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Screening for 2-quinolinone-derived selective androgen receptor agonists in doping control analysis

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Selective androgen receptor modulators (SARMs) represent a class of emerging drugs with high potential for misuse in sports, and therefore members of this group are banned as anabolic agents by the World Anti-Doping Agency. Preventive approaches to restrict their use include early implementation of target analytes into doping control screening assays and evaluation of the mass spectrometric behavior of these drugs to allow their unequivocal identification as well as the characterization of structurally related compounds and metabolic products. Four model SARMs with the 6-alkylamino-2-quinolinone structure, including the advanced drug candidate LGD-2226, were synthesized. Fragmentation pathways after positive electrospray ionization and collision-induced dissociation were studied using an LTQ Orbitrap mass analyzer, and diagnostic product ions and common dissociation pathways were employed to establish a screening procedure targeting intact quinolinone-based SARMs as well as putative metabolic products such as dealkylated analogues. Therefore, features of a triple quadrupole mass analyzer such as multiple reaction monitoring and precursor ion scanning were utilized. Sample preparation based on commonly employed liquid-liquid extraction and subsequent liquid chromatographic/tandem mass spectrometric measurement allowed for detection limits of 0.01–0.2 ng/mL, and intra- and interday precisions between 3.2 and 8.5% and between 6.3 and 16.6%, respectively. Recoveries varied from 81 to 98%, and tests for ion suppression or enhancement effects were negative for all analytes. Copyright © 2007 John Wiley & Sons, Ltd.

The search for anabolic agents with reduced androgenic side effects has been of great interest ever since the beneficial effects of anabolic steroids such as testosterone and its synthetic derivatives became evident. Steroid replacement therapies have been employed to counteract symptoms of wasting diseases such as cancer and AIDS, for the treatment of osteoporosis, but also in cases of hypogonadism and testosterone deficiencies,[1–4] as endogenous androgens (e.g. testosterone and its metabolite dihydrotestosterone) are of particular importance for the masculinization of males including both, primary and secondary characteristics.[5] In particular, the latter comprise numerous aspects that are of paramount importance for body conformation and performance such as the growth of muscles and bones, the predisposition of protein anabolism and erythrocyte production.[6]

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The considerable number of side effects associated with therapies based on steroid replacement, particularly with synthetic derivatives of testosterone, has led to the development of non-steroidal agents that selectively activate the androgen receptor in target tissues without affecting other organs. These so-called selective androgen receptor modulators (SARMs) provide interesting clinical benefits and are intended for the prevention and treatment of osteoporosis, sarcopenia, frailty, muscle wasting, but also for use as male contraceptives without causing undesired effects related to the administration of anabolic steroids such as liver toxicity, prostate hypertrophy and acne. Currently, hundreds of drug candidates based on various chemical structures are in preclinical or clinical trials. A rough categorization allows the classification of SARMs into propionanilides, hydantoins, quinolines, tetrahydroquinolines and benzimidazole derivatives, as reviewed in recent articles.[7,8] Drugs belonging to the first-mentioned group have recently completed phase II clinical trials, and representatives of all other classes have also demonstrated efficient receptor binding and muscle tissue selectivity. Due to their great anabolic potential and

reduced androgenic side effect profile, doping control authorities expect that SARMs will have a considerable impact on drug abuse in sports in the near future.[8–10] Doping control analytical assays were reported recently for arylpropionamide- and hydantoin-derived SARMs,[11,12] and the present study is focused on representatives of quinoline-based compounds that proved oral bioavailability and efficient androgen receptor activation in bones and muscles.[13] The dissociation behavior of four model compounds (Scheme 1) after electrospray ionization (ESI) and high resolution/high accuracy tandem mass spectrometry was studied, and a sports drug testing assay was established based on diagnostic product ions and characteristic dissociation routes as measured on triple quadrupole instruments.

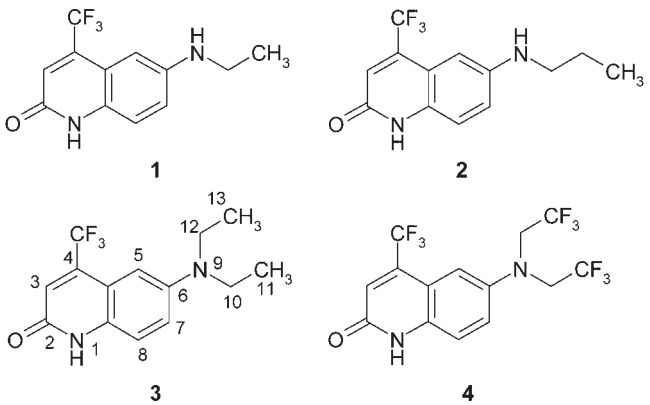
EXPERIMENTAL

Chemicals and reagents

Ethyl-4,4,4-trifluoroacetoacetate (98%), aniline (99.5+%), palladium on charcoal (10%), propionaldehyde (97%), trifluoroacetic acid (>99%) and sodium cyanoborohydride (95%) were purchased from Sigma (Deisendorf, Germany). Ethanol (abs., p.a.), acetic acid (100%, p.a.) and sulphuric acid (95–97%, p.a.) were obtained from Merck (Darmstadt, Germany). Potassium carbonate (p.a.) and sodium hydrogen carbonate (anhydrous, p.a.) were purchased from Merck. *t*-Butyl methyl ether (distilled before use) was obtained from KMF (St. Augustin, Germany). The *β*-glucuronidase (*E. coli*) enzyme was supplied by Roche Diagnostics (Mannheim, Germany), and sodium dihydrogen phosphate monohydrate (p.a.) and disodium hydrogen phosphate dihyhdrate (p.a.) were from Sigma. Deionized water used for sample preparation and buffer solutions was of MilliQ grade.

Synthesis and characterization of model compounds

In order to allow method development aiming at preventive doping research, therapeutics in clinical studies are of considerable interest and should be implemented in new screening and confirmation assays as soon as possible. Hence, four model SARMs with 6-bisalkylamino-2-quinolinone structure were prepared according to procedures published elsewhere,[14,15] providing substances with physicochemical properties resembling those of potential drug targets in future doping control analyses. Briefly, the common nucleus of all quinolinone-based SARMs was prepared by condensing aniline with ethyl-4,4,4-trifluoroacetoacetate followed by 6-nitrosylation of the resulting 4-trifluoromethylquinolin-2(*1H*)-one. The nitro residue was reduced to its corresponding amino function, which was subsequently alkylated to 6-*N*-monoethyl-, monopropyl-, bisethyl- and bistrifluoroethylamino-4-trifluoromethylquinolin-2(*1H*)-ones (Scheme 1, **1**, **2**, **3** and **4**, respectively). The final products were purified by flash chromatography on silica gel using *n*-hexane and ethyl acetate (4:1, v/v).



Scheme 1. Chemical structures of four 2-quinolinone-derived model SARMs: **1** (mol wt = 256), **2** (mol wt = 270), **3** (mol wt = 284), and **4** (LGD2226, mol wt = 392).

The synthesized compounds were characterized by nuclear magnetic resonance (NMR) spectroscopy with ^{1}H, H,H-COSY, H,C HMQC, and H,C HMBC experiments employing a Bruker AVANCE II 600 MHz instrument (Bruker, Karlsruhe, Germany) equipped with a 5 mm TBI probehead (z-gradient coil). Approximately 10 mg of each compound was dissolved in deuterated dimethyl sulfoxide (d_6-DMSO) or d_4-methanol, and spectra were recorded at room temperature. In addition, the elemental composition of each substance was determined using high resolution/high accuracy mass spectrometry utilizing an LTQ Orbitrap (Thermo, Bremen, Germany) at a resolving power of 30 000.

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 were 1 mg/mL and 1 μg/mL, respectively. Over a period of 4 weeks no degradation was observed in either of the solutions.

Electrospray ionization tandem mass spectrometry

ESI-MS(/MS) was performed on a Thermo LTQ Orbitrap mass spectrometer. The instrument was operated in positive ion mode and calibrated using the manufacturer's calibration mixture (consisting of caffeine, MRFA and Ultramark). Mass accuracies of <2 ppm (calculated from 30 averaged spectra) were achieved for the period of analysis. The 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 3500 V, the capillary temperature was set to 290°C, and the precursor ions (protonated molecules) were isolated using a width of 2 *m/z* units. The protonated species were dissociated at normalized collision energies between 25 and 35. The damping gas in the linear ion trap was helium (purity grade 5.0), and gas supplied to the curved linear ion trap (CLT) was nitrogen obtained from a nitrogen generator (CMC Instruments, Eschborn, Germany).

Liquid chromatography/tandem mass spectrometry

All analyses were performed using an Agilent 1100 Series liquid chromatograph (Waldbronn, Germany) coupled to an Applied Biosystems API 4000 QTrap mass spectrometer

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(Darmstadt, Germany) with an electrospray ionization (ESI) source. The liquid chromatograph was equipped with a Macherey-Nagel Sphinx column (4.0 × 70 mm, 5 μm particle size), 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 starting at 30% B increasing to 100% B within 4.5 min followed by re-equilibration at 30% B for 1.5 min. The flow rate was set to 800 μL/min. The ion source was operated in the positive ion mode at 550°C using a spray voltage of 5500 V. All four analytes (Scheme 1) as well as the internal standard (ISTD, methyltestosterone) were detected by means of characteristic product ions formed from the protonated molecules by collision-induced dissociation (CID) utilizing the multiple reaction monitoring (MRM) mode as listed in Table 1. Nitrogen was employed as curtain and collision gas (5×10^{-3} Pa) delivered from a CMC nitrogen generator, and collision offset voltages were optimized for each product ion (Table 1).

Sample preparation

The sample preparation was performed according to a method previously described for the detection of selected anabolic androgenic steroids.[16] Briefly, a volume of 2 mL of urine was buffered to pH 7.0 (750 μL of a sodium phosphate buffer (0.2 M, Na_2HPO_4/NaH_2PO_4, 1:2, w/w)) and 200 ng of methyltestosterone (20 μL of a 10 ng/μL solution) and 20 μL of β-glucuronidase were added. After incubation at 50°C for 1 h, liquid-liquid extraction (LLE) was performed at pH 9.6 (established by the addition of 200 mg of a mixture of K_2CO_3 and $NaHCO_3$ (2:1, w/w)) with 4 mL of *t*-butyl methyl ether by shaking the sample for 15 min and centrifuging at 620 *g* for 5 min. The organic layer was transferred into a fresh test tube, evaporated to dryness employing a rotary evaporator at reduced pressure, and the dry residue was reconstituted in 100 μL of water/acetonitrile (4:1, v/v). The solution was transferred to HPLC vials and 10 μL was injected into the LC/MS/MS system.

Assay validation

The qualitative determination of quinolinone-derived SARMs in human urine was validated regarding specificity, recovery, lower limit of detection (LLOD), and intra- and interday precision according to ICH guidelines.[17] The respective items were defined and tested as follows:

Specificity

Ten different blank urine specimens (6 male and 4 female urine samples) were prepared as described in order to probe for interfering peaks in the selected ion chromatograms at the expected retention times for all target analytes.

Recovery

The recovery of all analytes was determined at 100 ng/mL. Ten blank specimens were fortified with the target analytes before sample preparation, and another ten blank samples were extracted according to the described protocol followed by addition of the analytes into the final sample extract. To both sets of samples, 200 ng of methyltestosterone (ISTD) were spiked into the ether layer before evaporation. Recoveries were calculated by comparison of mean peak area ratios of analytes and ISTD of samples fortified prior to and after LLE.

Lower limit of detection (LLOD)

The LLOD was defined as the 'lowest content that can be measured with reasonable statistical certainty'[18] at a signal-to-noise (S/N) ratio ≥3. Ten blank urine samples were spiked with the ISTD only. Ten additional blank urine specimens were fortified with 1 ng/mL of all four quinolinone derivatives. The samples were prepared and analyzed according to the established protocol, providing the data necessary to estimate the LLOD.

Intraday precision

Within 1 day, ten urine samples of low (1 ng/mL), medium (10 ng/mL), and high (100 ng/mL) concentrations of all model SARMs were prepared and analyzed, and the intraday precision was calculated for each concentration level.

Interday precision

On three consecutive days, a total of 30 urine samples of low, medium and high concentrations (1, 10 and 100 ng/mL, respectively) were prepared and analyzed randomly, and the assay precision was calculated for each concentration level.

Table 1. MS parameters for multiple reaction monitoring of four model SARMs

Compound	Declustering potential (V)	Ion transition (*m*/*z*)	Collision offset voltage (V)	Dwell time (ms)
1	85	257 – 200	85	40
	85	257 – 224	85	40
	85	257 – 228	80	40
2	91	271 – 224	43	40
	91	271 – 228	37	40
	91	271 – 229	27	40
3	21	285 – 213	63	40
	21	285 – 241	47	40
	21	285 – 256	29	40
4	76	393 – 203	87	40
	76	393 – 223	69	40
	76	393 – 241	57	40
Methyltestosterone	70	303 – 109	40	40

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Test for ion suppression/enhancement effects

In order to estimate ion suppression effects possibly caused by matrix interference, four different blank urine samples and solvent only were analyzed with continuous co-infusion of the target analytes (solution concentration 0.5 pg/μL, flow rate 5 μL/min) via a T-connector according to literature recommendations.[19,20]

Administration study urine samples

Assay validations commonly include analyses of administration study urine specimens. As the presented compounds have not yet entered the pharmaceutical market, no authentic urine samples but only spiked specimens were measured.

RESULTS AND DISCUSSION

The development of doping control screening methods for emerging drugs necessitates adequately characterized reference compounds that allow the validation of procedures and proof of concept that the established method is capable of detecting the analytes relevant for doping controls. However, the unknown metabolic fate as well as the chemical diversity and, thus, virtually unlimited variety of related drugs, complicate a targeted screening. Generalized and comprehensive methods as for instance enabled by precursor ion or neutral loss scan experiments using product ions characteristic of common SARMs nuclei might become necessary.

Synthesis and characterization of model SARMs

The syntheses yielded the intended structures with overall yields ranging from 6–18%, which provided sufficient amounts of pure analytes necessary for method development and structural characterization. All four model SARMs were characterized by NMR analyses: compound **1** (^{1}H NMR, 600 MHz, d_6-DMSO) δ: 12.02 (s, 1H, H-1), 6.86 (s, 1H, H-3), 6.69 (m, 1H, H-5), 7.03 (dd, $J = 8.9$ Hz, 2.3 Hz, 1H, H-7), 7.24 (d, $J = 8.9$ Hz, 1H, H-8), 5.83 (m, 1H, H-9), 3.03 (m, 2H, H-10), 1.18 (t, 3H, H-11); compound **2** (^{1}H NMR, 600 MHz, d_6-DMSO) δ: 12.01 (s, 1H, H-1), 6.86 (s, 1H, H-3), 6.69 (m, 1H, H-5), 7.04 (dd, $J = 9.0$ Hz, 2.4 Hz, 1H, H-7), 7.23 (d, $J = 8.9$, 1H, H-8), 5.87 (t, 1H, H-9), 2.96 (m, 2H, H-10), 1.57 (m, 2H, H-11), 0.94 (t, 3H, H-12); compound **3** (^{1}H NMR, 600 MHz, d_4-methanol) δ: 6.96 (s, 1H, H-3), 6.93 (m, 1H, H-5), 7.25 (dd, $J = 9.3$ Hz, 2.6 Hz, 1H, H-7), 7.35 (d, $J = 9.2$, 1H, H-8), 3.44 (dd, 2H, H-10), 1.19 (t, 3H, H-11), 3.44 (dd, 2H, H-12), 1.19 (t, 3H, H-13); compound **4** (^{1}H NMR, 600 MHz, d_6-DMSO) δ: 12.19 (s, 1H, H-1), 6.96 (s, 1H, H-3), 7.16 (m, 1H, H-5), 7.57 (dd, $J = 9.2$ Hz, 2.7 Hz, 1H, H-7), 7.37 (d, $J = 9.0$, 1H, H-8), 4.38 (q, 2H, H-10), 4.38 (q, 2H, H-12).

Electrospray ionization tandem mass spectrometry

The CID behavior of model target analytes **1**–**4** was studied employing high resolution/high accuracy (tandem) mass spectrometry using a hybrid linear ion trap-orbitrap instrument. The characterization of dissociation pathways of new emerging therapeutics is of particular importance in sports drug testing as the willingness of some athletes to misuse compounds with or without clinical approval has often been reported.[21–23] Drugs that have never been marketed as well as designer steroids were determined, demonstrating that doping control laboratories need sensitive screening assays with a comprehensive knowledge about mass spectrometric fragmentation pathways of distinct categories of therapeutics to identify new or unknown substances in urine specimens.

Common dissociation routes of all four model compounds were elucidated based on MS^n experiments and accurate mass measurements. Product ion mass spectra are depicted in Figs. 1(a)–1(d), calculated elemental compositions of product ions as obtained from MS^n measurements are listed in Table 2, and major fragmentation pathways are presented using compound **3**. The protonated molecule of **3**, $[M+H]^+$ at *m/z* 285, dissociates efficiently under CID conditions, as shown in Fig. 1(c). Protonation is suggested to occur at the bis-alkylated amino function initiating the homolytic or heterolytic cleavage of a C–N bond yielding the product ions $[M+H-29]^+$ and $[M+H-28]^+$ at *m/z* 256 and 257, respectively (Scheme 2). The formation of odd-electron product ions from an even-electron precursor ion is usually not favored under ESI-CID conditions but is explained by an extensive conjugated π-electron system that comprises the entire quinolinone structure of the analytes. The resulting radical cation at *m/z* 256 subsequently eliminated a methyl radical (15 Da) yielding *m/z* 241, as demonstrated in MS^3 experiments (Table 2), which subsequently released a methylimine radical (28 Da, Table 2). Essentially identical behavior was observed with the bis-trifluoroethylated analogue (compound **4**) that underwent consecutive losses of trifluoroethyl (83 Da, yielding *m/z* 310) and trifluoromethyl (69 Da) radicals giving rise to the common product ion at *m/z* 241 (Fig. 1(d), Table 2). However, the precursor ion at *m/z* 393 demonstrated considerably higher stability than its non-halogenated counterpart **3** due to the exchange of mobile hydrogen by fluorine atoms, and higher collision energies were required to generate the spectrum shown in Fig. 1(d). In contrast to these common features of **3** and **4**, the elimination of ethylene (28 Da) from **3** yielding the even-electron product ion at *m/z* 257 was not observed with substance **4**. However, the product ion at *m/z* 257 resembles the protonated precursor of compound **1**. The neutral loss of C_2H_4 did not trigger the loss of a methyl radical from the remaining alkyl side chain in MS^3 experiments but another neutral loss of ethylene producing the protonated 6-amino-quinolin-2-one at *m/z* 229 (Scheme 2, Table 2). This behavior was also observed in MS/MS measurements of **1**, presumably comprising the same composition and structure as the product ion at *m/z* 257 derived from **3**. This product ion gave rise to further dissociation products originating from the elimination of water (18 Da, *m/z* 239) as well as the release of a trifluoromethyl radical (69 Da, *m/z* 188), as illustrated in the inset of Fig. 1(a). A summary of the proposed dissociation pathway is presented in Scheme 2, and the major outcome of the mass spectrometric study of dissociation pathways of these model SARMs is the generation of common product ions at *m/z* 241 and 229 from bis-alkylated and mono-alkylated 6-amino-2-quinolinone-derived SARMs, respectively.

The use of triple quadrupole/QTrap analyzers, however, yielded product ion mass spectra differing considerably in

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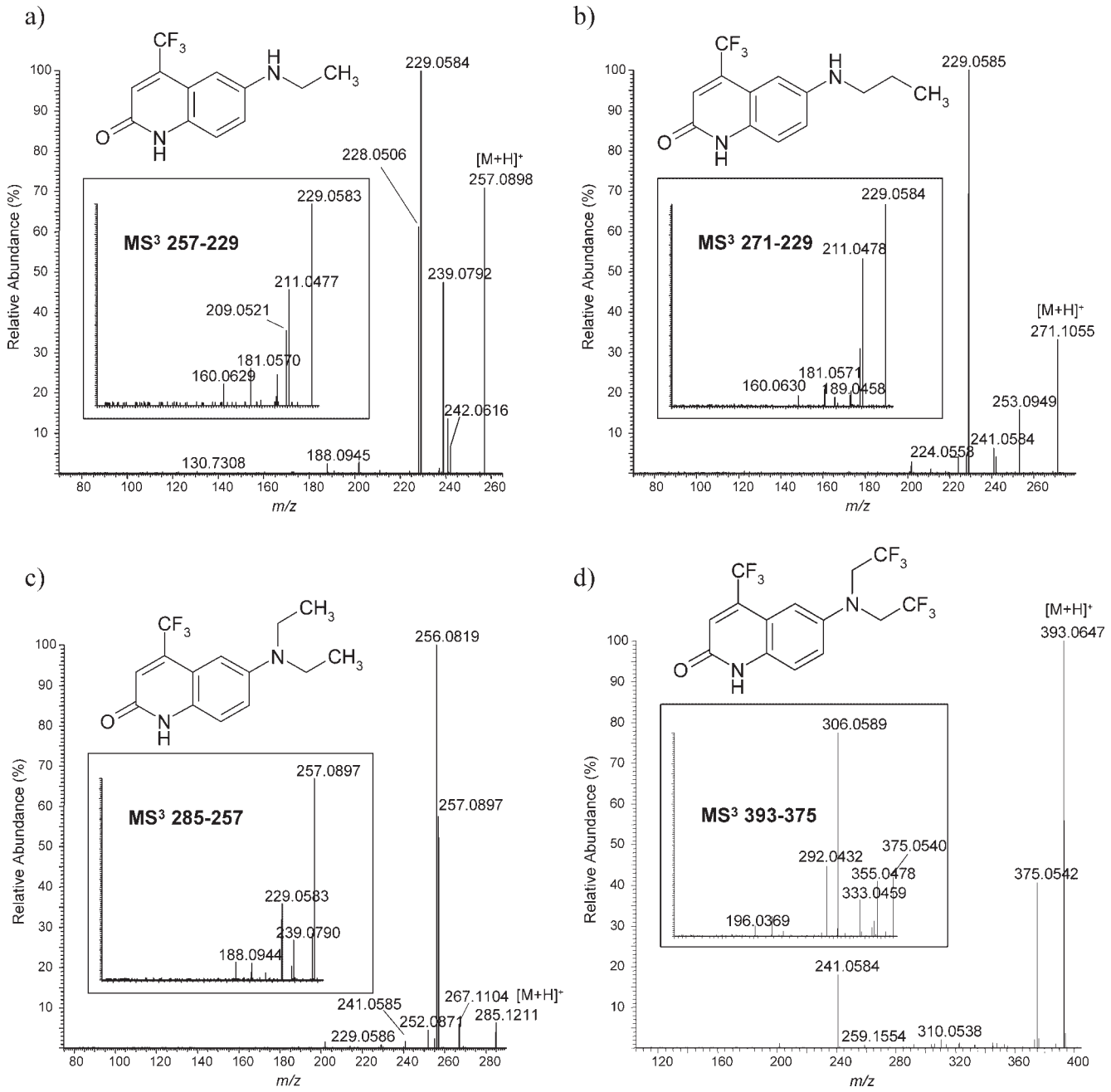

Figure 1. ESI product ion spectra of protonated molecules of four 2-quinolinone-derived model SARMs measured on an LTQ-Orbitrap: (a) compound **1**, collision energy = 30 arbitrary units; (b) compound **2**, collision energy = 25 arbitrary units; (c) compound **3**, collision energy = 25 arbitrary units; and (d) compound **4**, collision energy = 40 arbitrary units. MS^3 experiments yielded spectra shown as insets in respective MS/MS product ion spectra.

the relative abundances of product ions, as illustrated in Figs. 2(a)–2(d). While the even-electron ion at *m/z* 229 was the base peak in the LTQ Orbitrap product ion spectra of mono-alkylated SARMs (Figs. 1(a) and 1(b)), the corresponding radical cation at *m/z* 228 was primarily observed in triple quadrupole CID spectra (Figs. 2(a) and 2(b)). Also for bis-alkylated analogues the dissociation behavior was found to be different in triple quadrupole analyzers, yielding a base peak at *m/z* 241 (Figs. 2(c) and 2(d)), the intensity of which was considerably lower in LTQ Orbitrap product ion spectra (Figs. 1(c) and 1(d)). Consequently, precursor ion scans were conducted utilizing product ions of superior abundance and identification power such as *m/z* 228 and 241, and MRM experiments for targeted analyses were based on ion transitions providing the greatest S/N ratios (Table 1). The different behavior of the analytes in ion trap or triple quadrupole tandem mass spectrometry is attributed to the reactive character of the generated radical cations that undergo further dissociations when stored in the ion trap device.

Assay validation

Based on the mass spectrometric data, an assay for the qualitative determination of SARMs with 2-quinolinone structure was established and validated for doping control purposes. The validation results are summarized in Table 3.

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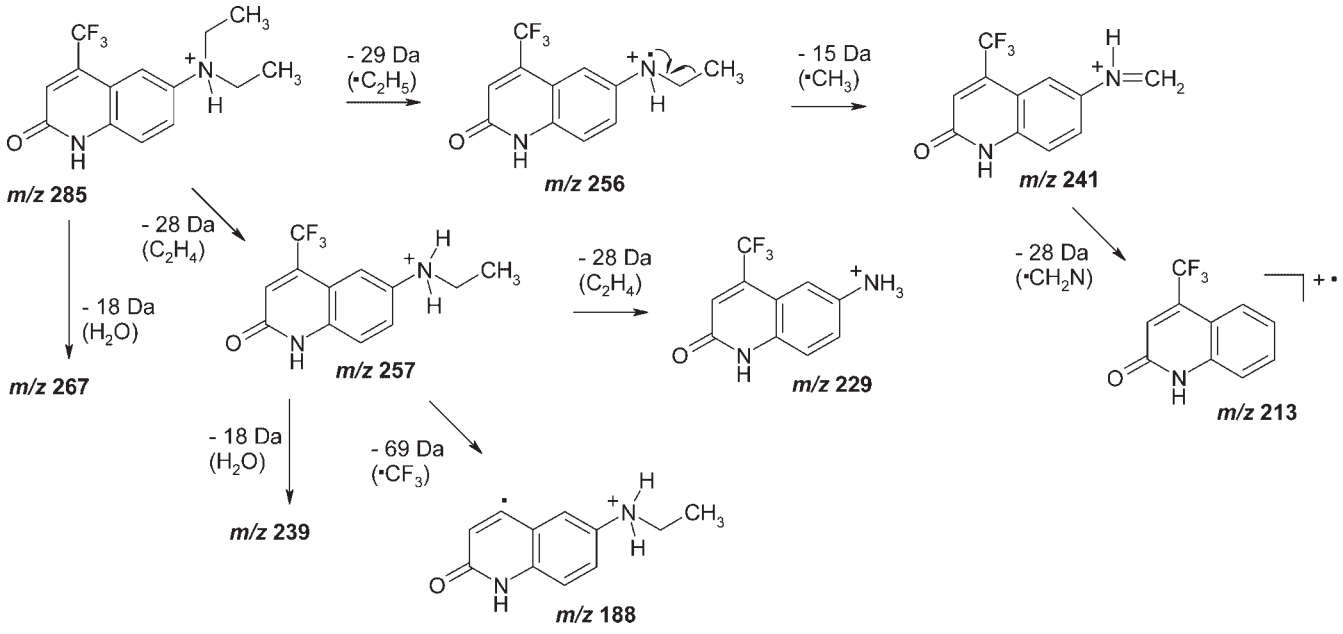
Table 2. Elemental compositions of protonated molecules of **1–4** and resulting product ions using high resolution/high accuracy MS^n experiments

Compound	Precursor ion (*m/z*)		Elemental comp. (exp.)	Error (ppm)	Collision energy (arb. units)	Product ion (*m/z*)	Elemental comp. (exp.)	Error (ppm)	Cleaved species
	MS^2	MS^3							
1	257.0898		$C_{12}H_{12}ON_2F_3$	0.6	30	239.0792	$C_{12}H_{10}N_2F_3$	0.4	H_2O
						229.0584	$C_{10}H_8ON_2F_3$	0.3	C_2H_4
						228.0506	$C_{10}H_7ON_2F_3$	0.6	C_2H_5
						188.0945	$C_{11}H_{12}ON_2$	0.3	CF_3
		239.0790	$C_{12}H_{10}N_2F_3$	0.1	20	224.0555	$C_{11}H_7N_2F_3$	−0.2	CH_3
						211.0478	$C_{10}H_6N_2F_3$	0.0	C_2H_4
		229.0583	$C_{10}H_8ON_2F_3$	0.1	25	211.0477	$C_{10}H_6N_2F_3$	−0.1	H_2O
						209.0521	$C_{10}H_7ON_2F_2$	−0.1	HF
		228.0505	$C_{10}H_7ON_2F_3$	−0.1	20	200.0555	$C_9H_7N_2F_3$	−0.5	CO
2	271.1054		$C_{13}H_{14}ON_2F_3$	0.4	25	253.0949	$C_{13}H_{12}N_2F_3$	0.8	H_2O
						241.0584	$C_{11}H_8ON_2F_3$	0.5	C_2H_6
						229.0584	$C_{10}H_8ON_2F_3$	0.3	C_3H_6
		253.0948	$C_{13}H_{12}N_2F_3$	0.3	15	224.0556	$C_{11}H_7N_2F_3$	0.2	C_2H_5
						211.0478	$C_{10}H_6N_2F_3$	0.1	C_3H_6
		229.0584	$C_{10}H_8ON_2F_3$	0.3	25	211.0478	$C_{10}H_6N_2F_3$	0.2	H_2O
						209.0521	$C_{10}H_7ON_2F_2$	−0.1	HF
						160.0630	$C_9H_8ON_2$	−0.7	CF_3
3	285.1209		$C_{14}H_{16}ON_2F_3$	0.1	25	267.1103	$C_{14}H_{14}N_2F_3$	−0.3	H_2O
						257.0895	$C_{12}H_{12}ON_2F_3$	−0.4	C_2H_4
						256.0818	$C_{12}H_{11}ON_2F_3$	0.2	C_2H_5
						241.0584	$C_{11}H_8ON_2F_3$	0.3	C_3H_8
		257.0897	$C_{12}H_{12}ON_2F_3$	0.1	20	239.0791	$C_{12}H_{10}N_2F_3$	0.0	H_2O
						229.0584	$C_{10}H_8ON_2F_3$	0.2	C_2H_4
						228.0505	$C_{10}H_7ON_2F_3$	−0.1	C_2H_5
						188.0944	$C_{11}H_{12}ON_2$	−0.2	CF_3
		256.0818	$C_{12}H_{11}ON_2F_3$	0.2	20	241.0584	$C_{11}H_8ON_2F_3$	0.1	CH_3
		241.0583	$C_{11}H_8ON_2F_3$	−0.2	25	221.0520	$C_{11}H_7ON_2F_2$	−0.3	HF
						213.0396	$C_{10}H_6ONF_3$	−0.2	CH_2N
4	393.0642		$C_{14}H_{10}ON_2F_9$	−0.6	40	375.0543	$C_{14}H_8N_2F_9$	1.2	H_2O
						310.0539	$C_{12}H_8ON_2F_6$	1.0	$C_2H_2F_3$
						241.0585	$C_{11}H_8ON_2F_3$	0.8	$C_3H_2F_6$
		375.0540	$C_{14}H_8N_2F_9$	0.5	20	355.0478	$C_{14}H_7N_2F_8$	0.7	HF
						306.0590	$C_{13}H_8N_2F_6$	1.1	CF_3
						292.0432	$C_{12}H_6N_2F_6$	0.8	$C_2H_2F_3$
		306.0590	$C_{13}H_8N_2F_6$	0.8	20	237.0635	$C_{12}H_8N_2F_3$	0.3	CF_3
						223.0479	$C_{11}H_6N_2F_3$	0.5	$C_2H_2F_3$
		241.0584	$C_{11}H_8ON_2F_3$	0.4	20	223.0479	$C_{11}H_6N_2F_3$	0.2	H_2O



Scheme 2. Proposed dissociation pathway of 2-quinolinone-derived SARMs as illustrated by means of compound **3**.

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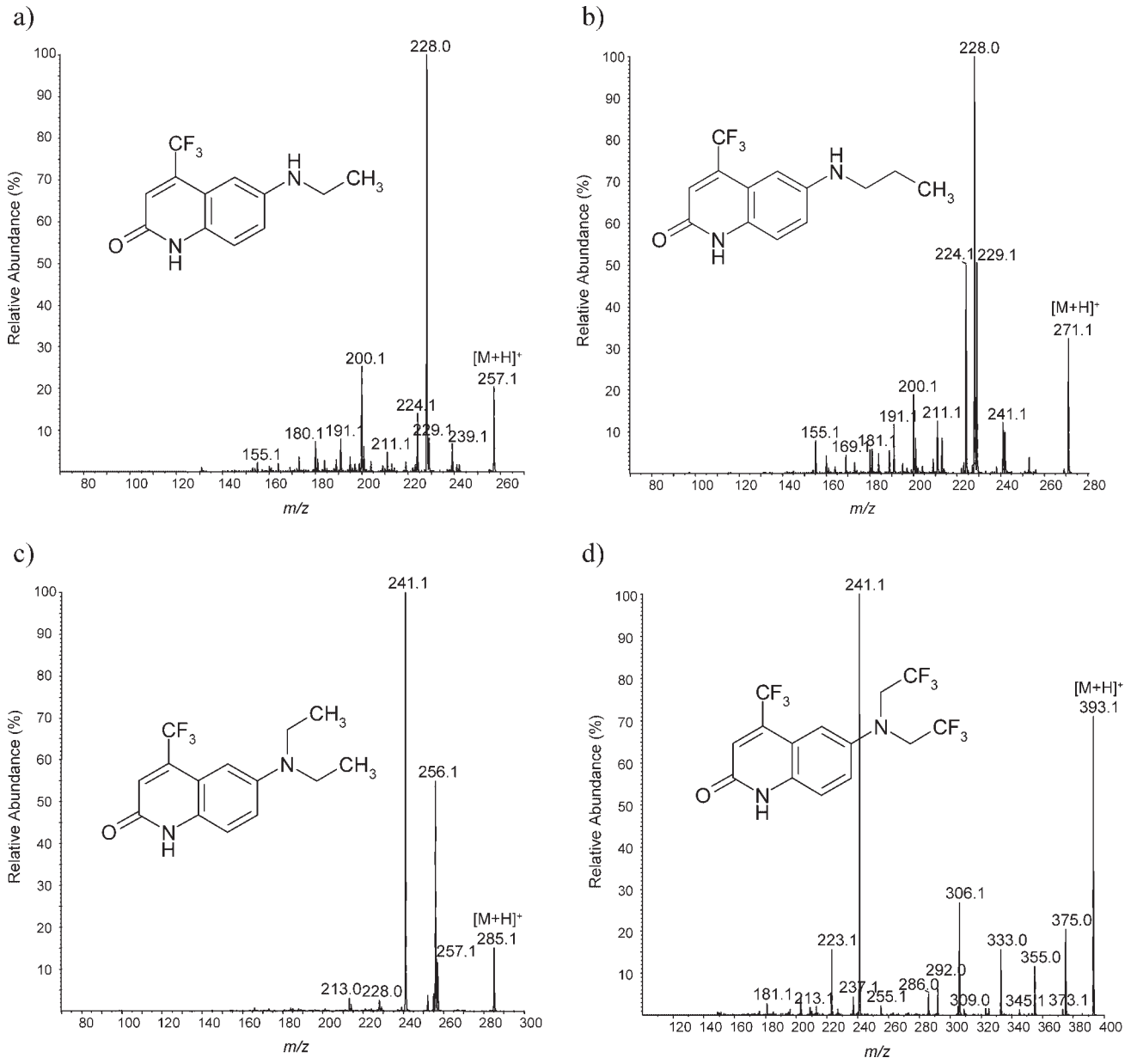

Figure 2. ESI product ion spectra of protonated molecules of four 2-quinolinone-derived model SARMs measured on an Applied Biosystems API 4000 QTrap: (a) compound **1**, collision offset voltage = 40 V; (b) compound **2**, collision offset voltage = 40 V; (c) compound **3**, collision offset voltage = 35 V; and (d) compound **4**, collision offset voltage = 50 V.

Table 3. Summary of assay validation results

Compound			Intraday precision (n = 30)		Interday precision (n = 90)	
	LLOD (ng/mL)	Recovery (%) at 100 ng/mL	Concentration (ng/mL)	CV (%)	Concentration (ng/mL)	CV (%)
1	0.05	98	1	6.4	1	16.6
			10	5.1	10	12.7
			100	8.5	100	13.9
2	0.05	96	1	6.2	1	13.9
			10	3.2	10	11.5
			100	6.6	100	11.0
3	1	95	1	5.3	1	9.5
			10	4.1	10	7.5
			100	5.9	100	6.3
4	0.05	81	1	8.0	1	16.6
			10	8.5	10	15.1
			100	5.8	100	11.4

CV: coefficient of variance.

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RCM

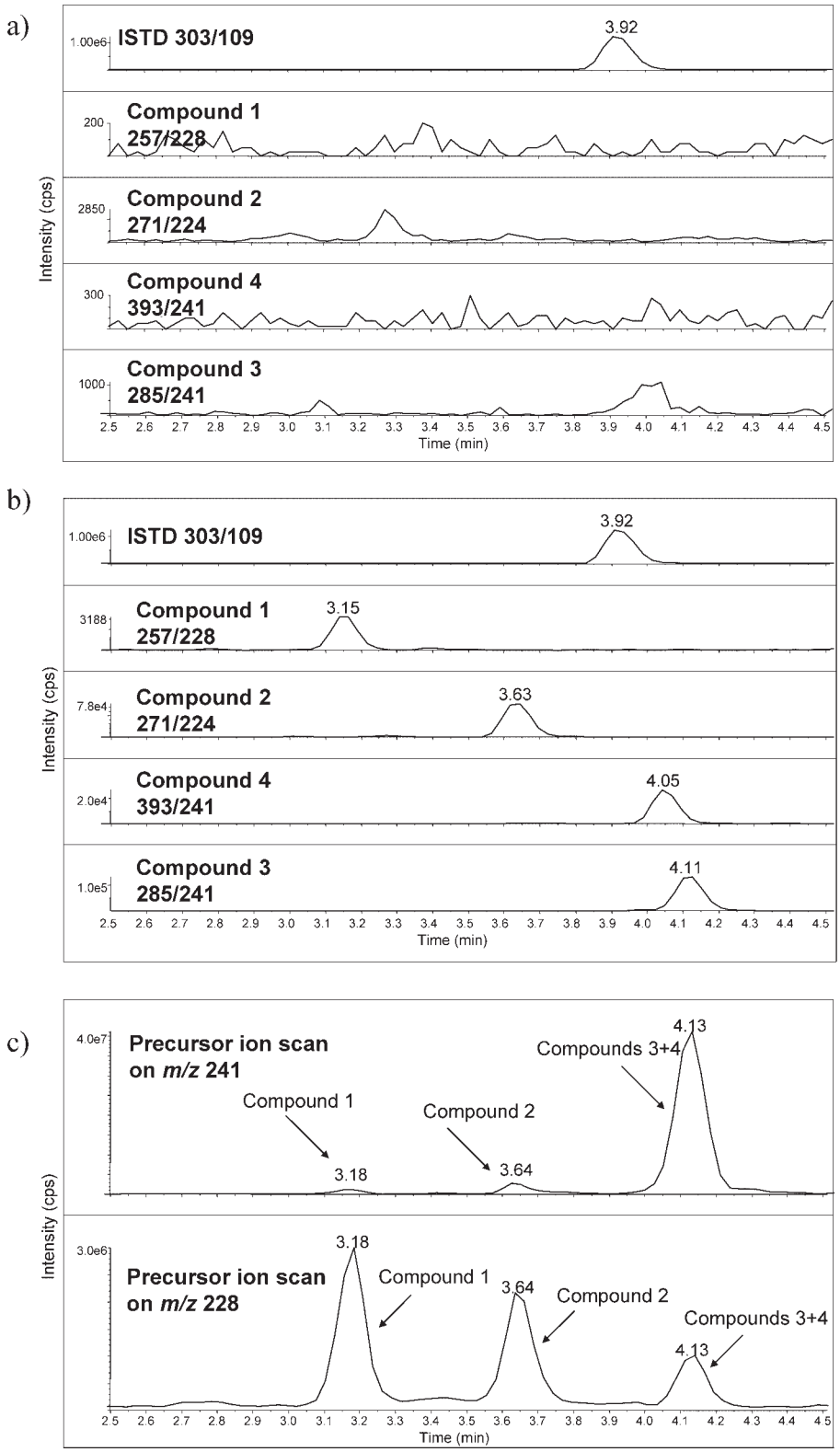

Figure 3. Extracted ion chromatograms of (a) blank urine containing the internal standard (methyltestosterone) only and (b) urine specimen spiked with 1 ng/mL of each target compound measured on an Agilent 1200 HPLC interfaced to an Applied Biosystems API 4000 QTrap. All analytes were determined using three diagnostic ion transitions employing multiple reaction monitoring. Precursor ion scan experiments were conducted on *m/z* 229 and 241 and required approx. 50 ng/mL of analyte in order to generate abundant signals, as illustrated in (c).

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In order to include this new class of target compounds into existing sports drug testing procedures, the sample preparation strategy was adapted from established screening and confirmation methods applied for designer anabolic steroids.[16] The target analytes were analyzed by LC/MS/MS employing an Agilent 1100 Series liquid chromatograph coupled to an Applied Biosystems API 4000 QTrap mass spectrometer in multiple reaction monitoring (MRM) mode, and typical extracted ion chromatograms obtained from a blank urine specimen and a sample enriched with 1 ng/mL of each compound are illustrated in Fig. 3. Moreover, two precursor ion scan experiments were implemented utilizing the common core ions of 6-amino-2-quinolinone-based SARMs, *m/z* 228 and 241, which should allow a broad and comprehensive screening for structurally related or modified drugs and respective metabolic degradation products. This strategy has frequently been used in metabolite identification studies as well as in doping control procedures.[11,24–27]

Lower limits of detection

The model SARMs **1**, **2** and **4** were identified at estimated LLODs of 0.01 ng/mL urine using one diagnostic ion transition at a S/N ratio $\geq$3 while for the detection of compound **3** a minimum concentration of 0.2 ng/mL was required. According to WADA guidelines, three characteristic product ions (Table 1) are required for confirmation purposes, which led to estimated limits of identification at 0.05 and 1 ng/mL, respectively.

Recovery

The recoveries of all tested compounds ranged from 81–98%, as outlined in detail in Table 3.

Intraday precision

The intraday precision was determined at three concentrations of all target analytes and ranged from 5.3–8.0%, 3.2–8.5% and 5.9–8.5% for low (1 ng/mL), medium (10 ng/mL) and high (100 ng/mL) concentrations, respectively (Table 3).

Interday precision

The interday precision was also determined at three concentrations of SARMs **1–4** and varied from 9.5–16.6%, 7.5–15.1% and 6.3–13.9% for low (1 ng/mL), medium (10 ng/mL) and high (100 ng/mL) concentrations, respectively (Table 3).

Specificity

In ten blank urine specimens, no interfering signals were observed at the retention times of the investigated compounds.

Test for ion suppression/enhancement effects

Four different blank urine samples (2 male, 2 female donors) were prepared for analysis and measured as described. Via T-connector all four analytes were continuously infused and ion suppression/enhancement effects were less than 10% at the retention times of the target compounds.

Administration study urine samples/metabolite identification

Administration study urine samples are common and important items of method validations in order to demonstrate the applicability of new procedures to authentic specimens. In this particular case, such samples are not available as the drugs are still undergoing clinical trials and are not yet approved to enter the market. However, preliminary results obtained by *in vitro* metabolism experiments have demonstrated that *N*-dealkylation of the selected model SARMs appears to be a major degradation pathway, as observed with numerous other drugs bearing mono- or bis-alkylated amino functions.[28–32] Thus, the established screening procedure based on MRM and precursor ion scan experiments should enable the sensitive detection of known target analytes using specific and diagnostic product ions as well as the determination of unknown related drugs and/or metabolites due to characteristic product ions resulting from the common nuclei of this class of therapeutics.

CONCLUSIONS

The temptation to artificially increase physical performance is omnipresent in elite sports, and, at least partially, powered by pharmaceutical innovations developed for the treatment of serious diseases. New therapeutic agents are frequently released and several possess considerable potential for misuse in sports. Doping control laboratories need to expand their screening and monitoring capacities and preventive doping research is required that includes method development for those drugs entering the public market in the near future. Doping with anabolic agents has been a serious issue for many years and these compounds resemble the most frequently observed category of prohibited drugs in doping control samples. Thus, sports drug testing authorities expect SARMs to become a major problem. In the presented study a screening procedure for the detection of 6-amino-2-quinolinone-derived SARMs was described as these compounds have shown anabolic properties with tissue selectivity and significantly reduced biomedical side effect profiles. Model compounds currently under preclinical or clinical investigation were synthesized and included into a validated method to provide chromatographic and mass spectral data to doping control laboratories for future reference if compounds such as LGD 2216, an advanced drug candidate representing 2-quinolinone-based SARMs, are clinically approved and become commercially available. Dissociation pathways of target analytes were studied in detail as unambiguous mass spectrometric results are of utmost importance for reliable and unequivocal doping control analyses, in particular for new classes of drugs with virtually unlimited modification options.

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