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Analysis of exemestane and 17β-hydroxyexemestane in human urine by gas chromatography/mass spectrometry: development and validation of a method using MO-TMS derivatives

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Trimethylsilylation of anabolic agents and their metabolites is frequently achieved by using the derivatization mixture *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA)/NH_4I/2-mercaptoethanol. Nevertheless, artifacts were formed when this mixture was employed in the monitoring of exemestane and its main metabolite 17β-hydroxyexemestane prior to GC-MS analysis. These artifacts were identified as the *N*-methyltrifluoracetamide (MTFA) and trimethylsiloxyethylmercapto products of the respective trimethylsilyl (TMS) derivatives. Furthermore, artifact formation was evaluated taking the structure (1,4-diene-3-keto-6-exomethylene) of the compounds into account. Although these artifacts are relevant for investigations regarding the derivatization process and may be of interest in many fields, they are detrimental to cope with the requirements of the World Anti-Doping Agency (WADA) in terms of the limits of detection (LODs) required. To overcome this issue, a method using an alternative derivatization was proposed: formation of methyloxime-TMS derivatives through double derivatization using *O*-methylhydroxylamine/pyridine and MSTFA/TMS imidazole after enzymatic hydrolysis and liquid-liquid extraction. Samples from an excretion study after administration of exemestane to healthy volunteers were analyzed by the proposed method and detection of both exemestane and its main metabolite was possible. This method showed excellent results for both analytes meeting the LODs required for antiestrogenic agents (50 ng/mL) established by WADA. The method was validated for the main metabolite, it was robust and cost-effective for qualitative and quantitative purposes, with LOD and LOQ of 10 ng/mL and 25 ng/mL, respectively.

Exemestane (6-methylenandrosta-1,4-diene-3,17-dione) is a steroidal aromatase inhibitor structurally related to the natural substrate of aromatase, androstenedione (4-androstene-3,17-dione). Exemestane binds covalently to the active site of aromatase, causing irreversible inactivation. This type of inactivation is called 'suicidal' inhibition.[1–3] Aromatase is an enzyme complex responsible for the conversion of androgens (androstenedione and testosterone) into oestrogens (estrone and estradiol, respectively). Therefore, inhibition of oestrogen biosynthesis by aromatase inhibitors is an important treatment option in postmenopausal women with hormone-dependent breast cancer.[4]

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Male athletes may use aromatase inhibitors to avoid side effects caused by large amounts of anabolic androgenic steroids administration, such as gynaecomastia and to increase testosterone levels by inhibiting the feedback regulation. Hence, this class of compounds has been included in 2001 and 2005, for males and females, respectively, in the World Anti-Doping Agency (WADA) list of prohibited substances and methods. Its main metabolite is 17β-hydroxyexemestane and it is extensively metabolized and excreted by urinary and faecal routes.[5]

Several doping control laboratories monitor antiestrogenic agents and anabolic steroids by gas chromatography/mass spectrometry (GC/MS) after derivatization with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), ammonium iodide (NH_4I) and 2-mercaptoethanol. This derivatization mixture show good results in doping control screening, allowing the detection of several anabolic steroids, β_2-agonists and endogenous glucocorticosteroids.[6–8] The for-

mation of *O*-trimethylsilyl (*O*-TMS) enol ethers can be achieved by using this derivatization mixture due to hydriodic acid (HI) formation *in situ*, thus promoting the acidic catalysis of the reaction. The oxygen protonation in the α,β-unsaturated ketone group is the first step of this reaction.[9] Addition of reducing agents such as 2-mercaptoethanol, ethanethiol or dithioerythritol minimizes iodine formation, hampering the derivatization mixture degradation.[10] However, this derivatization mixture did not show satisfactory results in the derivatization of exemestane and 17β-hydroxyexemestane. Opfermann *et al*. have already reported the extensive formation of exemestane and 17β-hydroxyexemestane artifacts when using an analogous derivatization mixture; MSTFA/NH_4I/ethanethiol, prior to GC/MS analysis.[11] This artifact formation also occurs when 2-mercaptoethanol is used instead of ethanethiol. In order to circumvent this problem, liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) analysis is the currently used methodology in many laboratories for detection of exemestane and 17β-hydroxyexemestane. However, GC/MS still remains an interesting choice since it is less expensive and also the most available technique in clinical and doping control laboratories.[12]

The main goals of this work were: (i) Evaluation of the formation of artifacts with the derivatization mixture MSTFA/NH_4I/2-mercaptoethanol applied for these structures (androsta-1,4 diene-3-keto-6-exomethylene); (ii) to employ an alternative derivatization (*O*-methylhydroxylamine/pyridine-TMSimidazole/MSTFA) avoiding exemestane and 17β-hydroxyexemestane artifact formation; (iii) to validate a method for detection of 17β-hydroxyexemestane by GC/MS.

EXPERIMENTAL

All analytical and managerial procedures were accredited for the ISO/IEC 17025 standards, by the Brazilian National Metrological Institute (INMETRO)[13] jointly with WADA International Standards for Laboratories.[14]

Aromatase inhibitor

Exemestane (Aromasin®) was obtained from Pfizer (Ascoli Piceno, Italy). Each tablet contains 25 mg of exemestane.

Chemical and reagents

All chemical reagents used were of analytical grade: *tert*-butylmethyl ether (TBME), methanol and pyridine from Tedia (Farfield, OH, USA); *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) from Chem. Fabrik Karl Bucher-Gmbh (Waldstetten, Germany); ammonium iodide (NH_4I), 2-mercaptoethanol, trimethylsilylimidazole (TMSimid) and β-glucuronidase from *E. coli* (500 000 units) from Sigma (St. Louis, MO, USA); *O*-methylhydroxylamine hydrochloride and potassium bicarbonate from Spectrum (New Brunswick, NJ, USA); disodium hydrogen phosphate, sodium dihydrogen phosphate and potassium carbonate from Merck KGaA (Darmstadt, Germany); boldenone (androsta-1,4-diene-17β-ol-3-one) (internal standard, I.S.) from Steraloids (Newport, RI, USA); 17β-hydroxyexemestane from Australian National Measurement Institute (NMI, Lindfield, NSW, Australia). Deuterated androsterone glucuronide was a kind gift from The Institute of Biochemistry, Germany Sports University Cologne (Cologne, Germany).

Stock solutions of 17β-hydroxyexemestane and internal standard were prepared in methanol at a concentration of 1 mg/mL. These solutions were further diluted to appropriate working solutions. All solutions were sealed and kept at −15°C until use.

Excretion study urine samples

An excretion study was performed with four healthy male volunteers (age: 20–30 years, weight: 75–80 kg). An informed consent was signed by each volunteer and the study was approved by the local ethical committee (Hospital Universitário Clementino Fraga Filho – Universidade Federal do Rio de Janeiro – protocol number 020/00).

One exemestane tablet was orally administered after a meal. Urine samples were collected for 14 days. On day 1 all urine was collected. On day 2 the urine was collected at intervals of 4 h. On days 3–14 just the early morning urine was collected. The blank urine was collected 8 h before exemestane administration. All urine samples were stored at −15°C until analysis.

Sample preparation

The urine samples were prepared using the screening method for anabolic steroids described by Schänzer and Donike[15] with few modifications,[16] and then analyzed by GC/MS.

An urine aliquot of 2 mL was taken. The urine samples were fortified with 1 μg of internal standard (boldenone) and 1 μg of [2,2,3,4,4-$^2H_5$]-androsterone glucuronide for hydrolysis efficiency evaluation. The pH was adjusted to 7 with 750 μL of a freshly prepared aqueous solution of 0.8 M phosphate buffer (Na_2HPO_4 and NaH_2PO_4) and mixed briefly on a vortex-mixer. Then, 1000 units of β-glucuronidase from *E. coli* were added and hydrolysis was performed for 1 h at 50°C. The mixture was alkalinized with 500 μL of aqueous buffer solution containing K_2CO_3 and $KHCO_3$ 20% each (pH 10). The analytes were extracted with 5 mL of TBME, and the mixture was shaken for 5 min and centrifuged at 3000 rpm for 5 min. The ethereal phase was transferred to another fresh glass tube and evaporated to dryness under mild nitrogen flow at 40°C. The residues were dried in a vacuum oven for at least 30 min and then derivatized with 100 μL of MSTFA/NH_4I/2-mercaptoethanol (1000:2:6 v/w/v, 60°C/30 min). A volume of 3 μL of each sample was injected into the GC/MS system.

Alternative derivatization strategy

Alternatively the residues were derivatized with 50 μL of *O*-methylhydroxylamine hydrochloride/pyridine (8:100 w/v, 60°C/30 min) followed by evaporation of pyridine under nitrogen flow at 40°C, the solvent was further removed in a vacuum oven for at least 30 min and 50 μL of MSTFA/TMSimid (100:2 v/v) were added and heated at (60°C/20 min).

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GC/MS procedure

Urine sample extracts were analyzed by GC/MS employing a model 7890A gas chromatograph (Agilent, Palo Alto, CA, USA), equipped with a 7693 autosampler and coupled to a model MS 5975 C single quadrupole mass spectrometer (Agilent, Palo Alto, CA, USA).

GC conditions: carrier gas, helium at 1 mL/min constant flow mode; column used, Ultra-1® capillary column (100% methylpolysiloxane, 17 m × 0.2 mm × 0.11 μm; J&W Scientific, Agilent Technologies, Inc.); injector temperature, 280°C; injection mode: pulsed split, split ratio 1:10, pulse pressure/time 50 psig/0.80 min; injection volume 3 μL; the GC oven temperature was programmed to increase from 140°C to 180°C (40°C/min), then set to increase from 180°C to 230°C (3°C/min) and finally set to increase from 230°C to 300°C (40°C/min) (3 min hold).

MS conditions: electron ionization (EI), ionization voltage, 70 eV; ion source temperature, 220°C; quadrupole temperature, 150°C; transfer line temperature, 280°C. Detection was made using full scan mode; mass range, 50–700 Da and selected ion monitoring simultaneously.

Method validation

A quantitative method validation was performed for 17β-hydroxyexemestane. The parameters analyzed were repeatability, reproducibility, robustness, recovery and specificity. Limits of detection and quantification (LOD and LOQ) were obtained through successive analyte dilution until signal-to-noise ratios were 3 and 10, respectively. The calibration curve was built using five concentration levels (25, 35, 45, 55 and 65 ng/mL). Each level was analyzed in triplicate. The curves were prepared by addition of 120 ng of boldenone as internal standard and different analyte concentrations into 2 mL blank urine. Linearity was determined by linear regression using the least-squares method. Repeatability was evaluated using relative deviation standard (RSD %) obtained in three concentration levels (25, 45 and 65 ng/mL), reproducibility and robustness were evaluated by repetition of the curves on two days with different analysts. Analyte recovery was evaluated using 12 samples, 6 of them spiked in blank urine before extraction and the other 6 spiked after that step. Method specificity was demonstrated by analysis of 10 different blank urine samples.

RESULTS AND DISCUSSION

Exemestane and 17β-hydroxyexemestane artifacts

Volatility and thermal stability of compounds are required in GC/MS analysis. Derivatization is mandatory for polar and thermolabile compounds for making them liable to be analyzed by gas chromatography. Anabolic agents and their metabolites are examples of compounds which have polar groups. They do not show good chromatography behavior because of the presence of hydroxyl and keto groups in their structures. For this reason the derivatization process is strongly recommended for these compounds prior to GC analysis.[17] The most used derivatization reaction for the analysis of anabolic agents is trimethylsilylation, often achieved by the use of MSTFA. However, this reagent alone is not capable of derivatizing keto groups and tertiary alcohols, which makes the presence of a catalyst necessary.[18] Many anabolic agents and their metabolites have keto groups in their structures. Reaction of these groups with MSTFA plus a catalyst causes the formation of their trimethylsilyl (TMS) derivatives through the enol form. This is highly useful in order to increase molecular mass and to avoid background interferences and several authors describe the mixture MSTFA/NH_4I/2-mercaptoethanol as an useful tool for taking this process on.[19,20] Unfortunately, enolization of keto groups should be controlled when side reactions (artifacts formation) are possible.[21]

In this particular case after protonation of the 3-keto group of androsta-1,4-diene-3-keto-6-exomethylene steroids delocation of electron pairs by a resonance effect occurs and several electrophilic sites become available. Usually, 1,4-diene-3-keto steroids, such as methandienone and boldenone, have the possibility to eliminate the hydrogen next to the positive charge and reestablish the double bond. However, exemestane and its main metabolite do not have hydrogens near the positive charge available to be extracted and recover system conjugation due to the 6-exomethylene in their structure. Thus, during this derivatization reaction these compounds are susceptible to addition by nucleophilic agents.

The GC/MS analysis of exemestane and 17β-hydroxyexemestane in urine samples after derivatization with MSTFA/NH_4I/2-mercaptoethanol was not successful, as their corresponding signals were not detected (Fig. 1). Furthermore, multiple unexpected signals (artifacts) were observed in all excretion urine samples as well as spiked urine samples at a concentration of 200 ng/mL. Two artifacts arising from nucleophilic attack were detected in high amounts in urine samples, these ones are formed from *N*-methyltrifluoroacetamide (MTFA) and trimethylsiloxyethylmercapto groups incorporation to bis *O*-TMS derivatives (Figs. 2(A) and 2(B), respectively).

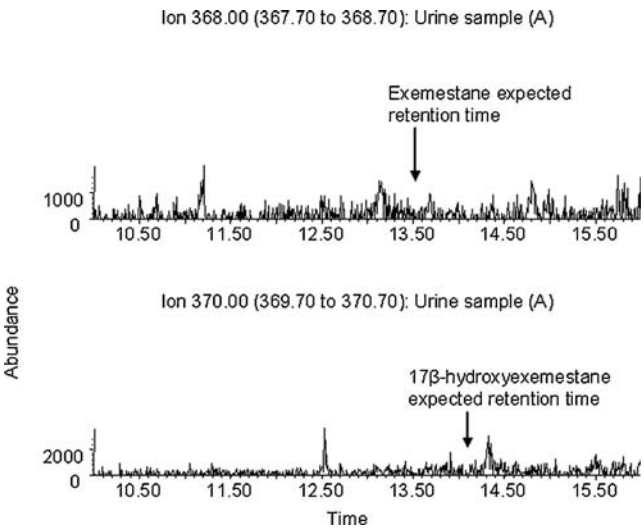

Figure 1. Extracted ion chromatograms of exemestane (*m/z* 368) and 17β-hydroxyexemestane (*m/z* 370) *O*-TMS derivatives, respectively, from an excretion urine sample. Note the signals relative to them were not observed on their chromatograms.

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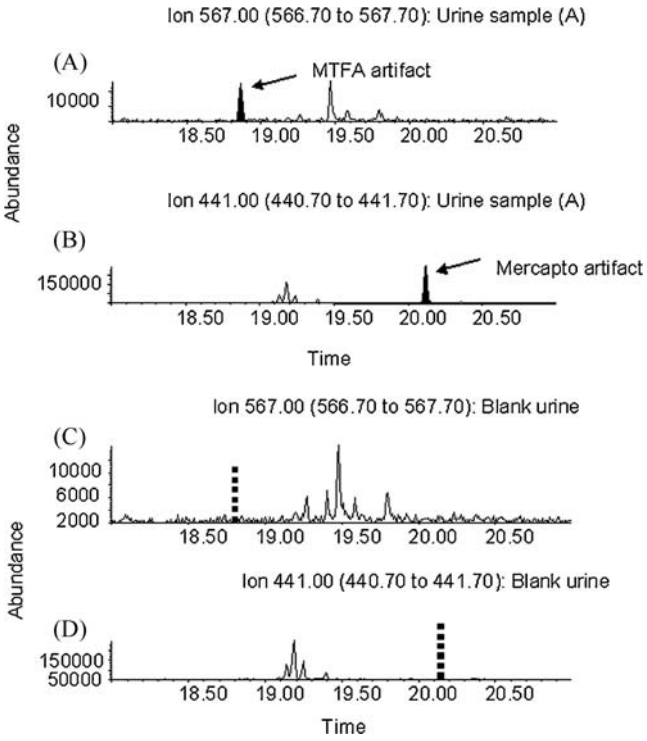

Figure 2. Extracted ion chromatograms of exemestane artifacts (*m/z* 567 and 441) after trimethylsilation from an excretion urine sample (A and B) and blank urine from the same subject (C and D). (A) MTFA artifact chromatogram and (B) mercapto artifact chromatogram.

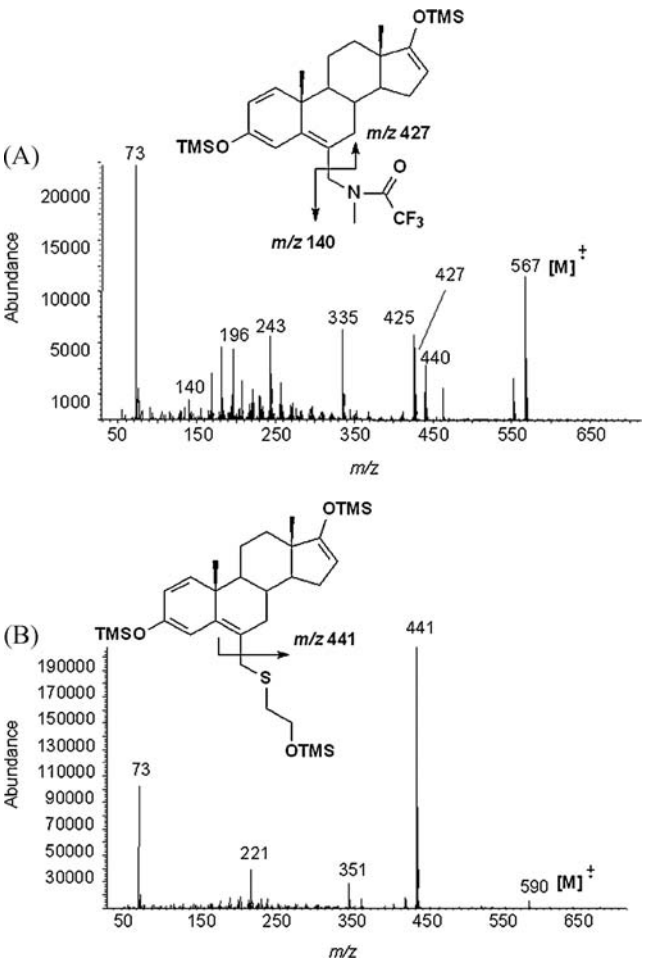

Figure 3. Electron impact mass spectra of exemestane artifacts after trimethylsilation. (A) MTFA artifact mass spectrum and (B) mercapto artifact mass spectrum.

According to Opfermann *et al*. the most probable site for MTFA or trimethylsiloxyethylmercapto/ethylmercapto addition is at C3.[11] However, it is possible to observe in the exemestane MTFA artifact mass spectrum two diagnostic ion fragments (*m/z* 427 and 140) which indicate that the most probable site for this addition is the exomethylene in C6 (Fig. 3(A)). Besides this, exomethylene is more likely than C3 to undergo a nucleophilic addition.

In the mass spectrum of the MTFA artifact the fragment ion *m/z* 427 derives from the heterolytic cleavage (β from nitrogen atom) between the C6 and the methylene. The fragment ion *m/z* 140 derives from the same cleavage but represents the side chain (acetamide moiety). The fragment ion *m/z* 440 arises from a neutral loss of MTFA from the molecular ion and the fragment ion *m/z* 425 represents subsequent loss of a methyl radical.

Regarding the trimethylsiloxyethylmercapto artifact (Fig. 3(B)) the exact trimethylsiloxyethylmercapto position in the molecule could not be determined, but again the most available eletrophilic site is the exomethylene. Hence, this position would be the most probable site for this addition as well. The dominant ion fragment in its mass spectrum is *m/z* 441, which could be explained by the trimethylsiloxyethylmercapto radical loss. The 17β-hydroxyexemestane artifacts are analogous to those of the parent compound, differing by a 2 Da increase in molecular weight, due to the 17-keto reduction.

Artifact formation and the consequent loss of sensitivity are particularly critical when legal LODs are to be reached according to the field in which the method is going to be applied. Unfortunately, in this special case this method was not applicable for both qualitative and quantitative doping purposes, because the *O*-TMS derivatives from exemestane and 17β-hydroxyexemestane were not detected. Additionally, their artifacts were not observed in the LOD required for WADA and they did not show a linear behavior. This observation is in accordance with the 17β-hydroxyexemestane and exemestane artifacts unstability reported by Opfermann *et al*. [11] This artifact unstability should be considered in doping and clinical testing.

Alternative derivatization with formation of MO-TMS derivatives

An interesting tool to circumvent electrophilic site formation during the enolization process is to previously protect the keto group with alkyloximes. The use of methyloxime (MO) derivatives of keto steroids for analytical and structural studies by GC/MS was first recommended by Fales and Luukkainen.[22] Marques *et al*. achieved good results using this alternative derivatization in the analysis of synthetic 19-norsteroids with a 3-keto-4,9,11-triene

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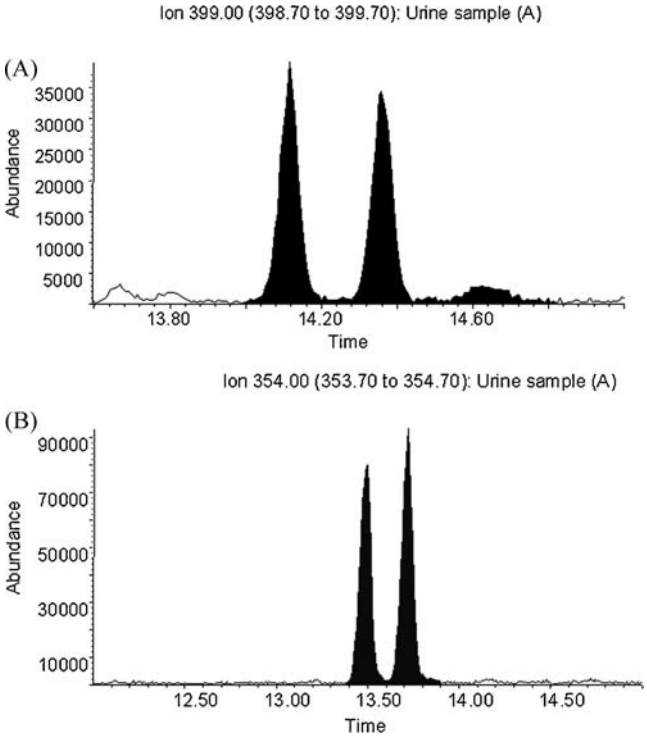

Figure 4. Extracted ion chromatograms of (A) 17β-hydroxyexemestane (*m/z* 399) and (B) exemestane (*m/z* 354) MO-TMS derivatives, from an excretion urine sample.

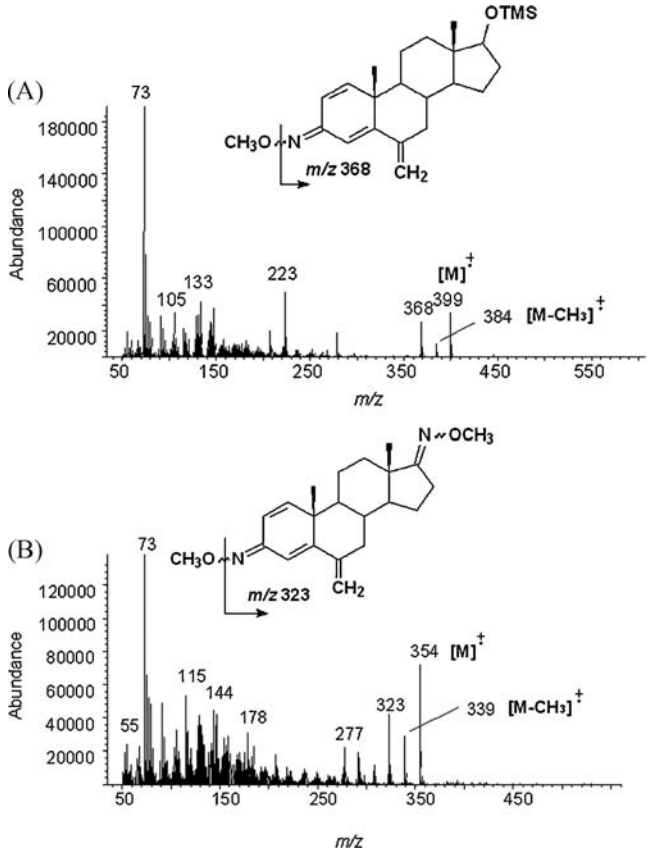

Figure 5. Electron impact mass spectra of 17β-hydroxyexemestane (A) and exemestane (B) MO-TMS derivatives.

ring system.[23] In fact, methyloxime formation and trimethylsilation of hydroxyl groups are used in many confirmatory procedures in the analysis of anabolic steroids.[24] Generally, to improve chromatographic behavior, a second derivatization step after methyloxime (MO) derivative formation is used. This alternative derivatization was used in this work and the second step was performed with MSTFA/TMSimid aiming at the formation of methyloxime-trimethylsylil (MO-TMS) derivatives. This alternative derivatization allowed the detection of exemestane and its main metabolite in urine samples at an LOD of 10 ng/mL and no artifacts were formed. As well as oxime, methyloximes show two geometric isomers (anti and syn). Hence, two signals were expected on the chromatogram for 17β-hydroxyexemestane (Fig. 4(A)) and four signals were expected for exemestane. However, for the latter, two of the geometric isomers formed were not detectable at low concentrations and only two isomers were detected in urine samples (Fig. 4(B)). The exemestane and 17β-hydroxyexemestane isomer signals showed baseline resolution and were symmetrical, thus the geometric isomer formation did not interfere with the quantitation process. The three diagnostic ions of 17β-hydroxyexemestane (Fig. 5(A)) and exemestane (Fig. 5(B)) MO-TMS derivatives were: the molecular fragment ions (*m/z* 399 and 354, respectively), and the fragment ions arising from loss of methyl radical (*m/z* 384 and 339, respectively) and loss of methoxy radical (*m/z* 368 and 323, respectively). Furthermore, it was possible to observe the fragment ion *m/z* 223 on the mass spectrum of 17β-hydroxyexemestane that derives from B-ring cleavage.

Validation results

Validation parameters

The method was validated for the 17β-hydroxyexemestane MO-TMS derivative. The calibration curve was prepared on two different days by two different analysts with five concentration levels of the analyte (25, 35, 45, 55 and 65 ng/mL) in triplicate. The analyte was spiked into blank urine, as well as boldenone, which was used as internal standard. The molecular ion signal area ratios (analyte/internal standard) were correlated by linear regression. Using the least-squares method, the equations obtained were: $y = 0.0178x + 0.2496$ (first day), $y = 0.0284x - 0.1189$ (second day). The determination coefficients (r^2) were determined: 0.9988 (first day) and 0.9932 (second day). The Cochran test

Table 1. RSD % for three concentration levels of 17β-hydroxyexemestane MO-TMS derivative measured on two days by two different analysts

	Repeatability	
	Concentration level (ng/mL)	RSD %
Day 1	25	3.81
	45	4.85
	65	1.42
Day 2	25	1.66
	45	2.49
	65	2.00

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was applied for verification of homocedasticity. The C_{calc} was lower than C_{tab} in both situations, thus the curves were homocedastic. The reproducibility and robustness of the method were also evaluated by a two-factor analysis of variance (ANOVA). Different days and analysts were equivalent. Repeatability was evaluated by the RSD, as shown in Table 1.

The limit of detection (LOD) found for the 17β-hydroxyexemestane MO-TMS derivative in urine was 10 ng/mL. The limit of quantification (LOQ) was 25 ng/mL. The LOD achieved meets the Minimum Required Performance Level (MRPL) established by WADA.[25]

Analyte recovery was evaluated using 12 samples, 6 of them spiked in blank urine before extraction and the other 6 spiked after that step. The recovery obtained was 89% with RSD 6.6%.

To check the specificity, 10 different blank urines were compared with the urine sample spiked with 17β-hydroxyexemestane. No interferent signals were found in the 17β-hydroxyexemestane retention time. One sample from a proficiency test containing 17β-hydroxyexemestane was analyzed by this method and the quantitative and qualitative results obtained were satisfactory.

CONCLUSIONS

The conventional derivatization mixture MSTFA/NH_4I/2-mercaptoethanol is widely used to derivatize anabolic agents and other substances, such as endogenous glucocorticosteroids, prior to GC/MS analysis and usually shows excellent results. Nevertheless, the 1,4-androsta-3-keto-6-exomethylene steroids, like exemestane and its main metabolite, did not show good behavior when this derivatization mixture was employed: the 6-exomethylene in their structures seems to play an important role on the artifact formation. Their presence was detected through their artifacts. However, these were not acceptable for both quantitative and qualitative purposes and did not meet the requirements of WADA.

The alternative strategy for derivatization with methyloxime and MSTFA/TMSimid was quite useful for detection of exemestane and its main metabolite in urine by GC/MS, avoiding artifact formation. This method was developed and validated for quantification of the main exemestane metabolite in urine samples. The method was robust, reliable and achieved the LOD criteria determined by WADA for antiestrogenic substances.

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