvents were ammonium formiate buffer 10 mM pH 4 in water (A) and acetonitrile (B). The gradient percentage of organic solvent (B) started with 5% for 0.75 min, changed linearly to 80% during a period of 4.25 min, followed by an isocratic elution for 1 min. After returning to initial conditions, the system was equilibrated for 1.5 min. The flow rate was set to 350 μL/min, and the injected volume was 10 μL. The column temperature and the autosampler tray were set at 25 °C and 12 °C, respectively. The mass spectrometer was operated in negative ionisation mode, collision offset voltage was set at 22 V and 18 V for SARM S-4 and M5 metabolite, respectively. A single transition was followed for each compound, i.e., *m*/*z* 440.1 → 261.1 (SARM S-4) and 307.1 → 205.0 (M5 metabolite). The first (Q1) and third (Q3) quadrupoles were set at 0.7 amu mass resolution. The ionisation conditions were the following: the capillary temperature 320 °C and ESI spray voltage 3 kV. The sheath and auxiliary gas (nitrogen) flow rate was 30 and 15 (arbitrary units), respectively. The tube lens voltages were set to 110 V and the Q2 collision gas (argon) pressure was 0.2 Pa.

2.3. Confirmation UPLC–QTOF-MS and MS/MS

Two different extracts were analyzed for confirmation purpose. One was prepared according to the steps described in the screening section and the other was treated as follow. Urine sample was thawed at 37 °C and centrifuged at 1350 × *g* for 5 min before sample treatment. Internal standard mefruside was added to 2 mL of urine (125 ng/mL). 1 mL phosphate buffer 0.8 M pH 7.0 was added prior to hydrolysis with 50 μL of β-glucuronidase from *E. coli* at 50 °C for 60 min. Then, 200 mg of solid carbonate buffer (Na_2CO_3/$NaHCO_3$, 1:10 (w/w) was added to reach a pH of 8.5–9.0. A liquid–liquid extraction was performed using 5 mL of TBME. The organic layer was isolated using liquid N_2 and evaporated to dryness under an air flow in a water bath at 50 °C. The residue was dissolved in 125 μL of ammonium formiate buffer 10 mM pH 4/acetonitrile (80/20).

UHPLC–QTOF-MS analyses were performed on a Micromass-Q-Tof Premier mass spectrometer with an electrospray ionization (ESI) interface coupled with an Acquity UPLC system (both from Waters, Milford, MA, USA).

Separations were carried out on a Waters Acquity UPLC column (BEH C_{18} 100 mm × 2.1 mm, 1.7 μm) at 30 °C and 400 μL/min. A Van Guard (Waters) pre-column (BEH C_{18} 50 mm × 2.1 mm, 1.7 μm) was placed prior to the column. The mobile phase was (A) 0.1% formic acid in water, and (B) 0.1% formic acid in acetonitrile. The gradient started linearly from 5% to 40% B over 15 min, then to 95% in 2 min. The column was re-equilibrated with 5% B for 3 min. The injection volume was fixed at 10 μL and the samples were maintained at 4 °C in the autosampler.

Detection was performed in the negative ion mode over an *m*/*z* range of 100–1000 in the centroid mode. The ESI conditions were as follows: the desolvation gas flow was 850 L/h at a temperature of 360 °C, the capillary voltages were defined as

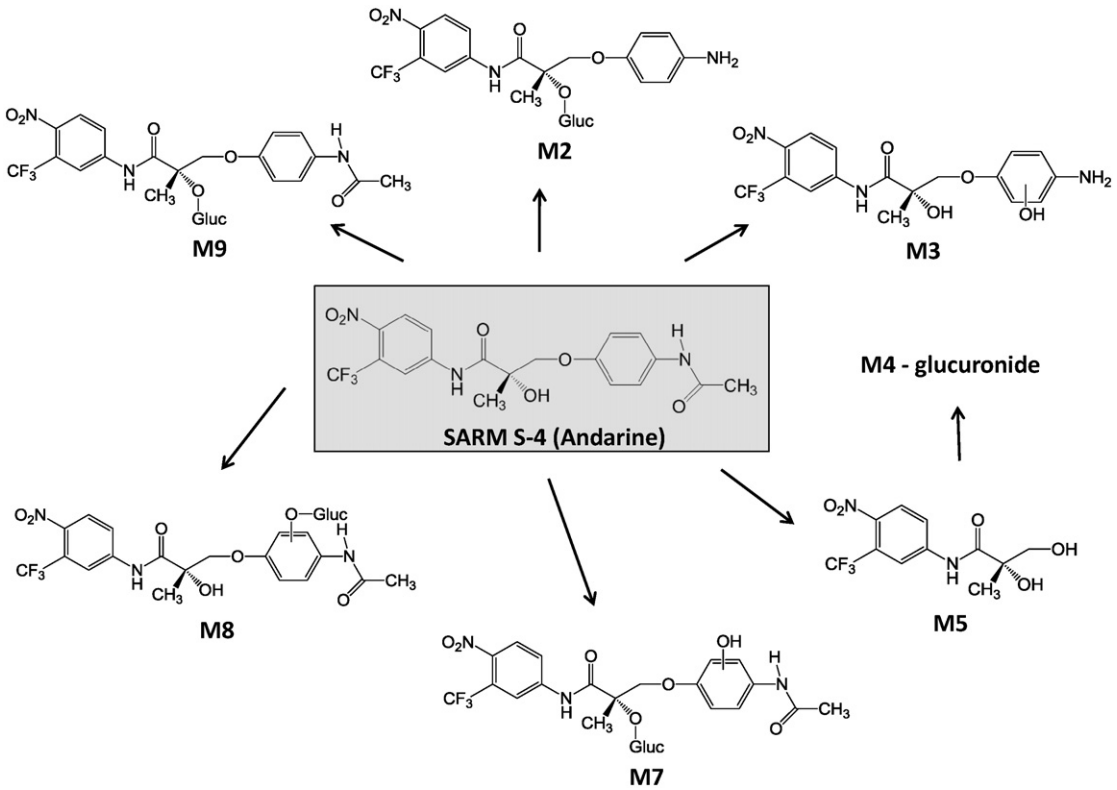

Fig. 1. Chemical structures of SARM-S4 (Andarine) and its metabolites detected in the athlete's urine sample. A complete scheme of SARM S-4 metabolism has been published elsewhere [17].

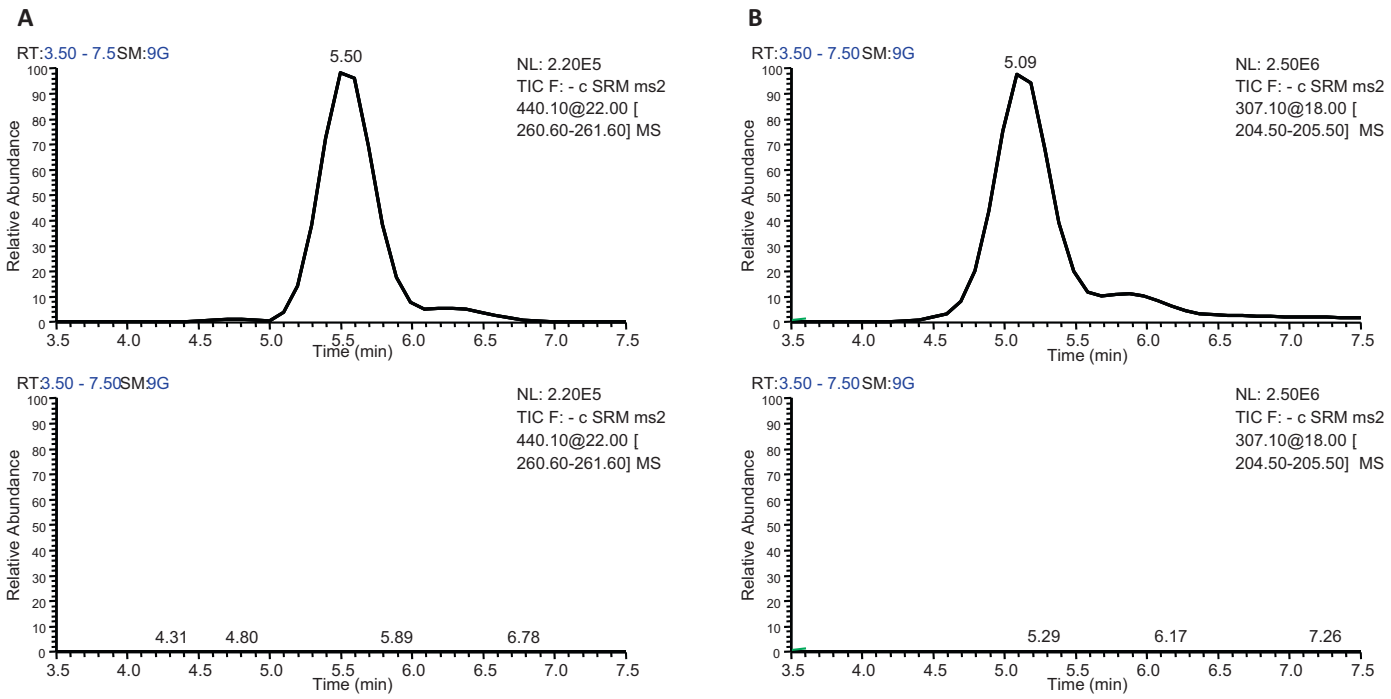

Fig. 2. Screening results of (A) SARM S-4 (m/z 440.1 → 261.1) and (B) M5 metabolite (m/z 307.1 → 205.0) for the athlete's sample (top panels) and the negative urine control (bottom panels).

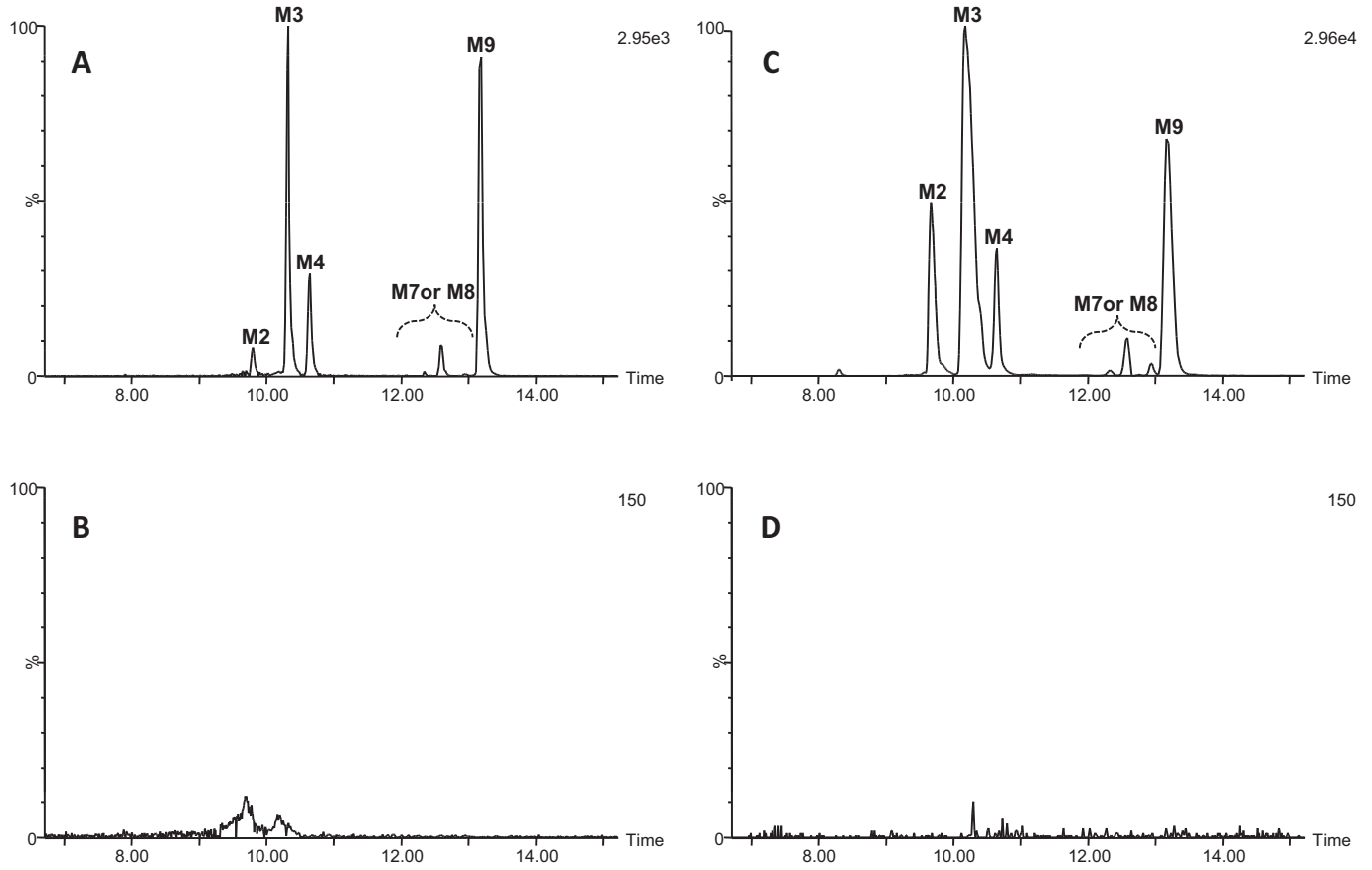

Fig. 3. Sum of the glucuronide metabolites (M2, M3, M4, M7, M8 and M9) extracted ions $[M-H]^-$ found in an excretion study urine (A) and in the athlete's sample (C) after a liquid–liquid extraction. Negative urine control and hydrolyzed extract athlete's sample are depicted in B and D, respectively.

Table 1
Theoretical and experimental data for SARM S-4 and metabolites obtained by UHPLC–QTOF-MS in negative ESI mode.

Compound	Elemental composition	Theoretical mass (*m/z*)	Experimental mass (*m/z*)	Error (ppm)	Retention time (min)
S-4	$C_{19}H_{17}F_3N_3O_6$	440.1069	440.1072	0.7	15.68
M2	$C_{23}H_{23}F_3N_3O_{11}$	574.1285	574.1306	3.7	9.67
M3	$C_{23}H_{23}F_3N_3O_{12}$	590.1234	590.1226	−1.4	10.18
M4	$C_{17}H_{18}F_3N_2O_{11}$	483.0863	483.0849	−2.9	10.65
M5	$C_{11}H_{10}F_3N_2O_5$	307.0542	307.0543	0.3	12.05
M7	$C_{25}H_{25}F_3N_3O_{13}$	632.1339	632.1328	−1.7	12.59
M8	$C_{25}H_{25}F_3N_3O_{13}$	632.1339	632.1347	1.3	13.25
M9	$C_{25}H_{25}F_3N_3O_{12}$	616.1390	616.1381	−0.9	13.18

2.4 kV, and the cone voltage was kept constant at 40 V. The micro-channel plates (MCPs) were operated at 1750 V, the source temperature was 120 °C, the cone gas flow and the collision gas flow were set to 10 L/h and 0.25 mL/min, respectively. For the dynamic range enhancement (DRE) lockmass, a solution of leucine–enkephalin at 2 ng/mL (Sigma–Aldrich, Buchs, Switzerland) was infused through the Lock Spray probe at 5 μL/min.

Confirmation of SARM S4 and its glucuronide was performed on MS/MS mode, using *m/z* 440.10 as parent ion for SARM S4 and *m/z* 616.13 for the glucuronide (negative mode). The collision energies were set to 17 and 26 eV respectively.

2.4. Excretion study sample

A spot urine sample coming from an excretion study was provided by the World Association of Anti-Doping Scientists (WAADS) in the context of an educational program in 2009.

3. Results and discussion

The recent developments of the Selective Androgen Receptor Modulator as therapeutics as well as the increasing internet availability have led the WADA to introduce these synthetic compounds on the prohibited list. Since the metabolic pathway description of the arylpropionamide-derived molecule named as SARM S-4 [17], we report here the first adverse analytical finding in the fight against doping.

After reception to the laboratory, the athlete's urine sample followed the usual screening procedures for in-competition testing. SARMs compounds were analyzed by LC–MS/MS after the sample preparation described above, allowing the extraction of conjugated and aglycon moieties. Traces of the specific transitions for SARM S-4 and its metabolites M5 (Fig. 1) showed the presence of these two compounds in the screening sample whereas no peaks were detected in the negative control (Fig. 2).

According to the international standard for laboratories (ISL) [18], a suspicious screening result needs to be confirmed with a particular assay targeting the investigated analyte and/or metabolites. The presence of SARM S-4 and M5 metabolite was validated by LC–MS/MS in product scan mode (data not shown) in regards to the identification criteria for qualitative assay edited by the WADA [19]. Moreover, the concentration of SARM S-4 in the confirmation sample was estimated at about 1 μg/mL.

Further investigations on the athlete's confirmation sample were performed by UHPLC–QTOF-MS in ESI negative ionization mode. This technique allows a sensitive full MS range acquisition and accurate mass determination on molecular and fragment ions [20]. SARM S-4 and its metabolites [17], described in Table 1, were identified thanks to exact mass measurement using a mass chromatogram window of 5 ppm. The calculated mass error between the theoretical and experimental mass was less than 3.7 ppm.

The extracted ion chromatograms corresponding to the different glucuronide metabolites described by Thevis et al. [17] were highlighted in Fig. 3A for the excretion study and Fig. 3C for the athlete's sample. No signal was detected in the negative urine control (Fig. 3B), indicating that this molecule could be related to the SARM S-4 metabolism. In order to verify that the identified metabolites are glucuro-conjugated, the athlete's urine was subjected to an additional extraction including a hydrolysis step with β-glucuronidase. As shown in Fig. 3D, the analysis of this hydrolyzed extract by UHPLC–QTOF-MS did not reveal any signal related to M2, M3, M4, M7, M8 and M9 compounds. Moreover, after hydrolysis, the aglycon compounds such as SARM S4 and M5 were considerably enhanced. These results proved the presence of the glucuronide conjugated metabolites.

Additional evidences were given through the MS/MS experiments performed on the UHPLC–QTOF-MS system. Fig. 4A shows an example of the MS/MS fragmentation pattern of compound M9 corresponding to the SARM S-4 glucuronide. The exact mass measurement enabled the molecular formula assignment of the parent (*m/z* 616.1381, $C_{25}H_{25}F_3N_3O_{12}$, calculated mass 616.1390, error −0.9 ppm) and the fragments. The neutral loss of the dehydrated glucuronic acid (176.0312, $C_6H_8O_6$) leads to the fragment ion *m/z* 440 (*m/z* 440.1069, $C_{19}H_{17}F_3N_3O_6$, calculated

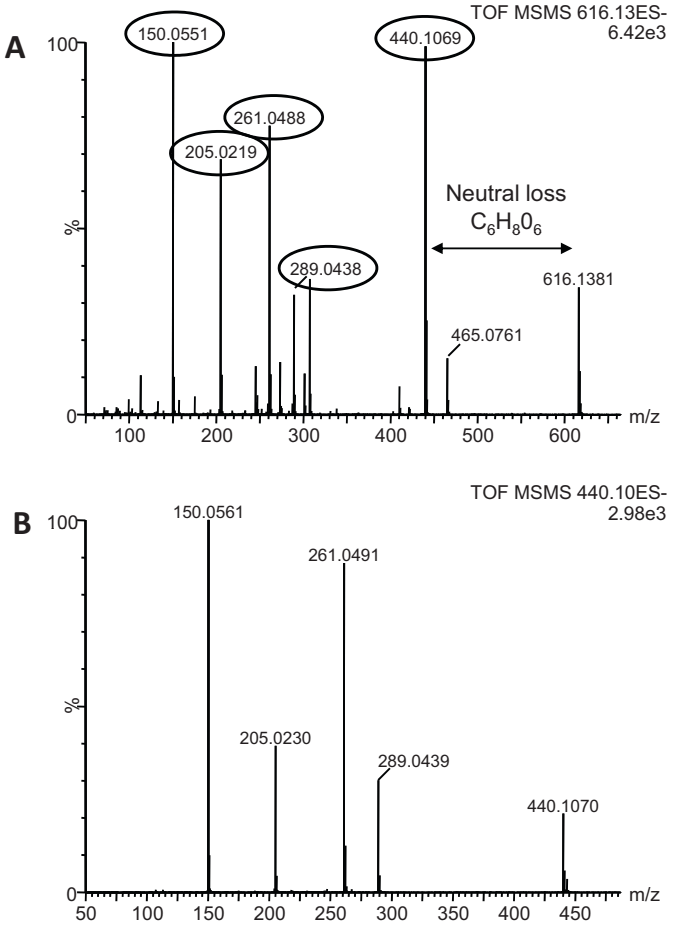

Fig. 4. MS/MS spectra comparison between SARM S-4 glucuronide metabolite M9 (A) and the parent compound (B).

mass 440.1069, error 0.0 ppm) corresponding to the SARM S-4. Diagnostic ions at m/z 150.0551, m/z 205.219, m/z 261.0488 and m/z 289.04338 match with the fragmentation pattern of the SARM S-4, as illustrated in Fig. 4B and previously described in literature [17,21]. The same behavior was observed for the other glucuronide metabolites (data not shown).

An additional metabolic pathway of the SARMs is related to the sulfo-conjugation [17]. The theoretical masses corresponding to the different sulfo-conjugated metabolites were extracted in the athlete's sample chromatogram and did not show the presence of any of these metabolites. This result is probably due to the extraction solvent not compatible with the polarity of such molecules. A dilute and shoot approach could be an attractive alternative for the sample preparation to detect the sulfo-conjugated fraction.

Interestingly, a blood sample was withdrawn on the same athlete during a doping control testing procedure that took place one day before the urine collection. The plasma was thus extracted with a dedicated sample preparation and analyses were performed with the same analytical tools used for urine investigations. The screening results for the blood sample did not show any trace of the SARM S-4. This negative finding was confirmed by the Center for Preventive Doping Research of the German Sport University in Cologne. Thus, the athlete must have taken the forbidden product between the blood withdrawal and the urine collection time. This hypothesis correlates with the estimated high amount of the SARM S-4 and its metabolites found in urine.

4. Conclusions

This case report presents the first adverse analytical finding regarding a Selective Androgen Receptor Modulator, SARM S-4 also named Andarine, classified in the S1 class of the WADA prohibited list. Established screening procedures revealed the presence of the parent compound and the M5 metabolite. State-of-the-art mass spectrometric techniques allowed the confirmation and the identification of SARM S-4 and several metabolites previously described following in vivo and in vitro studies. Moreover, plasma was also investigated for the presence of SARM S-4 but MS analyses did not reveal any traces of the investigated compound.

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References

[1] J.T. Dalton, A. Mukherjee, Z. Zhu, L. Kirkovsky, D.D. Miller, Discovery of nonsteroidal androgens, Biochem. Biophys. Res. Commun. (1998) 1–4.
[2] J. Chen, J. Kim, J.T. Dalton, Discovery and therapeutic promise of selective androgen receptor modulators, Mol. Interv. (2005) 173–188.
[3] M.L. Mohler, C.E. Bohl, A. Jones, C.C. Coss, R. Narayanan, Y. He, D.J. Hwang, J.T. Dalton, D.D. Miller, Nonsteroidal selective androgen receptor modulators (SARMs): dissociating the anabolic and androgenic activities of the androgen receptor for therapeutic benefit, J. Med. Chem. (2009) 3597–3617.
[4] M. Thevis, W. Schanzer, Synthetic anabolic agents: steroids and nonsteroidal selective androgen receptor modulators, Handb. Exp. Pharmacol. (2010) 99–126.
[5] W. Gao, J.D. Kearbey, V.A. Nair, K. Chung, A.F. Parlow, D.D. Miller, J.T. Dalton, Comparison of the pharmacological effects of a novel selective androgen receptor modulator, the 5alpha-reductase inhibitor finasteride, and the antiandrogen hydroxyflutamide in intact rats: new approach for benign prostate hyperplasia, Endocrinology (2004) 5420–5428.
[6] W. Gao, P.J. Reiser, C.C. Coss, M.A. Phelps, J.D. Kearbey, D.D. Miller, J.T. Dalton, Selective androgen receptor modulator treatment improves muscle strength and body composition and prevents bone loss in orchidectomized rats, Endocrinology (2005) 4887–4897.
[7] S. Bhasin, O.M. Calof, T.W. Storer, M.L. Lee, N.A. Mazer, R. Jasuja, V.M. Montori, W. Gao, J.T. Dalton, Drug insight: testosterone and selective androgen receptor modulators as anabolic therapies for chronic illness and aging, Nat. Clin. Pract. Endocrinol. Metab. (2006) 146–159.
[8] S. Bhasin, R. Jasuja, Selective androgen receptor modulators as function promoting therapies, Curr. Opin. Clin. Nutr. Metab. Care (2009) 232–240.
[9] D. Yin, W. Gao, J.D. Kearbey, H. Xu, K. Chung, Y. He, C.A. Marhefka, K.A. Veverka, D.D. Miller, J.T. Dalton, Pharmacodynamics of selective androgen receptor modulators, J. Pharmacol. Exp. Ther. (2003) 1334–1340.
[10] World Anti-Doping Agency, The Prohibited List – International Standard. http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/WADA_Prohibited_List_2008_EN.pdf, 2011 (accessed 19.05.11).
[11] M. Thevis, W. Schanzer, Mass spectrometry of selective androgen receptor modulators, J. Mass Spectrom. (2008) 865–876.
[12] T. Kuuranne, A. Leinonen, W. Schanzer, M. Kamber, R. Kostiainen, M. Thevis, Aryl-propionamide-derived selective androgen receptor modulators: liquid chromatography–tandem mass spectrometry characterization of the in vitro synthesized metabolites for doping control purposes, Drug Metab. Dispos. (2008) 571–581.
[13] M. Thevis, M. Kohler, A. Thomas, N. Schlorer, W. Schanzer, Doping control analysis of tricyclic tetrahydroquinoline-derived selective androgen receptor modulators using liquid chromatography/electrospray ionization tandem mass spectrometry, Rapid Commun. Mass Spectrom. (2008) 2471–2478.
[14] M. Thevis, M. Kohler, A. Thomas, J. Maurer, N. Schlorer, M. Kamber, W. Schanzer, Determination of benzimidazole- and bicyclic hydantoin-derived selective androgen receptor antagonists and agonists in human urine using LC–MS/MS, Anal. Bioanal. Chem. (2008) 251–261.
[15] M. Thevis, M. Kohler, N. Schlorer, M. Kamber, A. Kuhn, M.W. Linscheid, W. Schanzer, Mass spectrometry of hydantoin-derived selective androgen receptor modulators, J. Mass Spectrom. (2008) 639–650.
[16] M. Thevis, H. Geyer, M. Kamber, W. Schanzer, Detection of the arylpropionamide-derived selective androgen receptor modulator (SARM) S-4 (Andarine) in a black-market product, Drug Test. Anal. (2009) 387–392.
[17] M. Thevis, A. Thomas, G. Fussholler, S. Beuck, H. Geyer, W. Schanzer, Mass spectrometric characterization of urinary metabolites of the selective androgen receptor modulator andarine (S-4) for routine doping control purposes, Rapid Commun. Mass Spectrom. (2010) 2245–2254.
[18] World Anti-Doping Agency, The World Anti-Doping Code – International Standard – Laboratories. http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_Int.Standard_Laboratories_2009_EN.pdf, 2011 (accessed 23.06.11).
[19] World Anti-Doping Agency, WADA Technical Document – TD2010IDCR. http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_TD2010IDCRv1.0_Identification%20Criteria%20for%20Qualitative%20Assays_May%2008%202010_EN.doc.pdf, 2011 (accessed 23.06.11).
[20] R. Plumb, J. Castro-Perez, J. Granger, I. Beattie, K. Joncour, A. Wright, Ultra-performance liquid chromatography coupled to quadrupole-orthogonal time-of-flight mass spectrometry, Rapid Commun. Mass Spectrom. (2004) 2331–2337.
[21] M. Thevis, M. Kamber, W. Schanzer, Screening for metabolically stable arylpropionamide-derived selective androgen receptor modulators for doping control purposes, Rapid Commun. Mass Spectrom. (2006) 870–876.