Journal of Steroid Biochemistry & Molecular Biology 127 (2011) 248–254

Contents lists available at SciVerse ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Detection of new exemestane metabolites by liquid chromatography interfaced to electrospray-tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 23 April 2011
Received in revised form 19 July 2011
Accepted 14 August 2011

Keywords:
Exemestane metabolism
LC–MS/MS analysis
Doping control

ABSTRACT

Exemestane is an irreversible aromatase inhibitor used for anticancer therapy. Unfortunately, this drug is also misused in sports to avoid some adverse effects caused by steroids administration. For this reason exemestane has been included in World Anti-Doping Agency prohibited list. Usually, doping control laboratories monitor prohibited substances through their metabolites, because parent compounds are readily metabolized. Thus metabolism studies of these substances are very important. Metabolism of exemestane in humans is not clearly reported and this drug is detected indirectly through analysis of its only known metabolite: 17β-hydroxyexemestane using liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) and gas chromatography coupled to mass spectrometry (GC–MS). This drug is extensively metabolized to several unknown oxidized metabolites. For this purpose LC–MS/MS has been used to propose new urinary exemestane metabolites, mainly oxidized in C6-exomethylene and simultaneously reduced in 17-keto group. Urine samples from four volunteers obtained after administration of a 25 mg dose of exemestane were analyzed separately by LC–MS/MS. Urine samples of each volunteer were hydrolyzed followed by liquid–liquid extraction and injected into a LC–MS/MS system. Three unreported metabolites were detected in all urine samples by LC–MS/MS. The postulated structures of the detected metabolites were based on molecular formulae composition obtained through high accuracy mass determination by liquid chromatography coupled to hybrid quadrupole-time of flight mass spectrometry (LC–QTOF MS) (all mass errors below 2 ppm), electrospray (ESI) product ion spectra and chromatographic behavior.

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1. Introduction

Exemestane (6-methyleneandrosta-1,4-diene-3,17-dione) is an orally active third generation irreversible aromatase inhibitor, structurally related to the endogenous steroid androstenedione [1]. This drug is capable of promoting a “suicidal” inactivation, binding irreversibly to aromatase (*CYP19A1*) through an intermediate provenient from a NADPH-dependent mechanism [2]. Aromatase is a cytochrome P450 enzyme which catalyzes estrogen formation (estrone and estradiol) from androgens (androstenedione and testosterone). This drug is used clinically in postmenopausal women with advanced hormone dependent breast cancer refractory to tamoxifen treatment [3,4].

Unfortunately, in sports competition, male athletes are encouraged to treat some adverse effects caused by extensive abuse of anabolic steroids (e.g., gynaecomastia) using aromatase inhibitors. Furthermore, in this case, negative feedback promoted by estradiol on Luteinizing Hormone (LH) production is reduced, increasing endogenous testosterone levels. Hence, since 2001 and 2005 the use of aromatase inhibitors has been prohibited by the World Anti-Doping Agency (WADA) for male and female athletes, respectively [5].

Steroids are usually metabolized to active or inactive metabolites and excreted into urine. Unaltered steroids are generally excreted only in a brief period after their administration. Therefore, knowledge of steroid metabolism must be considered so as to detect steroid abuse indirectly for a longer period after administration. Generally, doping control laboratories monitor steroid abuse indirectly through some target metabolites. The metabolism of exemestane in men is not clearly described and the only known metabolite is the 17β-hydroxyexemestane. Mareck et al. have reported detection and quantitation of this metabolite by LC–MS/MS [6].

Aiming at a better detection of exemestane abuse in sports competition, our research group has been working on a possible route to exemestane metabolism (Fig. 1).

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doi:10.1016/j.jsbmb.2011.08.014

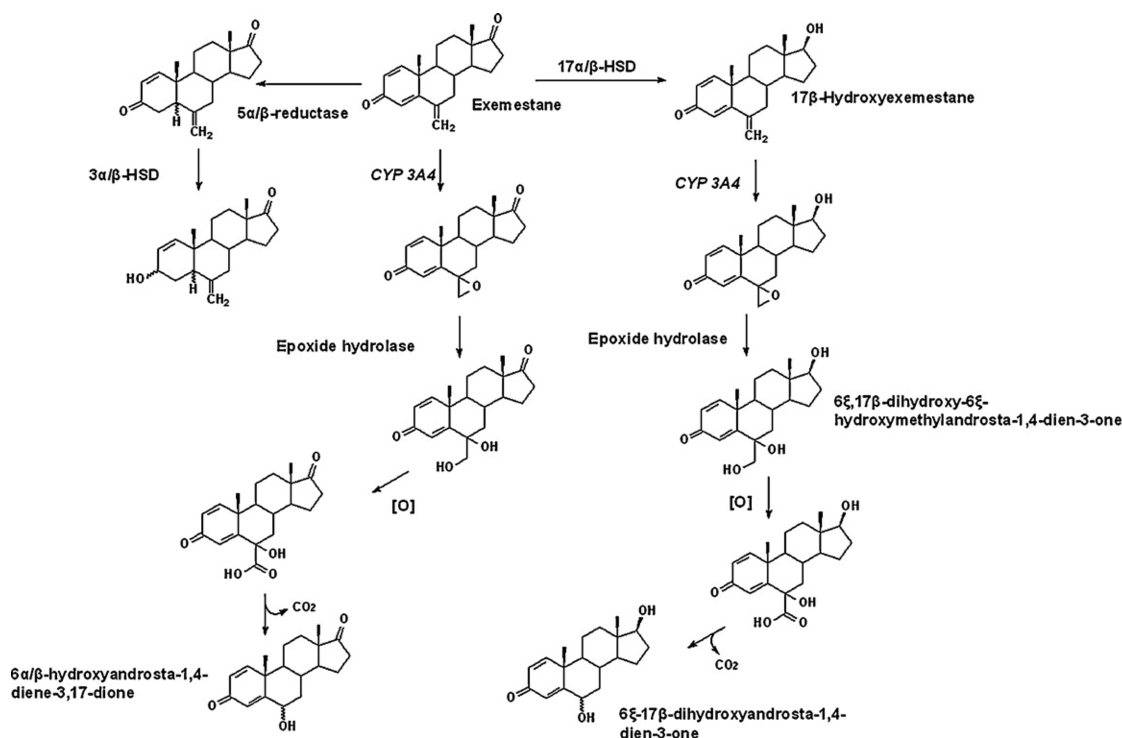


Fig. 1. Proposed exemestane metabolic pathway (HSD, hidroxysteroid-dehydrogenase). Metabolites that were detected by GC–MS: 6ξ-hydroxy-6ξ-hydroxymethylandrosta-1,4-diene-3,17-dione, 6ξ-hydroxyandrosta-1,4-diene-3,17-dione and 3ξ-hydroxy-5ξ-androst-1-ene-6-methylene-17-one. The metabolites 6ξ, 17β-dihydroxy-6ξ-hydroxymethylandrosta-1,4-dien-3-one,6ξ-17β-dihydroxyandrosta-1,4-dien-3-one and 6α/β-hydroxyandrosta-1,4-diene-3,17-dione (isomer forms) were detected by LC–MS/MS.

Three metabolites (6ξ-hydroxy-6ξ-hydroxymethylandrosta-1,4-diene-3,17-dione, 6ξ-hydroxyandrosta-1,4-diene-3,17-dione and 3ξ-hydroxy-5ξ-androst-1-ene-6-methylene-17-one) have been detected by gas chromatography interfaced to mass spectrometry (GC–MS) [7]. However, this technique was not capable of detecting some proposed metabolites with 17-keto group reduction and simultaneous oxidation in C6-exomethylene group.

Liquid chromatography interfaced to tandem mass spectrometry (LC–MS/MS) has been a useful tool to perform metabolism studies [8,9]. Furthermore, this technique shows more sensibility and lower detection limits when compared to GC–MS [10].

Excretion studies are generally performed by the administration of the drug to volunteers followed by the comparative analysis of pre and post-administration samples. This allows the location of signals which are absent on the pre-administration samples and appear on the post-administration ones which are probably metabolites from the administered drug. Also, mass spectra have been very useful on identification and structural elucidation of drug metabolites in doping analysis through fragment interpretation and correlation with structural characteristics [11].

The aim of this study was to identify unreported exemestane metabolites specially those oxidized in 6-exomethylene group and simultaneously reduced in 17-keto group by LC–MS/MS. Liquid chromatography interfaced to hybrid quadrupole time of flight mass spectrometry (LC–QTOF MS) was employed in the characterization of molecular formulae composition. Thus, controlled excretion studies were performed and evaluated.

2. Experimental

All analytical and managerial procedures were accredited for the ISO/IEC 17025 standards, by the Brazilian National Metrological Institute (INMETRO) [12] jointly with WADA International Standards for Laboratories [13].

2.1. Excretion study urine samples

An excretion study was performed with four healthy male volunteers (age: 23–29 years old; 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 (25 mg) was orally administered to each of the four volunteers after a meal. Urine samples were collected for 14 days. On day 1, all urine fractions were collected. On day 2, urine fractions were collected each 4 h. From day 3 to 14, only the early morning urine was collected. Blank urine was collected 8 h before exemestane administration. All urine samples were stored at −20 °C until analysis.

2.2. Chemicals and reagents

The following chemicals and reagents were used: acetonitrile (ACN), formic acid, *tert*-butylmethylether (TBME) and methanol, all pesticide grade from Tedia (Farfield, OH, USA). β-Glucuronidase from *Escherichia coli* from Sigma (St. Louis, MO, USA). Potassium bicarbonate from Spectrum (New Brunswick, NJ, USA). Di-sodium hydrogen phosphate, sodium dihydrogen phosphate and potassium carbonate from Merck KGaA (Darmstadt, Germany).

2.3. Sample preparation

The urine samples were prepared using the screening method for anabolic steroids described by Schanzer and Donike [11] with few modifications [14], and then analyzed by LC–MS/MS and LC–QTOF MS. The urine samples of each volunteer were collected and analyzed separately.

An urine aliquot of 2 mL was taken. The pH was adjusted to 7 with 750 μL of 0.8 M aqueous phosphate buffer (Na_2HPO_4 and NaH_2PO_4) and mixed briefly on a vortex-mixer. Then, 1000 units of β-Glucuronidase from *Escherichia 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 $KHCO_3$ 10% each (pH 10). The analytes were extracted with 5 mL of TBME, and the mixture was stirred for 5 min and centrifuged at c.a. 1500 × *g* for another 5 min. The ethereal phase was transferred to another glass tube and evaporated to dryness under mild nitrogen flow at 40 °C. The residues were resuspended with 200 μL of mobile phase (1:1, v/v, ACN/H_2O; 0.1% formic acid). 10 μL of each sample were injected separately into LC–MS/MS and LC–QTOF MS systems.

2.4. LC–MS/MS analysis

HPLC Agilent 1200 series (Santa Clara, CA, USA) was interfaced to a MS AB 4000 Qtrap (AB Sciex, Carlsbad, CA, USA) using the electrospray (ESI) interface. Compound separation was performed using a Zorbax C18 column (150 mm × 4.6 mm i.d., 5.0 μm) at flow rate of 1 mL/min, with a Zorbax C18 guard column (10 mm × 4.6 mm, 5.0 μm). The column was kept inside the oven at 30 °C. The mobile phase used was acetonitrile/water with the addition of formic acid at 0.1% (v/v). A gradient mode was performed as follows: 20% ACN (0 min), 80% ACN (20.00 min), 20% ACN (20.10 min) until 24.00 min.

Spray voltage was used in positive ionization mode at 5500 V, curtain gas pressure 15 psi, capillary temperature 550 °C, and declustering potential 30 V. The collision energies applied were optimized for each metabolite. Product ion scans were performed by fragmenting the pseudomolecular ions which were proposed in exemestane metabolic pathway (Fig. 1). Multiple reactions monitoring (MRM) was applied using one transition for each metabolite. The transitions were: *m/z* 333–147 for metabolite: 6ξ,17β-dihydroxy-6ξ-hydroxymethylandrosta-1,4-dien-3-one; *m/z* 303–285 for metabolite: 6ξ-17β-dihydroxyandrosta-1,4-dien-3-one and *m/z* 301–283 for metabolite: 6α/β-hydroxyandrosta-1,4-diene-3,17-dione. Data processing was done using AB Sciex Analyst software version 1.5.

2.5. ESI-accurate mass measurements (LC–QTOF MS)

HPLC Agilent 1200 (Cernusco sul Naviglio, MI, Italy) series was interfaced to a hybrid quadrupole-time of flight mass spectrometer (QTOF MS) Agilent 6520 (Cernusco sul Naviglio, MI, Italy). Time of flight mass detector temperatures: gas temperature, 330 °C; Drying gas flow, 10 L/min; Fragmentor voltage, 150 V; Skimmer, 60 V, capillary voltage, 4000 V. The ions were acquired in SCAN mode, mass range 100–1000 Da. All other MS parameters (transfer optic voltage, voltage of the ion focus and octapole lens for optimizing the beam shape as it enters the TOF analyzer, TOF voltages, and detector voltage) were automatically optimized by the instrument autotuning procedure, performed daily. The mass calibration was also performed daily before starting the analysis using a calibration solution provided by the manufacturer.

Purine with an $[M+H]^+$ ion at *m/z* 121.0509 and an Agilent proprietary compound (HP0921) yielding an ion at *m/z* 922.0098 were simultaneously introduced via a second orthogonal sprayer, and these ions were used as internal calibrants along all the analysis. All aspects of instrument control, tuning, method setup and parameters, sample injection and sequence operation were controlled by the Agilent Technologies Mass Hunter software.

Liquid chromatography conditions: column Zorbax C18 (100 mm × 2.1 mm × 1.8 μm), the column was kept inside the oven at 25 °C. The separation was performed in gradient mode as follows: 10% ACN (0 min), 30% ACN (10 min), 40% ACN (15 min), 40% ACN (18 min), 100% ACN (22 min) until 25 min. Flow was set to 250 μL/min.

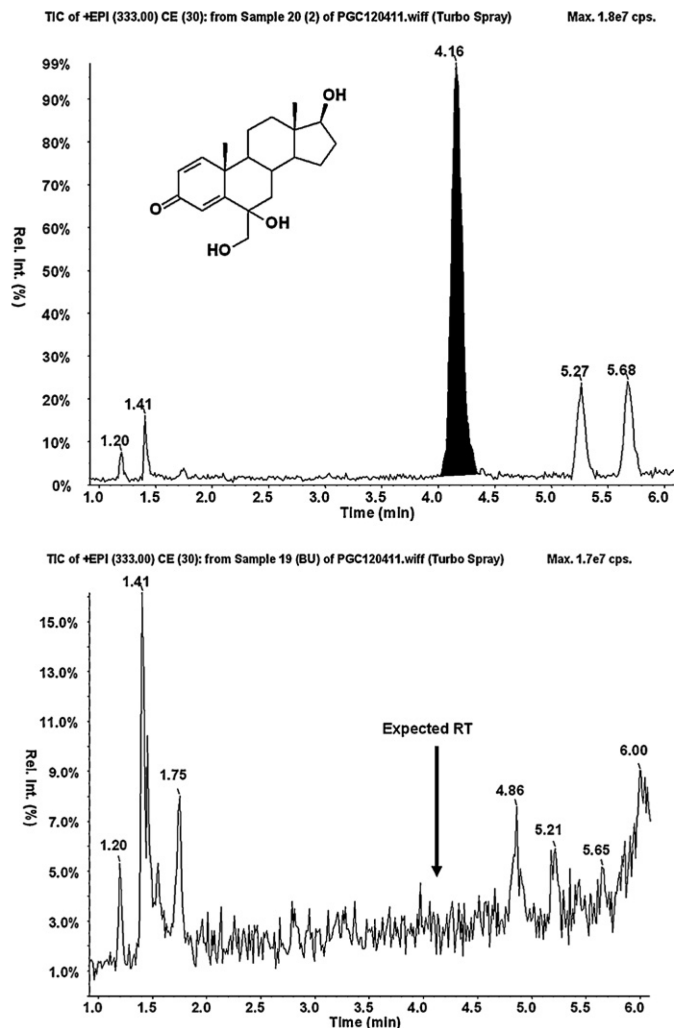
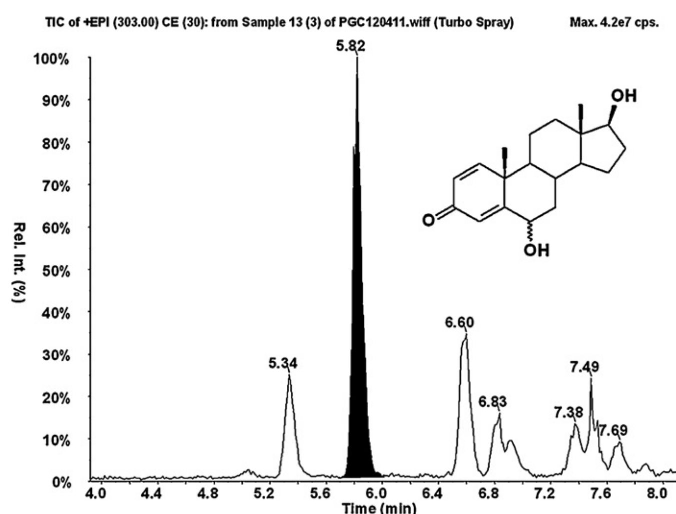


Fig. 2. Total ion chromatogram of enhanced product ion *m/z* 333 (TIC of EPI *m/z* 333) from urine excretion sample and blank urine (BU), respectively.

3. Results

A recent excretion study performed by our research group has detected three new urinary exemestane metabolites employing GC–MS. Two of them oxidized in C6 exomethylene group (6ξ-hydroxy-6ξ-hydroxymethylandrosta-1,4-diene-3,17-dione, 6ξ-hydroxyandrosta-1,4-diene-3,17-dione and the other one reduced in A-ring (3ξ-hydroxy-5ξ-androst-1-ene-6-methylene-17-one). However, in this previous study the proposed metabolites which should be C6-oxidized and C17-reduced simultaneously could not be observed [7]. LC–MS/MS was employed because tandem mass spectrometry shows great selectivity, sensibility and allows a variety of mass experiments to be performed (e.g.: precursor ion, product ion scans and multiple reactions monitoring). As the proposed metabolites could not be detected by GC–MS, LC–MS/MS was also the logical alternative technique. Furthermore, this technique does not require a derivatization step and thus, it makes the sample preparation procedure faster than the one used for GC–MS analysis also eliminating the possibility of artifact formation as related by our group [15].



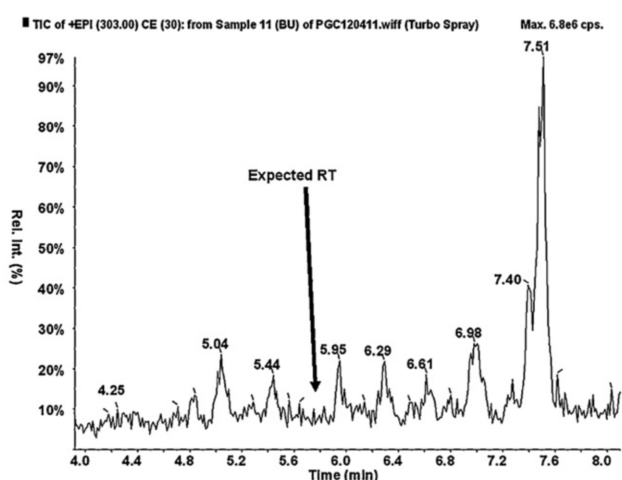
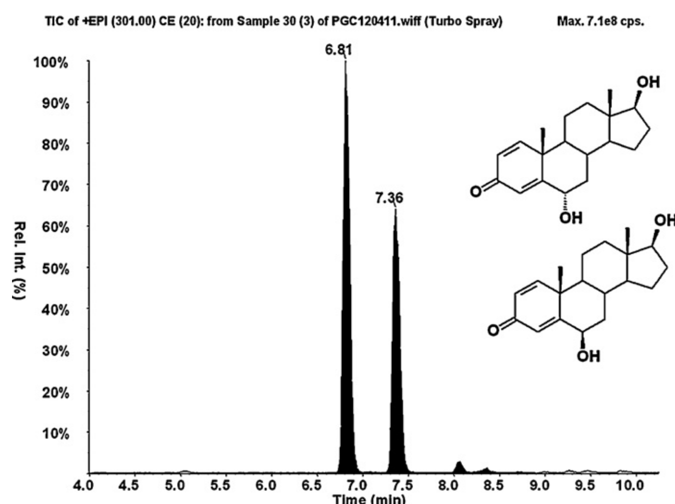


Fig. 3. Total ion chromatogram of enhanced product ion m/z 303 (TIC of EPI m/z 303) from urine excretion sample and blank urine (BU), respectively.



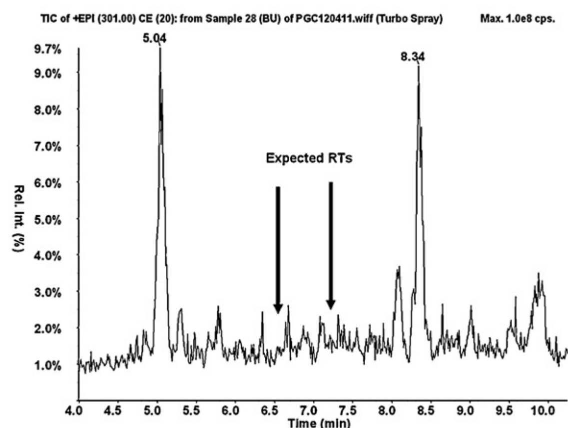


Fig. 4. Total ion chromatogram of enhanced product ion m/z 301 (TIC of EPI m/z 301) from urine excretion sample and blank urine (BU), respectively.

Product ion scans were performed by selecting the pseudomolecular ions in the first quadrupole (Q1) according to the metabolites proposed in Fig. 1. Three suspect signals were detected in all urine samples except in blank urines from the same subjects (Figs. 2–4) and their ESI product ion mass spectra are shown (Figs. 5–7). All these metabolites were characterized through their accurate mass measurement by LC–QTOF MS, obtaining deviations below 2 ppm compared to the theoretical mass (Table 1).

4. Discussion

The total ion chromatogram (TIC) of enhanced product ion m/z 333 showed an intense signal at the retention time (RT) of 4.16 min. Observing its). Their urinary excretion profiles are presented as well (Fig. 8 early RT and high m/z value a feasible structure is in agreement with a very polar metabolite proposed herein: 6ξ,17β-dihydroxy-6ξ-hydroxymethylandrosta-1,4-dien-3-one (Fig. 2). This metabolite was not detected by GC–MS.

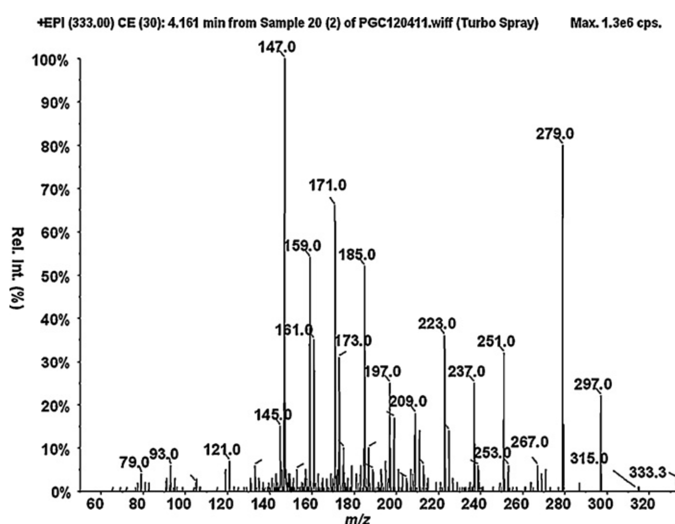


Fig. 5. Electrospray (ESI) product ion spectrum of m/z 333.

Table 1
Accurate masses and mass deviations of proposed exemestane metabolites obtained by LC–QTOF MS.

Compound	Elemental composition	Calc. mass	Theor. mass	Error (ppm)
6ξ,17β-Dihydroxy-6ξ-hydroxymethylandrosta-1,4-dien-3-one	$C_{20}H_{29}O_4$	333.2060	333.2065	1.50
6ξ,17β-Dihydroxyandrosta-1,4-diene-3-one	$C_{19}H_{27}O_3$	303.1957	303.1960	0.99
6α/β-Hydroxyandrosta-1,4-diene-3,17-dione	$C_{19}H_{25}O_3$	301.1709	301.1804	1.99

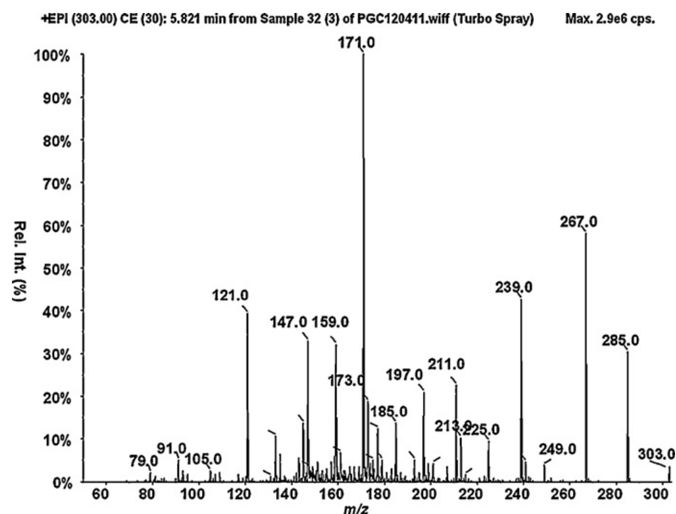


Fig. 6. Electrospray (ESI) product ion spectrum of m/z 303.

Its electrospray (ESI) product ion spectrum shows pseudomolecular ion $[M+H]^+$ m/z 333 and fragment ions arised from one, two and three neutral water losses $[M+H-18-36-54]^+$ as m/z 315, m/z 297 and m/z 279, respectively. A characteristic fragment ion m/z 267 arised from neutral loss of formaldehyde (−30 Da) [16,17] from fragment ion m/z 297 appears as well, suggesting a C6-hydroxymethyl group (Fig. 5).

Another suspect signal related to m/z 303 was observed at 5.82 min (Fig. 3). Its ESI product ion spectrum presents two fragment ions m/z 285 and m/z 267 which are originated from two consecutive water neutral losses. A fragment ion m/z 239 seems to be formed by the two water neutral losses followed by CO loss. The same fragmentation has been reported to 6β-hydroxytestosterone and 6α-hydroxyandrostenedione [17]. Another fragment ion m/z 171 is observed as well, as usually observed for C6 hydroxylated steroids [18]. All the features depicted above lead to the proposed metabolite 6ξ-17β-dihydroxyandrosta-1,4-dien-3-one. Another evidence is observed on the ESI product ion spectrum of 3-keto-1,4,6-triene steroids (17α/β-hydroxy-androsta-1,4,6-triene-3-one), its fragment ions are identical [19] with the fragment ions observed in ESI product ion spectrum of compound 6ξ-17β-dihydroxyandrosta-1,4-dien-3-one: m/z 159, m/z 171, m/z 173 and m/z 197, suggesting that the water neutral loss from C6 hydroxy group leads to a 3-keto-1,4,6-triene structure identical to the ones mentioned above (Fig. 6).

Two close signals are observed around 6.81 min and 7.37 min related to m/z 301 (Fig. 4) and their ESI product ion spectra show the same fragment ions (Fig. 7). Its ESI fragmentation features are pretty similar to another 3-keto-1,4,6-triene steroids (androsta-1,4,6-triene-3,17-dione) [19]. Due to fragment ions associated with the compound mass and early RT the metabolites 6α/β-hydroxyandrosta-1,4-diene-3,17-dione (6α/β-hydroxyexemestane) were proposed. One of them has already been reported by GC–MS, however only one isomer was detected by that technique [7]. This fact can be explained by the

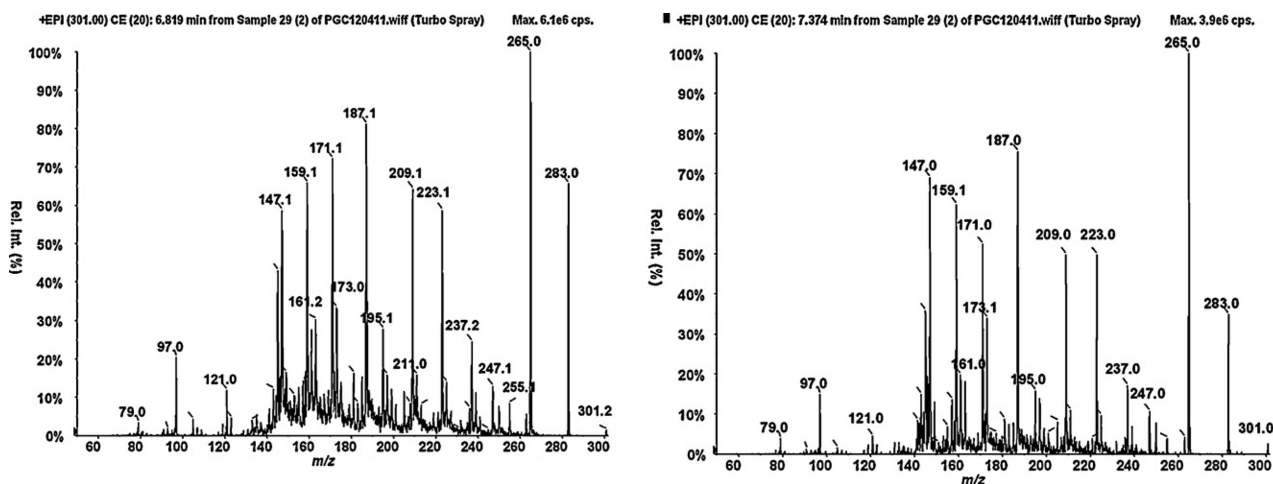


Fig. 7. Electrospray (ESI) product ion spectra of m/z 301 (isomer form).

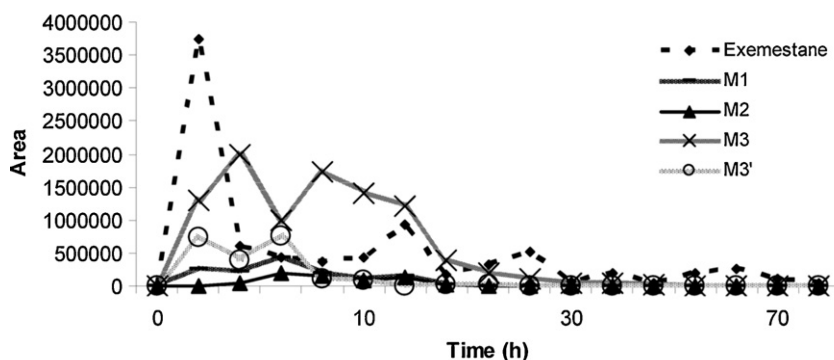


Fig. 8. Urinary excretion profile of exemestane and its proposed metabolites oxidized in C6 and reduced in C17. M1 = 6ξ,17β-dihydroxy-6ξ-hydroxymethylandrosta-1,4-dien-3-one, M2 = 6ξ-17β-dihydroxyandrosta-1,4-dien-3-one, M3/M3′ = 6α/β-hydroxyandrosta-1,4-diene-3,17-dione.

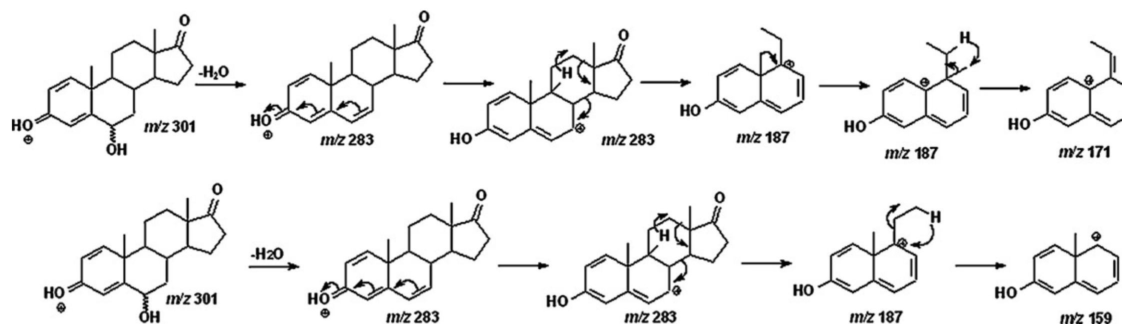


Fig. 9. Proposed ESI fragmentation pathway of 6α/β-hydroxyandrosta-1,4-diene-3,17-dione.

derivatization employed as in these conditions the 3-5-dienol derivatives are preferably formed [20].

Formation of the main fragment ions observed in ESI product ion spectrum of ion *m*/*z* 301 is proposed (Fig. 9). A multiple stage mass spectrometry (MS/MS/MS experiment) was carried out and it was observed that *m*/*z* 159 and *m*/*z* 171 derive from *m*/*z* 187. The protonation occurs preferably in 3-keto group (high proton affinity) because it is conjugated with double bonds which stabilize the positive charge in the oxygen atom by resonance effect. At first a neutral water loss from C6 hydroxy group generates a highly conjugated system which is very stable (energetic favorable). Thus, the positive charge can be delocalized on carbon atom 8 (C8) resulting in the cleavage of C8–C14 and C12–C13 bonds followed by hydrogen rearrangement yielding the fragment ion *m*/*z* 187. This one undergoes further fragmentation generating other stable allyl fragment ions *m*/*z* 159 and *m*/*z* 171. This result is in agreement with MS/MS/MS experiments.

All metabolites reported by our previous GC–MS study were confirmed herein (data not shown), except the metabolite reduced in A-ring (3ξ-hydroxy-5ξ-androst-1-ene-6-methylene-17-one) [7], because this metabolite shows a poor proton affinity as reported to some fluoxymesterone metabolites [8].

The chromatographic behavior (in terms of RT) of the proposed metabolites is in agreement with the proposed structures. Note that metabolites oxidized in the C6-exomethylene, with greater dipolar moment, elute first (RT 4.16 min, 5.82 min, 6.81 or 7.36, respectively) than less polar compounds such as exemestane which elutes at RT 14.30 min and 17β-hydroxyexemestane eluting at 12.90 min in these chromatographic conditions.

A multiple reaction monitoring (MRM) method was developed using one transition for each metabolite (see Section 2). From MRM data urinary excretion profiles of exemestane metabolites were built. The maximum excretion rate was observed between 4 and 6 h after administration for all metabolites. One of the C6 hydroxy isomers seems to be excreted in higher amounts than the other. The 6ξ,17β-dihydroxy-6ξ-hydroxymethylandrosta-1,4-dien-3-one and 6ξ-17β-dihydroxyandrosta-1,4-dien-3-one metabolites are not detectable after 24–30 h after 25 mg of exemestane administration. One of the 6α/β-hydroxyandrosta-1,4-diene-3,17-dione isomers can be detected until 50 h while another isomer is detectable until 82 h after 25 mg of exemestane administration (Fig. 8).

Until now, only the 17β-hydroxyexemestane can be efficiently used for detection of exemestane misuse. These newly described metabolites (specially the 6α/β-hydroxyandrosta-1,4-diene-3,17-dione) could be used as alternative targets, making results more reliable and broadening the options for doping control analysis of this drug.

According to the data achieved in accurate mass experiments, differences between the theoretical and experimentally detected masses were acceptable (errors below 2 ppm), allowing the elemental composition assignment (Table 1).

5. Conclusion

Two unreported exemestane metabolites originated after the oxidation of the C6-exomethylene group and reduction of 17-keto group have been detected. Also, two unreported isomers oxidized in C6-exomethylene were observed. All these metabolites showed a good proton affinity and thus they are alternative targets to monitor exemestane abuse by LC–MS/MS. LC–MS/MS was able to detect three metabolites which were not detectable in previous GC–MS analysis, showing more sensibility than the last technique. ESI-product ion spectra of metabolites showed some characteristic fragment ions which were discussed and rationalized being in agreement with the proposed structures. These results add alternatives and reliability in doping control analysis of exemestane. Synthesis of standard compounds and further analysis could be performed for determination of absolute stereochemistry of these newly described metabolites.

Acknowledgements

The authors appreciate the support from CAPES, CNPq, FAPERJ and FUJB.

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