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Screening for metabolically stable aryl-propionamide-derived selective androgen receptor modulators for doping control purposes

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Anabolic agents have been among the most frequently detected drugs in amateur and professional sport. A novel class of therapeutics presumably complementing anabolic steroids in the near future includes so-called selective androgen receptor modulators (SARMs) that have been under clinical investigations for several years. Although not yet commercially available, their potential for misuse in sports is high. Four aryl-propionamide-derived SARMs were synthesized in order to establish a fast and robust screening procedure using liquid chromatography/electrospray ionization tandem mass spectrometry. Synthesized compounds were characterized by high-resolution/high-accuracy mass analysis employing a linear ion trap-Orbitrap hybrid mass spectrometer while routine analyses were conducted on a triple-quadrupole mass spectrometer. Characteristic product ions obtained by collision-induced dissociation were found at *m/z* 289 and 261 as well as *m/z* 269 and 241 representing the bisubstituted aniline residues of selected model compounds. Assay validation was performed regarding lower limit of detection (1 ng/mL), recovery (85–105%), intraday precision (7.6–11.6%) and interday precision (9.9–14.4%), and precursor ion scan experiments on diagnostic product ions enabled the detection of a structurally related compound at 50 ng/mL. Copyright © 2006 John Wiley & Sons, Ltd.

Ever since the beneficial effects of testosterone (T) on muscle, bone and physical performance were determined,[1] after the identification of its structure in 1935,[2–4] the search for even more potent analogues has been pursued for various purposes by pharmaceutical companies. While endogenous androgens are of utmost importance for male development and male secondary characteristics such as muscle mass, bone mass and body composition, anabolic-androgenic steroids have been employed for the treatment of anemia and several debilitating diseases, and more recently for the therapy of hypogonadal conditions or delayed puberty. In addition, their suitability for regulating male fertility has been the subject of several investigations.[5,6] However, limitations of anabolic-androgenic steroid therapies such as decreased levels of HDL cholesterol and negative influences on prostate and cardiovascular systems have led to the development of selective androgen receptor modulators (SARMs), which act as full agonists in anabolic target tissues (e.g. muscle and bone), but demonstrate only partial agonist activity in androgenic tissues such as prostate and seminal vesicles. Their main advantage over steroids in testosterone replacement therapies is that they do not represent substrates for 5α-reductases, one main route of metabolism of steroids related to T. The resulting metabolic product of T, dihydrotestosterone (DHT), is considered a more potent androgenic steroid than T, and, due to the significant amount of DHT locally produced from T in organs such as the prostate, DHT is believed to amplify the androgenic activity of T. This effect is excluded by synthetic SARMs that are not amenable to this particular metabolic pathway.

Currently investigated SARMs can be categorized by their chemical structures into four classes: (1) aryl-propionamide; (2) bicyclic hydantoin; (3) quinoline; and (4) tetrahydroquinoline analogues.[7] In 1998, the first report was published on a successful preparation of an aryl-propionamide-based SARM. It was derived from a closely related androgen receptor antagonist bicalutamide (Scheme 1),[8] and since then numerous structurally related compounds have been tested for anabolic and/or androgenic potency.[9–11] The most promising substances were obtained by the substitution of the thioether function by a regular ether function as this resulted in metabolic stability, and two products (Ostarine and Andarine, GTx, Inc., Memphis, TN, USA) have recently entered clinical trials. The principal structure of selected aryl-propionamide-based SARMs is depicted in Scheme 1, and it

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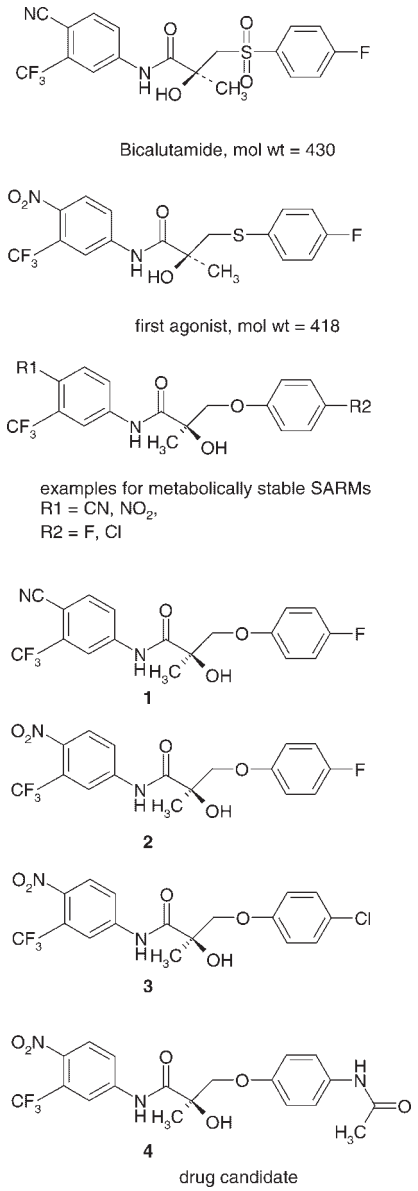


Scheme 1. Chemical structures of the androgen receptor antagonist bicalutamide, the first published SARM, and the synthesized model compounds **1–4** representing aryl-propionamide-derived SARMs.

consists of a *p*-substituted phenol residue linked to a 2-hydroxy-2-methylpropionamide functionality bearing a bisubstituted phenyl group.

Due to the promise shown by synthetic SARMs, in particular regarding anabolic properties such as muscle strength, body composition and bone density,[12] their potential for being misused in sports is extremely high, especially because of the considerably reduced side effects compared with conventional anabolic-androgenic steroids.[13] Hence, the need for screening procedures enabling doping control laboratories to detect and identify SARMs in human urine has become an important part of the fight against drug abuse in professional and amateur sport as the World Anti-Doping Agency (WADA) prohibits the use of any kind of anabolic agents.[14] Although SARMs are currently not officially available, doping control laboratories should get prepared to measure this class of compounds as incidences in the past have demonstrated that misused therapeutics are not necessarily clinically approved.[15–18] As a consequence, traditional doping control strategies have been complemented by preventive anti-doping research, an approach that includes the incorporation of new drugs or drug candidates into analytical screening protocols. In the present study we describe a novel assay to determine three selected aryl-propionamide-derived SARMs and related compounds in spiked urine specimens using liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) employing multiple reaction monitoring and simultaneous precursor ion scanning. The latter strategy in particular should provide comprehensive information on modified SARMs and metabolites as commonly employed in clinical metabolite research.[18–20]

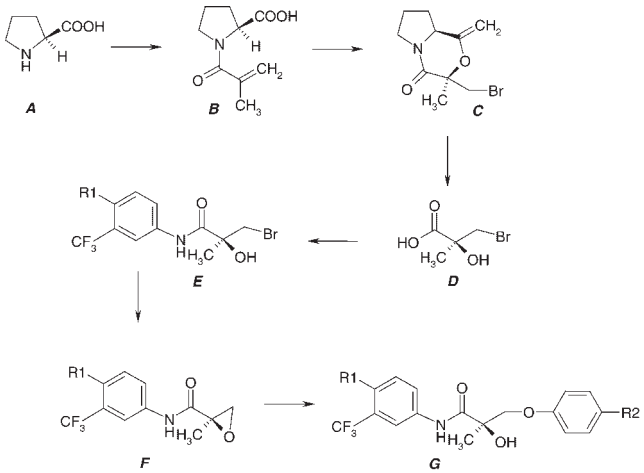
EXPERIMENTAL

Chemicals and reagents

S-Proline (99%), methacryloyl chloride (97%), *N*-bromosuccinimide (99%), thionyl chloride (99%), 4-nitro-3-(trifluoromethyl)aniline (98%), 4-cyano-3-(trifluoromethyl)aniline (97%), *N*,*N*-dimethylacetamide (absolute), 4-chlorophenol, acetaminophen (99%) and 4-fluorophenol (99%) were purchased from Sigma (Schnelldorf, Germany). Casodex (bicalutamide) was obtained from AstraZeneca (Wedel, Germany).

Synthesis of model compounds

The SARMs **1–4** (Scheme 1) were prepared in our laboratory according to established procedures,[10,21,22] as illustrated in a principal route of synthesis in Scheme 2. Briefly, S-proline (*A*) was converted into *N*-methacryloylproline (1-methacryloyl-pyrrolidine-2-carboxylic acid, *B*) using methacryloyl chloride. *N*-Bromosuccinimide was added to *B* yielding 3-bromomethyl-3-methyl-1-methylenetetrahydropyrrolo[2,1-c][1,4]oxazin-4-one (*C*), which was hydrolyzed using concentrated aqueous hydrochloric acid yielding 3-bromo-2-hydroxy-2-methylpropanoic acid (*D*). Substituted propionanilides (*E*) were obtained by converting *D* into its



Scheme 2. Principal route of synthesis to aryl-propionamide-based SARMs.

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Table 1. Measured elemental compositions of synthesized compounds **1–4**

Compound	Elemental composition (deprotonated species)	*m/z* (theor.)	*m/z* (exp.)	Error (ppm)
1	$C_{18}H_{13}O_3N_2F_4$	381.0857	381.0860	0.9
2	$C_{17}H_{13}O_5N_2F_4$	401.0749	401.0757	0.5
3	$C_{17}H_{13}O_5N_2F_3{}^{35}Cl$	417.0460	417.0462	0.6
4	$C_{19}H_{17}O_6N_3F_3$	440.1064	440.1067	0.6

corresponding propanoyl chloride and immediate reaction with respective anilines (e.g. 4-cyano-3-(trifluoromethyl)aniline). The epoxide *F* was prepared from *E* under alkaline conditions using potassium carbonate, and SARMs **1–4** (*G*) were obtained by reaction with appropriately *p*-substituted phenols.

Stock and working solutions

All solutions of target analytes were prepared in acetonitrile and stored at 2–8°C. The concentrations of stock and working solutions of SARMs were 1 mg/mL and 0.1 μg/mL, respectively, while the working solution of bicalutamide (internal standard, ISTD) was prepared at 1 μg/mL.

Electrospray ionization tandem mass spectrometry

ESI-MS(/MS) was performed on a ThermoFinnigan LTQ Orbitrap mass spectrometer (ThermoFinnigan, Bremen, Germany) employing negative ionization. The instrument was calibrated using the manufacturer's calibration mixture allowing for mass accuracies <6 ppm. Analytes were dissolved in acetonitrile/water (1:1, v/v) containing 0.1% formic acid at concentrations of 2 μg/mL and introduced into the mass spectrometer using a syringe pump at a flow rate of 5 μL/min. The ionization voltage was −3.5 kV, the capillary temperature was set to 300°C, and the $[M–H]^-$ precursor ions were dissociated using normalized collision energies between 25 and 35. The damping gas in the linear ion trap was helium 5.0, and the gas supplied to the curved linear ion trap (CLT) was nitrogen obtained from a CMC nitrogen generator (CMC Instruments, Eschborn, Germany).

Liquid chromatography/tandem mass spectrometry

LC/MS/MS analyses were performed on an Agilent 1100 Series HPLC system interfaced by ESI to an Applied Biosystems API3200 mass spectrometer. The LC system was equipped with a Macherey-Nagel C-18 Isis column (4 × 70 mm), and the eluents used were 5 mM ammonium acetate containing 0.1% acetic acid (mobile phase A) and acetonitrile (mobile phase B). A gradient was employed from 20% B to 100% B within 7 min, and after 1 min at 100% B the column was re-equilibrated at 20% B for 2.2 min. The flow rate was set to 800 μL/min, and the ion source was operated at 500°C. The analytes **1–3** and the ISTD biclautamide (Scheme 1) were detected by means of characteristic product ions generated from the deprotonated molecule by collision-induced dissociation (CID) utilizing the multiple reaction monitoring (MRM) mode. In addition, precursor ion scan experiments were carried out, using the product ions at *m/z* 261 and 241 as diagnostic for SARMs related to the model compounds **1–3**. The collision gas was nitrogen at 3.99e-3 Pa (obtained from a CTC nitrogen generator), and collision offset voltages (COV) for MRM analyses were optimized for each ion transition. In case of precursor ion scanning, the COV was set to −30 V, and the scan range was *m/z* 300–600 at a cycle time of 0.5 s. Target ion transitions of MRM experiments are listed in Table 2.

Urine sample preparation

Extraction of SARMs from human urine specimens was accomplished using solid-phase extraction (SPE) as described elsewhere.[23] Briefly, extraction cartridges (30 mg, 2 mL) were prepared from bulk PAD-1 material and preconditioned with 2 mL of methanol and 2 mL of deionized water. Then, a volume of 2 mL of urine (containing 10 ng/mL of ISTD) was added followed by a washing step with 2 mL of deionized water and an elution step with 2 mL of methanol. The methanolic layer was collected, evaporated to dryness,

Table 2. Characteristic product ions of $[M–H]^-$ of compounds **1–4** generated by CID. Determined masses represent average values (n = 30)

Compound	Precursor ion (*m/z*)	Product ions (*m/z*)	Elemental composition	*m/z* (theor.)	*m/z* (exp.)	Error (ppm)
1	381	381	$C_{18}H_{13}O_3N_2F_4$	381.0857	381.0866	2.3
		269	$C_{12}H_8O_2N_2F_3$	269.0532	269.0539	2.6
		241	$C_{11}H_8O_1N_2F_3$	241.0583	241.0592	3.5
		185	$C_8H_4N_2F_3$	185.0321	185.0331	5.5
2	401	401	$C_{17}H_{13}O_5N_2F_4$	401.0749	401.0757	0.5
		289	$C_{11}H_8O_4N_2F_3$	289.0431	289.0436	1.8
		261	$C_{10}H_8O_3N_2F_3$	261.0482	261.0488	2.4
		205	$C_7H_4O_2N_2F_3$	205.0220	205.0228	4.1
3	417	417	$C_{19}H_{17}O_6N_3F_3$	417.0460	417.0462	0.6
		289	$C_{11}H_8O_4N_2F_3$	289.0431	289.0436	1.9
		261	$C_{10}H_8O_3N_2F_3$	261.0482	261.0489	2.7
		205	$C_7H_4O_2N_2F_3$	205.0220	205.0228	4.1
4	440	440	$C_{17}H_{13}O_5N_2F_3Cl$	440.1064	440.1067	0.6
		289	$C_{11}H_8O_4N_2F_3$	289.0431	289.0436	1.8
		261	$C_{10}H_8O_3N_2F_3$	261.0482	261.0488	2.4
		205	$C_7H_4O_2N_2F_3$	205.0220	205.0228	4.1

the residue was reconstituted in 100 μL of acetonitrile, and a volume of 10 μL was injected into the LC/MS/MS system.

Assay validation for SARMs 1–3

In order to implement the model SARMs (**1**–**3**) into doping control routine analyses, the parameters lower limit of detection (LLOD), recovery, intra- and interday precision as well as specificity were determined according to ICH[24] guidelines and the requirements of WADA. Blank urine specimens necessary for the assay validation were obtained from ten different healthy volunteers (3 female, 7 male).

For screening purposes, the ion transitions *m/z* 381–241, 401–261 and 417–261 were chosen to detect the compounds **1**, **2** and **3**, respectively, while the confirmation of analytes was accomplished using the three ion transitions listed in Table 2. The ISTD was determined using the ion transition *m/z* 429–255. With all analyses, precursor ion scan experiments on the characteristic amide product ion (*vide infra*) were performed, as numerous modifications of aryl-propionamide-based SARMs are possible. These more general screening tools allow us to observe unknown or new chemically modified SARMs structurally related to the selected model compounds, as they are commonly used in metabolite identification or steroid analysis.

Lower limit of detection

The LLOD was defined as the 'lowest content that can be measured with reasonable statistical certainty'[25] at a signal-to-noise ratio ≥ 3. Ten different blank urine samples spiked with ISTD only, and another ten different blank urine specimens fortified with 1 ng/mL of each SARM model compound were prepared and analyzed according to the established protocol providing the data necessary to estimate the LLOD.

Recovery

The recovery of SARMs from human urine by SPE was determined at 10 ng/mL. Ten blank urine samples were fortified with **1**–**3** before sample preparation, and another ten blank urine specimens were extracted according to the described protocol followed by addition of 20 ng of **1**–**3** to the methanolic eluate. To both sets of samples, 20 ng of ISTD was spiked into the methanolic layer before evaporation. Recovery was calculated by comparison of mean peak area ratios of analyte and ISTD of samples fortified prior to and after SPE.

Intraday precision

During a 1-day period, ten urine samples of low (1 ng/mL), medium (5 ng/mL), and high (25 ng/mL) concentrations of SARMs were prepared and analyzed, and the intraday precision was calculated for each concentration level.

Interday precision

On three consecutive days, ten urine samples of low (1 ng/mL), medium (5 ng/mL), and high (25 ng/mL) concentrations of SARMs were prepared, analyzed randomly, and the assay interday precision was calculated for each concentration level.

Specificity

Twenty different urine specimens were prepared as described in order to probe for interfering peaks in the selected ion chromatograms at the expected retention times of **1**–**3**.

RESULTS AND DISCUSSION

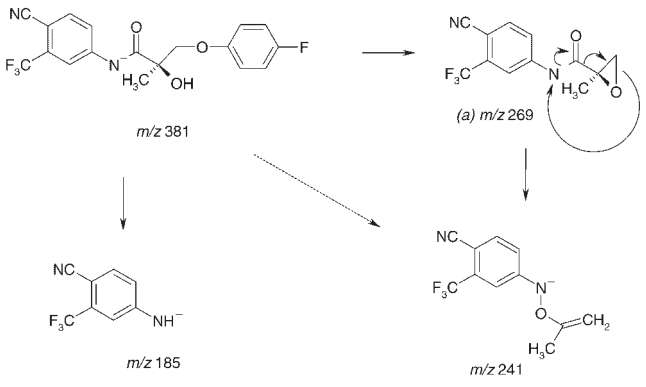
Synthesis of model compounds

The syntheses yielded compounds **1**–**4**, and accurate mass measurement employing ESI-MS on the LTQ Orbitrap mass analyzer allowed the determination of elemental compositions of protonated molecules (as shown in Table 1). The purity was greater than 90% as measured by means of LC/MS, and overall yields ranged from 5–12% of theory. The synthesis of these compounds has been optimized in several studies,[10,21,22] and a series of products resulted from the employed approach. Hence, structure confirmations of substances prepared in the present study using conventional strategies such as NMR or IR were not performed, and the determination of elemental compositions using high-resolution/high-accuracy mass analyses was considered sufficient.

Mass spectrometry

The product ion mass spectra of deprotonated SARMs (**1**–**4**) generated on the LTQ Orbitrap analyzer are depicted in Fig. 1. Following earlier studies, acidified solutions of SARMs were used for negative ionization as the addition of weak acids demonstrated favorable effects on the ionization of aryl-propionamide-based SARMs.[26]

The main fragmentation pathway of **1**–**3** yielded several abundant product ions, the proposed structures of which are illustrated in Scheme 3. The elimination of the substituted phenolic residue gave rise to *m/z* 269 or 289 for **1** or **2** and **3**, respectively, while a concomitant loss of carbon monoxide (−28 Da) generated the base peaks of respective product ion spectra (Fig. 1) at *m/z* 241 or 261. Based on high-resolution/accuracy mass analysis, the elemental compositions of these ions were determined (Table 2), substantiating the loss of CO and excluding the possibility of an elimination of ethylene. Further experiments were conducted with compound **1**, and, because *m/z* 241 was generated from *m/z* 269 in MS^3 in low



Scheme 3. Proposed dissociation pathway of SARM **1** yielding characteristic product ions at *m/z* 269, 241 and 185.

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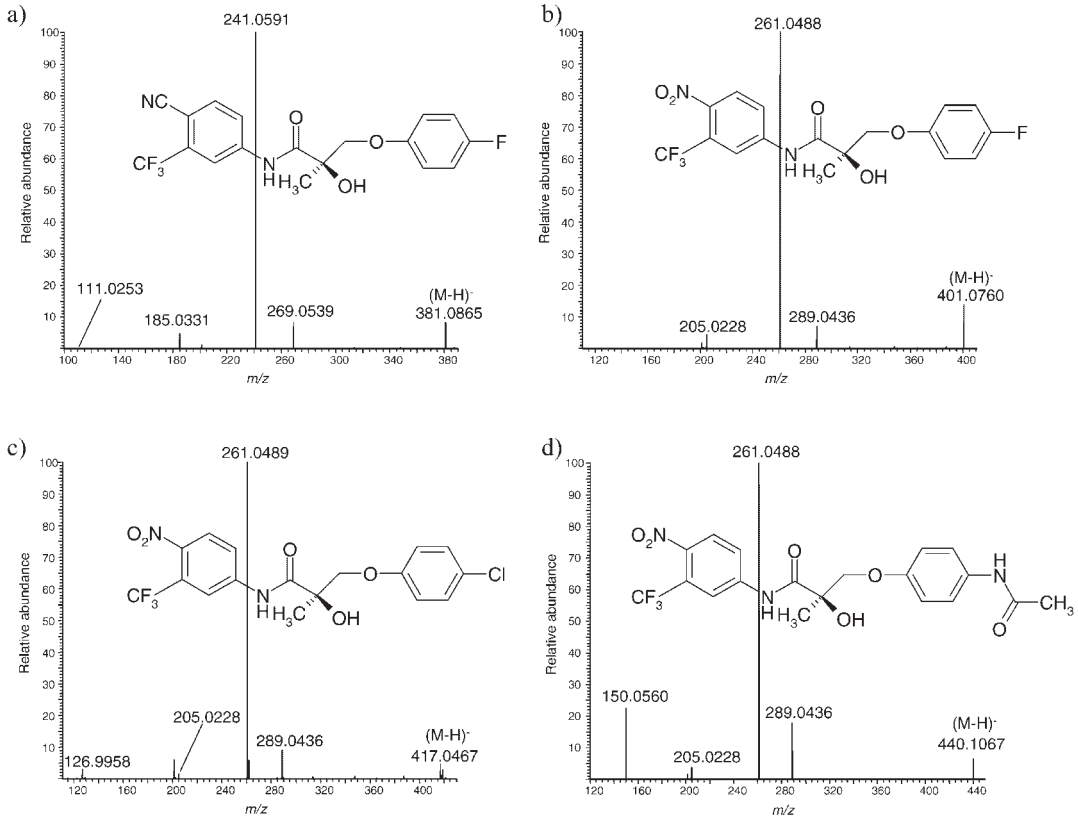

Figure 1. ESI product ion spectra of SARMs **1–4**. Negatively charged precursor ions $[M–H]^-$ were dissociated at normalized collision energies of 25 using an LTQ Orbitrap mass analyzer.

abundance only, a direct formation of *m/z* 241 from the deprotonated molecule is suggested as the major dissociation route. The ion at *m/z* 269 is proposed to have an epoxide-like structure corresponding to compound **1** (*a*, Scheme 3), which was obtained as an intermediate substance (Scheme 2) in the course of the synthesis of **1**. Hence, product ion mass spectra resulting from MS^2 and MS^3 experiments on the deprotonated epoxide (Scheme 3, *a*) and on *m/z* 269 generated from compound **1**, respectively, were compared. In both product ion spectra identical ions were observed (Table 3) substantiating the suggested structure, but different relative abundances were found using identical CID conditions.

Another ion in the product ion mass spectra of **1–3** was obtained from the cleavage of the amide bond yielding the deprotonated and bisubstituted amine, i.e. *m/z* 185 or 205 for **1** or **2** and **3**, respectively (Fig. 1, Scheme 3).

Assay validation for SARMs 1–3

Assay validation performed for human urine specimens provided sets of data demonstrating the excellent performance of the assay, thus allowing for the sensitive determination of model compounds **1–3** and related substances in applications employed for doping controls. The validation results are summarized in Table 4, and typical extracted ion chromatograms representing a positive control urine spiked at 1 ng/mL are presented in Fig. 2(a).

Specificity

Specificity was supported by the absence of any interfering signal at the expected retention times in the extracted ion chromatograms of diagnostic product ions of **1–3** (Table 4).

Table 3. Characteristic product ions of $[M–H]^-$ of the epoxide intermediate of compound **1** (Scheme 3, *a*) and *m/z* 269 obtained from compound **1** in MS^3 experiments. Determined masses represent average values (n = 30)

Compound	Precursor ion (*m/z*)	Product ions (*m/z*)	Elemental composition	*m/z* (theor.)	*m/z* (exp.)	Error (ppm)
Epoxide (*a*)	269	241	$C_{11}H_8O_1N_2F_3$	241.0583	241.0593	4.2
		227	$C_{10}H_6O_1N_2F_3$	227.0427	227.0437	4.5
		185	$C_8H_4N_2F_3$	185.0321	185.0332	5.8
1	318–269	241	$C_{11}H_8O_1N_2F_3$	241.0583	241.0591	3.3
		227	$C_{10}H_6O_1N_2F_3$	227.0427	227.0435	3.6
		185	$C_8H_4N_2F_3$	185.0321	185.0331	5.4

Table 4. Summary of validation results

			Intraday precision (n = 30)		Interday precision (n = 90)	
Compound	LLOD (ng/mL)	Recovery (%) at 100 ng/mL	Concentration (ng/mL)	CV (%)	Concentration (ng/mL)	CV (%)
1	1	105	1	9.8	1	11.3
			5	7.6	5	11.0
			25	9.7	25	10.3
2	1	85	1	8.9	1	12.8
			5	10.4	5	14.2
			25	11.6	25	11.6
3	1	87	1	8.7	1	14.4
			5	9.3	5	9.9
			25	10.5	25	10.8

Recovery

The comparison of peak area ratios of urine samples fortified with **1–3** prior to and after SPE allowed the calculation of recoveries as between 85 and 105% (Table 4).

Lower limit of detection

The LLODs of **1–3** were determined at 1 ng/mL using the three diagnostic ion transitions shown in Table 2.

Intraday and interday precision

The intraday precision ranged from 8.7–11.6% for all tested model compounds (**1–3**). The interday precision varied from 9.9–14.4%, as presented in Table 4.

Precursor ion scanning

In order to test the capability of the established assay to determine structurally related compounds, two precursor ion

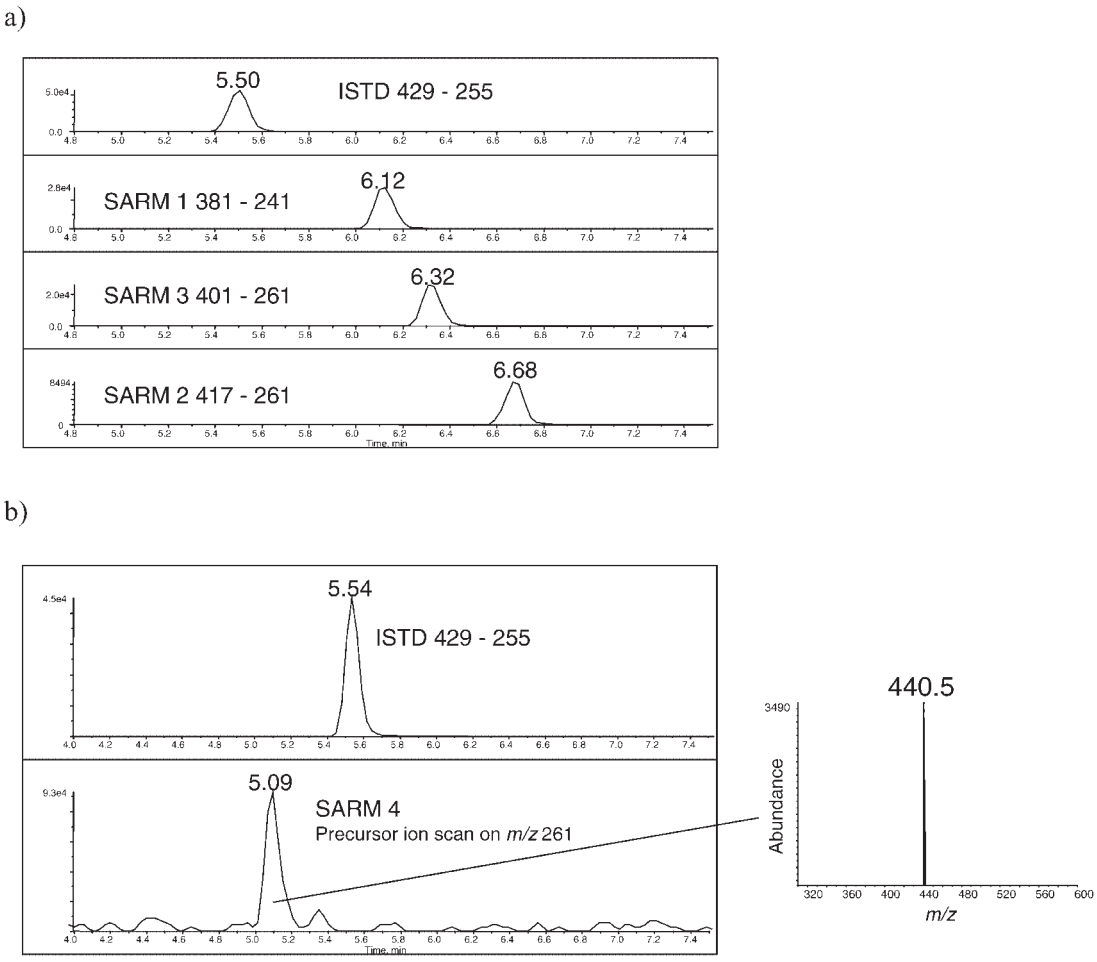

Figure 2. (a) Extracted ion chromatograms of a urine sample fortified with 1 ng/mL of SARMs **1–3**. Characteristic product ions generated from deprotonated molecules allow the unambiguous determination of the target compounds: SARM **1** (381–241), SARM **2** (417–261) and SARM **3** (401–261). (b) Total ion chromatogram of a precursor ion scan of a urine sample fortified with 50 ng/mL of SARM **4**. A distinct signal is observed at 5.1 min generated by the deprotonated molecule of **4** at *m/z* 440, as shown in the corresponding mass spectrum.

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scan experiments on the ions at *m/z* 261 and 241 were included in each analysis. These abundant ions are characteristic for aryl-propionamide-derived SARM structures and allow coverage of a large series of drug candidates described in the literature.[9–11] One selected candidate (Scheme 1, **4**), which has recently been tested as a promising SARM,[27] was synthesized according to the principal protocol described above and analyzed in urine specimens at 5 and 50 ng/mL using the precursor ion scan option. In all blank urine specimens analyzed for assay specificity, no signal was detected in both precursor ion scan chromatograms (*m/z* 261 and 241), and the remarkably low background noise was the basis of a sensitive screening method for substances related to the selected model compounds. While the sensitivity of the LC/MS/MS system was not sufficient to generate signal intensities allowing a mass spectrometric evaluation at a concentration of 5 ng/mL of **4**, 50 ng/mL of **4** gave rise to a considerable signal in a precursor ion scan chromatogram (Fig. 2(b)) allowing for follow-up analyses such as product ion scan experiments on detected precursor ions. According to metabolism studies, the main metabolic product of **4** is its deacetylated counterpart, the product ion spectrum of which also contains an abundant ion at *m/z* 269.[27] In addition, recent rat metabolism studies demonstrated the generation of numerous metabolites of **2** also yielding the diagnostic product ion at *m/z* 269.[28] Hence, it is expected that putative metabolites of aryl-propionamide-based SARMs should be detectable with the presented procedure.

CONCLUSIONS

Selective androgen receptor modulators (SARMs) are a promising class of anabolic therapeutics that are likely to be being misused in professional and amateur sports. Four model SARMs with aryl-propionamide nucleus have been synthesized and used to establish a fast, sensitive and robust screening assay using LC/MS/MS. In addition to common MS/MS target analyses, precursor ion scanning was included into the screening procedure allowing the detection of substances related to the prepared model compounds by means of diagnostic and abundant product ions. Detection limits of precursor ion scan experiments can only be estimated at approximately 50 ng/mL as physicochemical properties of analytes strongly influence their ionization as well as their CID efficiencies and thus resulting signal intensities in LC/MS/MS analyses. Although SARMs are not yet commercially available, sports drug testing facilities should become prepared for this new class of therapeutics as history has demonstrated that pharmaceutical products may be misused even before clinical trials are completed.

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