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ORIGINAL PAPER

Validation of a GC/MS method for the detection of two quinolinone-derived selective androgen receptor modulators in doping control analysis

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Abstract Selective androgen receptor modulators (SARMs) represent an emerging class of drugs likely to be abused in sport. For clinical applications, these substances provide a promising alternative to testosterone-replacement therapies and their advantages include oral bioavailability, androgen receptor specificity, tissue selectivity, and the absence of steroid-related side effects. Although not yet commercially available, since January 2008 SARMs have been included on the prohibited list issued yearly by the World Anti-Doping Agency (WADA), so control laboratories need to update their procedures to detect either the parent drugs or their metabolites. Within this context, two quinolinone SARM models were synthesized and automatically characterized to update the existing routine screening procedures. The conditions for the new target analytes are compatible with the existing laboratory protocols used for both in-competition and out-of-competition controls and can be included in them. Validation parameters according to ISO 17025 and WADA guidelines were successfully determined. For analytical determinations, spiked urine samples were hydrolyzed and extracted at pH 9.6 with 10 mL of *tert*-butyl methyl ether. Then, the analytes were subsequently converted into trimethylsilyl derivatives and detected by gas chromatography–mass spectrometry. The absence of interferents, together with excellent repeatability of both retention times and the relative abundances of diagnostic ions, allowed proper identification of all SARM analytes. The analytes' quantification was linear up to 500 ng/mL and precision criteria were satisfied (coefficient of variation less than 25% at 10 ng/mL). The limits of detection were 1 ng/mL for both SARMs, whereas recovery values were between 95.5 and 99.3%. The validated method can be efficiently used for urine screening of the 2-quinolinone-derived SARMs tested.

Keywords Gas chromatography–mass spectrometry · Doping control · Selective androgen receptor modulators · Urine screening · Validation

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Introduction

Endogenous androgens are essential for male development, spermatogenesis, and the maintenance of male secondary characteristics, such as bone mass, muscle mass, and body composition [1]. In recent years, testosterone has been used to treat hypogonadism, muscle wasting, osteoporosis, cancer cachexia, anemia, age-related frailty, for male contraception, and as hormone-replacement therapy in aging men [2–5]. The limited oral bioavailability of

testosterone as well as the need to differentiate desired anabolic from undesired androgenic effects led to the synthesis, and clinical testing, of numerous derivatives of anabolic androgenic steroids such as nandrolone decanoate and stanozolol that have been shown to increase bone mass by stimulation of bone formation [6, 7]. However, serious side effects associated with steroid-replacement therapies, including hepatic toxicity, decreased levels of HDL cholesterol, gynecomastia, and prostatic and cardiovascular illness [8–10], have driven research toward drugs with different mechanisms of action. From this research, selective androgen receptor modulators (SARMs) emerged as a new class of promising drugs.

SARMs represent a novel class of drugs for the treatment of various debilitating diseases, muscle wasting, osteoporosis, and for male contraception [11–17]. Major advantages of these drugs are the tissue-selective anabolic properties combined with considerably reduced side effects commonly associated with steroid-replacement therapies [18]. In fact, the metabolism of these drugs does not involve the enzymatic activities that are responsible for testosterone metabolism and, in particular, its transformation by aromatase and 5α-reductase enzymes [14, 19]. A series of orally bioavailable SARMs with various chemical structures were prepared and submitted to advanced clinical trials [20–22] as well as metabolic and spectrometric studies [23–28]. Currently investigated SARMs can be categorized by their common core structures into five classes: arylpropionamide, bicyclic hydantoins, quinolines, tetrahydroquinolines, and 4-azasteroids. Owing to their properties, SARMs are likely to be attractive to athletes, as these drugs offer the anabolic effects without the androgenic effects which are commonly associated with traditional anabolic androgenic steroid misuse. Although these drugs are not clinically approved yet, the availability of these substances on the black market was recently recognized [29]. On the basis of these facts, SARMs were added to the prohibited list issued annually by the World Anti-Doping Agency (WADA) since January 2008 [30]. Therefore, in recent years, several doping control analytical assays have been developed for arylpropionamide-, hydantoin-, quinoline-, and tetrahydroquinoline-derived SARMs [31–37] to establish new screening and confirmation procedures or to update existing assays with these target compounds. Moreover, additional in vivo and in vitro metabolism experiments were conducted and the detection of major metabolites was implemented in existing sport drug testing [25, 38, 39]. Although several methods for the detection of SARMs employing liquid chromatography–tandem mass spectrometry (MS) techniques were recently described, the analysis of this class of substances using conventional gas chromatography (GC)/MS approaches would be a useful complementary method as GC/MS systems are still an extensively employed tool in sport drug testing [40]. In the present work, two quinolinone-derived SARMs that possess tissue-selective androgen receptor agonist activity [41, 42], and not commercially available, were chemically synthesized (Fig. 1), and their mass-spectrometric behavior under electron ionization (EI) was studied. A procedure to detect these compounds in spiked urine specimens using GC/EI-MS employing selected ion monitoring (SIM) was developed and validated according to existing guidelines. Although the metabolism of these SARMs has not been described in the scientific literature yet, an in vitro metabolism study is under way in our laboratories [43].

Experimental

Chemicals and reagents

17α-Methyltestosterone, methanol, *tert*-butyl methyl ether (TBME), β-glucuronidase (from *Escherichia coli*), dithioerythritol, ammonium iodide (NH_4I), sodium hydrogen carbonate ($NaHCO_3$), sodium carbonate (Na_2CO_3), sodium phosphate dibasic dehydrate ($Na_2HPO_4·2H_2O$), and potassium phosphate monobasic (KH_2PO_4) were from Sigma-Aldrich (Milan, Italy). *N*-Methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) was obtained from Merck (Darmstadt, Germany). All solution and buffers were prepared using deionized water obtained from a Milli-Q system (Millipore, Billerica, USA). Phosphate buffer (0.1 M) was prepared by dissolving 4.63 g of KH_2PO_4 and 11.75 g of $Na_2HPO_4·2\ H_2O$ in 1 L of water, whereas carbonate buffer was prepared by dissolving 2.12 g of Na_2CO_3 and 6.72 g of $NaHCO_3$ in 1 L of water.

Synthesis and characterization of model compounds

Quinolinone-derived SARMs were prepared as described elsewhere according to established procedures

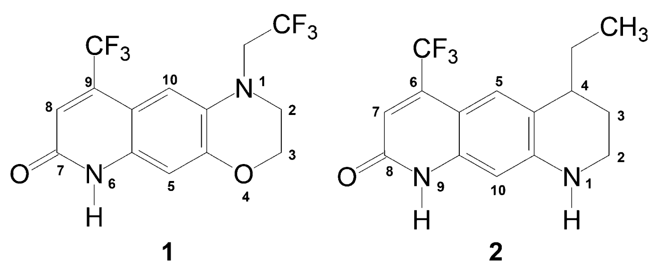

Fig. 1 Chemical structures of the compounds investigated: **1** US 6,462,038 (molecular weight 352), **2** LG 121071 (molecular weight 296)

[44, 45]. Target compounds were characterized by high-resolution/high-accuracy MS using an LTQ Orbitrap (Thermo, Bremen, Germany) employing an electrospray ionization source operating in positive mode, and nuclear magnetic resonance (NMR) spectroscopy with ^{1}H, distortionless enhancement by polarization transfer, and ^{13}C experiments employing a Bruker Avance 300 instrument (Bruker, Karlsruhe, Germany).

Stock and working solution

Stock standard solution were prepared in methanol at a concentration of 1,000 μg/mL and were stored at -20 °C until used. Working solutions were prepared at 10 μg/mL by dilution with methanol.

Gas chromatography/electron ionization–mass spectrometry

GC/MS determinations were performed using a 6890N gas chromatograph (Agilent Technologies, Milan, Italy) equipped with a 17-m fused-silica capillary column (J&W Scientific HP-1), of 0.2-mm inner diameter and 0.11-μm film thickness, for GC separation. Helium was employed as the carrier gas at a constant pressure of 20.16 psi. The gas chromatograph oven temperature was set at 120 °C for 3 min and then raised to 315 °C with a 15 °C/min heating rate. The total run time was 16 min. The gas chromatograph injector and transfer line were maintained at 280 °C. Fragmentation patterns of trimethylsilyl derivatives were investigated using a 5975 *inert* mass-selective detector (Agilent Technologies, Milan, Italy) with EI at 70 eV. Three diagnostic ions of each analyte were chosen and acquired for qualitative analyses using SIM at dwell times of 50 ms.

Sample preparation

The sample preparation involved minor modifications from the standard operating procedure described by *Donike et al.* [46] for the detection of anabolic steroids. Urine samples (3 mL) were fortified with 150 ng of the internal standard (ISTD) 17α-methyltestosterone, and then were buffered to pH 7.4 with 2 mL of a 0.1 M phosphate buffer. β-Glucuronidase (30 μL) was subsequently added and the mixture was incubated at 55 °C for 1 h. Once the hydrolysis was complete, the mixtures were cooled to room temperature and 2 mL of 0.1 M carbonate buffer was added to raise the pH to 9.6. Liquid–liquid extraction was performed by adding 10 mL of TBME and shaking the mixture in a multimixer for 10 min. After centrifugation at 2,200 rpm for 3 min, the organic layer was transferred into a vial and dried under nitrogen at 70 °C. The dry residue was derivatized with 50 μL of an MSTFA/NH_4I/dithioerythritol (1,000:2:4, v/w/w) solution for 30 min at 70 °C. A 1-μL aliquot was injected into the GC/MS system with a split ratio of 10:1.

Method validation

Method validation was performed according to ISO 17025 requirements and WADA and ICH guidelines [47, 48]. Therefore, the qualitative determination of the 2-quinolinone-derived SARMs in human urine was validated for linearity, specificity, limit of detection (LOD), precision, and recovery. Blank urine specimens required for the method validation were obtained from ten different healthy volunteers (four female, six male).

Specificity

Ten different blank urine samples were prepared as described already. The occurrence of possible interferences from endogenous substances or derivatization byproducts was tested by monitoring the selected-ion chromatograms, characteristic for each compound investigated, at the retention time interval expected for their elution.

Linearity

The linear calibration model was checked by analyzing blank urine samples spiked with standard solutions at concentrations of 5, 10, 25, 50, 100, 250, and 500 ng of each analyte per milliliter of urine. 17α-Methyltestosterone at a final concentration of 50 ng/mL was used as the ISTD. The linear calibration parameters were obtained using the least-squares regression method, whereas the correlation coefficient (R^2) was utilized to estimate linearity. Quantitative results from area counts were corrected using the ISTD signal.

Limit of detection

LODs were estimated as the analyte concentrations whose response provided a signal-to-noise (S/N) ratio of 3, as determined from the least abundant qualifier ions. The S/N ratios at the lowest concentration were used to extrapolate the theoretical LOD. These calculated LODs were then experimentally confirmed by analyzing urine samples spiked with all analytes at the LOD concentrations.

Recovery

The recovery of each compound was determined at 10, 100, and 500 ng/mL. Mean extraction recovery values were

obtained by comparing two experimental sets of data. In the first set, ten blank urine samples were spiked before the extraction step with target compounds, whereas in the second set, ten blank urine samples were spiked after the extraction step, with standard working solutions, at the same final concentration. For both sets of samples, the TBME layer was spiked with 150 ng of ISTD before evaporation. Recovery (%) was calculated as the ratio between the response (analyte peak area/ISTD peak area) obtained from the two separate series of samples. Recovery values exceeding 75% were considered satisfactory.

Intraday precision

Intraday precision, expressed as percent coefficient of variation (CV%), was assessed by extracting and analyzing, within 1 day, ten replicates of blank urine samples, spiked with the standard solutions at three concentrations (final concentrations of 10, 100, and 500 ng/mL for each analyte), performed by the same operator.

Interday precision

On three consecutive days, ten urine samples with low (10 ng/mL), medium (100 ng/mL), and high (500 ng/mL) concentrations of target compounds were prepared and analyzed by the same operator. Interday precision, expressed as CV%, was calculated for each concentration.

The interday/intraday precision was considered satisfactory when the CV% values were below 15% at high concentrations and below 25% at low concentration.

Results and discussion

Synthesis and characterization of model compounds

The syntheses provided sufficient amounts of the desired structures as pure analytes necessary for method development and structural characterization; the purities were greater than 95% in all cases. Accurate mass measurement allowed the determination of the elemental composition of protonated molecules: compound **1** elemental composition $C_{14}H_{11}O_2N_2F_6$, *m*/*z* (theoretical) 353.0719, *m*/*z* (experimental) 353.0725, error 1.7 ppm; compound **2** elemental composition $C_{15}H_{16}ON_2F_3$, *m*/*z* (theoretical) 297.1209, *m*/*z* (experimental) 297.1215, error 2.0 ppm.

Model SARMs were characterized by NMR analyses. Compound **1**: ^{1}H NMR [300 MHz, dimethyl-d_6 sulfoxide (d_6-DMSO)] δ 11.98 (br s, 1H, NH), 6.92 (s, 1H, 8-H), 6.84, 6.74 (2 s, 2×1H, 5-H and 10-H), 4.28 (t, 2H, *J*=4.2 Hz, 3-H), 4.20 (q, 2H, *J*=9.6 Hz,CH_2CF_3), 3.52 (t, 2 H, *J*=4.2 Hz, 2-H). ^{13}C NMR (75 MHz, d_6-DMSO) δ 159.8, 148.1, 136.0 (q, *J*=30.4 Hz), 134.1, 130.7, 125.6 (q, *J*=281.1 Hz), 122.6 (q, *J*=273.2 Hz), 118.4 (q, *J*=4.7 Hz), 107.9, 105.5, 102.8, 64.6, 51.9 (q, *J*=32.1 Hz), 47.8. Compound **2**: ^{1}H NMR (300 MHz, CD_3OD) δ 7.30 (s, 1 H, 5-H), 6.46 (s, 1H, 7-H), 6.37 (s, 1H, 10-H), 3.31 (m, 2H, 2-H), 2.69 (m, 1H, 4-H), 1.86 (m, 2 H, 3-H), 1.60 (m, 2H, CH_2CH_3), 0.99 (t, 3H, CH_2CH_3). ^{13}C NMR (75 MHz, CD_3OD/d_6-DMSO 1:1) δ 163.0, 149.4, 141.2, 138.9 (q, *J*=30,6 Hz), 124.9, 123.9 (q, *J*=275.6 Hz), 123.4, 112.7 (q, *J*=5.6 Hz), 104.9, 96.9, 38.2, 37.8, 28.8, 25.5, 11.5.

Interpretation of electron ionization mass spectra

A comprehensive assignment of the ultimate structures to EI mass fragments is beyond the scope of this study. However, a highly liable interpretation of EI mass spectra is made possible by comparison with definitive studies on homologue SARMs [26, 33]. The mass spectrum of the trimethylsilyl derivative of **1** (Fig. 2) shows a molecular ion at *m*/*z* 424. The base peak at *m*/*z* 409 is generated by the loss of a methyl radical (-15 u) from the trimethylsilyl residue. The subsequent elimination of trifluoroethane (-84 u) leads to a fragment ion at *m/z* 325. The release of a trifluoromethyl radical (-69 u) from the molecular ion produces the fragment ion at *m*/*z* 355. According to previous studies on 2-quinolinone-derived SARMs [26, 32, 33], the trifluoromethyl radical located on the aromatic ring is identified as the leaving group and cleavage of the cyclic structure was suggested. The subsequent loss of trifluoroethane (-84 u) or acetaldehyde (-44 u) possibly yields the fragment ion at *m*/*z* 271 or *m*/*z* 311, respectively.

Trimethylsilylation of **2** yielded a structure with a molecular mass of 440 u. Under EI conditions, the molecular ion decomposes to products ions at *m*/*z* 425, 395, 351, 411, and 337 (Fig. 3). The first three fragments most likely arise from loss of a methyl radical (-15 u) followed by the elimination of ethane (-30 u) or trimethylsilane (-74 u), giving the fragments at *m*/*z* 395 and *m*/*z* 351, respectively. The fragment at *m*/*z* 411 reasonably results from the loss of an ethyl radical (-29 u) from the molecular ion, whereas a consecutive elimination of a trimethylsilane molecule (-74 u) could be hypothesized to justify the fragment ion at *m*/*z* 337.

Method validation

On the basis of the mass-spectrometric data, three diagnostic ions of each analyte were chosen and acquired for qualitative analyses according to WADA guidelines: compound **1** *m*/*z* 409, 424, and 355 (Fig. 2); compound **2** *m*/*z*

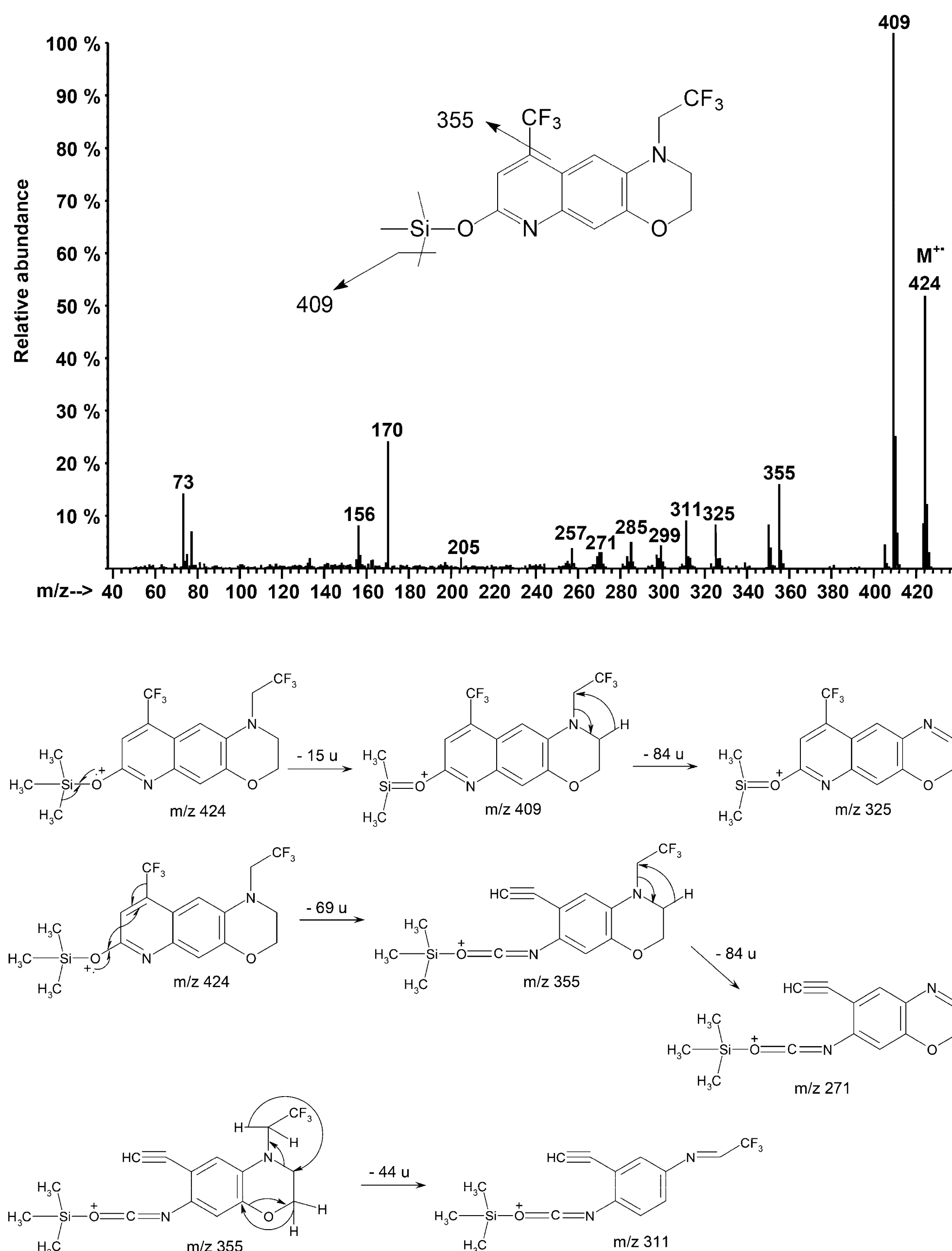

Fig. 2 Mass spectrum and proposed fragmentation routes of the trimethylsilyl derivative of compound **1** after electron ionization

440, 425, 411 (Fig. 3); ISTD *m*/*z* 301, 446, and 431. A SIM-GC/MS chromatogram obtained from a blank urine specimen fortified with 5 ng/mL of each compound is illustrated in Fig. 4.

Specificity

Evaluating ten blank urines for endogenous interferences, we observed no quantifiable analyte peaks (i.e., S/N ratio less than 3) at the expected retention time. This demonstrated that the method is selective for the compounds tested and is free from positive interference from urine components and column bleeding.

Linearity

The range of concentration studied was planned according to the approximate response factors obtained from the preliminary experiments with standard solutions. The calibration plots show good linearity for both SARMs under study in the interval 5–500 ng/mL. The coefficient of correlation (R^2) was 0.9983 and 0.9991, respectively, for compounds **1** and **2**.

Limit of detection

LODs for both compounds were estimated from calculation (see “Experimental”) at 1 ng/mL (Table 1). Estimated

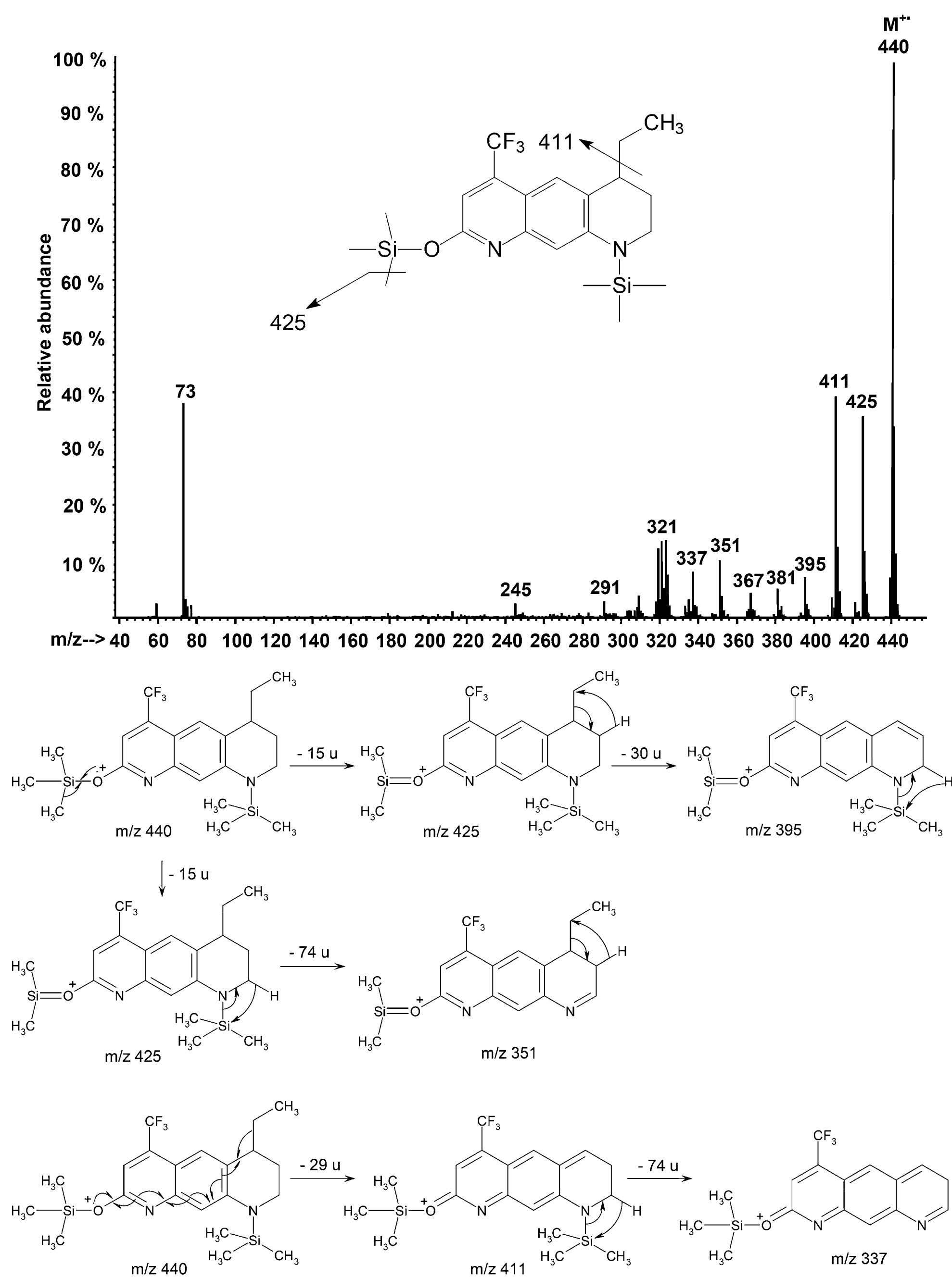

Fig. 3 Mass spectrum and proposed fragmentation routes of the trimethylsilyl derivative of compound **2** after electron ionization

LODs were experimentally confirmed by analyzing in triplicate a blank urine sample spiked with the target analytes at the LOD concentrations. All S/N ratios observed exceeded the critical value of 3, as expected. For this class of substances, the WADA requirements do not establish a limit for the minimum required performance, whereas for anabolic agents the fixed minimum required performance is 10 ng/mL [49]. Therefore, the LOD of 1 ng/mL measured for these SARMs appears satisfactory in comparison with what is required for anabolic steroids or experimentally determined for other anabolic agents, including other SARMs (0.2 ng/mL) [33].

Recovery

Recovery values are reported in Table 1. The recoveries of compounds **1** and **2** were 101.2 and 99.5%, respectively, for the low level, 99.3 and 95.5% for the medium level, and 96.7 and 96.6% for the high level. These high recovery values, obtained by applying a well-established extraction

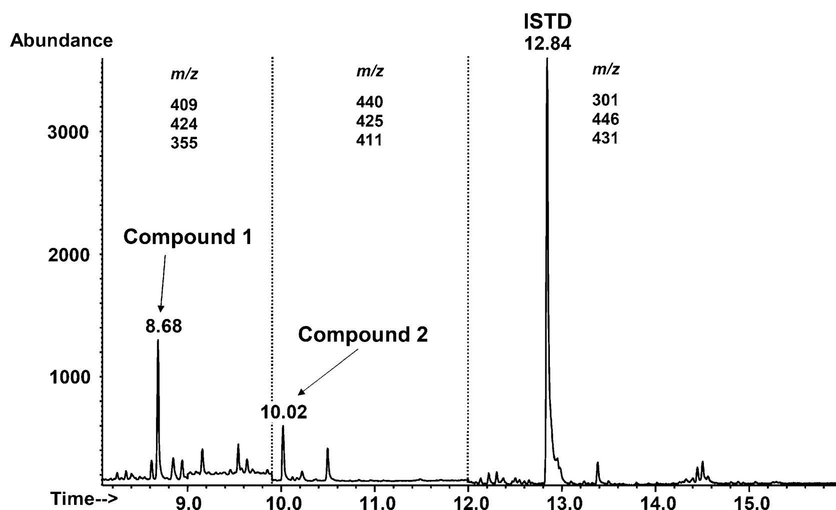

Fig. 4 Selected ion monitoring chromatogram from a gas chromatography–mass spectrometry run of a blank urine sample fortified with the target compounds at a concentration of 5 ng/mL and with 17α-methyltestosterone (*ISTD*) at a concentration of 50 ng/mL

procedure for anabolic steroids, clearly indicate that the new target analytes could be directly included in existing drug screening procedures.

Intraday and interday precision

The retention time and the relative abundances of the characteristic ions were respected for all tests. Repeatability of the retention times was excellent, with CV% values below 0.1% for the analytes at 10, 100, and 500 ng/mL and for the ISTD at 50 ng/mL. Repeatability of the relative abundances for the characteristic ions was satisfactory. At the three concentrations tested, for compound **1**, CV% values were below 5% for all ions monitored, whereas for compound **2** and ISTD, CV% values were below 2.5% for all ions monitored. The values obtained show a satisfactory intraday precision represented by CV% lower than 15% for the samples spiked at 10 and 100 ng/mL and 10% for the samples spiked at 500 ng/mL (Table 1). The interday precision of the method, expressed as CV%, was lower than 20% for the samples spiked at 10 and 100 ng/mL and 15% for the samples spiked at 500 ng/mL (Table 1). According to standard criteria taken from the literature that designate satisfactory intra-assay precision for qualitative screening methods when CV% values are below 15% at high concentrations and below 25% at lower concentrations [50], the method demonstrated satisfactory reproducibility.

Conclusions

In sport doping, the misuse of new drugs often anticipates the completion of their clinical trials and their industrial production and marketing. This is the case for several SARMs, whose administration induces anabolic/androgenic effects without the occurrence of most side effects typical of anabolic androgenic steroids. For this reason, it is necessary to continuously update the analytical procedures devoted to doping control, to include the new drugs as soon as they become available. In this study two model compounds representing quinolinone-based SARMs were synthesized and included in a validated GC/MS method to provide chromatographic and mass-spectral data useful to doping control laboratories. The analysis of these drugs in urine samples for antidoping purposes has never been published before. Future studies, including in vitro metabolism experiments, will provide further awareness about potential metabolic products to improve screening and confirmation methods in sport drug testing.

Table 1 Summary of assay validation results

Compound	LOD (ng/mL)	Concentration[a] (ng/mL)	Recovery (%)	Intraday precision CV (%)	Interday precision CV (%)
1	1	10	101.2	14.8	10.6
		100	99.3	10.1	14.2
		500	96.7	9.4	10.1
2	1	10	99.5	12.2	16.2
		100	95.5	8.7	15.1
		500	96.6	8.1	9.5

LOD limit of detection, *CV* coefficient of variation

[a] Concentrations for recovery and precision determination

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