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

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Introduction

Exemestane is an orally active third generation irreversible aromatase inhibitor, structurally related to the endogenous steroid androstenedione **[1]**.Unfortunately, in sports competition, male athletes are encouraged to treat some adverse effects caused by extensive abuse of anabolic steroids. The metabolism of exemestane in men is not clearly described. 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)**. 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 liquid chromatography coupled to electrospray tandem mass spectrometry (LC-ESI-MS/MS) and hybrid quadrupole time of flight mass spectrometry (LC-QTOFMS).

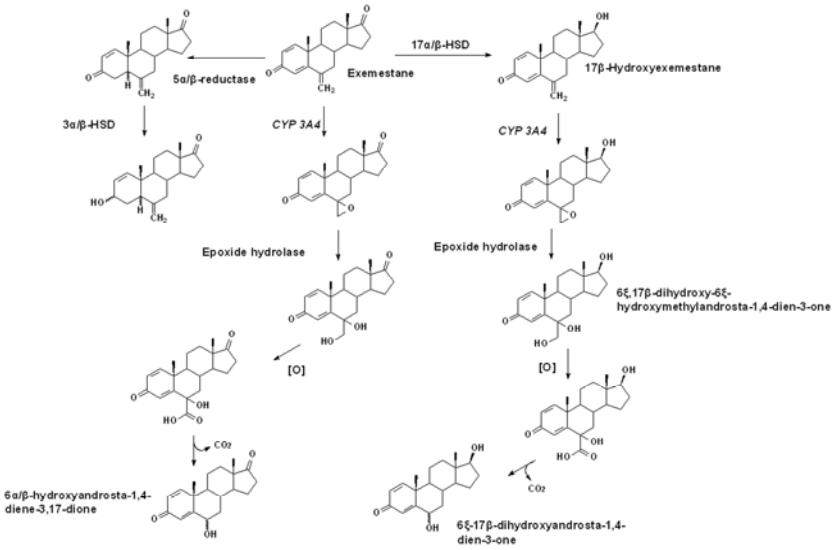

Figure 1: Proposed exemestane metabolic pathway.

Experimental

Excretion study urine samples

One exemestane tablet was orally administered to each of the four volunteers. All urine samples were stored at -20^{o}C until analysis. 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).

Sample preparation

The urine samples were prepared using the screening method for anabolic steroids described by Schänzer and Donike **[2]** with few modifications, and then analyzed by LC-ESI-MS/MS and LC-QTOFMS.

LC-ESI-MS/MS analysis

Compound separation was performed using a Zorbax C18 column (150 mm x 4.6 mm i.d, 5.0 μm) at a flow rate of 1 mL/min. 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^{o}C, declustering potential 30 V. The collision energies applied were optimized for each metabolite.

ESI-accurate mass measurements (LC-QTOFMS)

The chromatography conditions are the same depicted above. Time of flight mass detector temperatures: gas temperature, 330^{o}C; Drying gas flow, 10 L/min; Fragmentor voltage, 150V; skimmer, 60V, capillary voltage, 4000V.

Results

LC-ESI-MS/MS has been an useful tool to perform metabolism studies **[3]**, this technique shows more sensitivity and lower detection limits when compared to GC-MS **[4]**.Three suspect signals were detected in all urine samples except in blank urines from the same subjects **(Fig 2)** and their ESI product ion mass spectra are shown **(Fig 3)**. All these metabolites were also characterized through their accurate mass measurement by LC-QTOFMS **(Table 1)**.

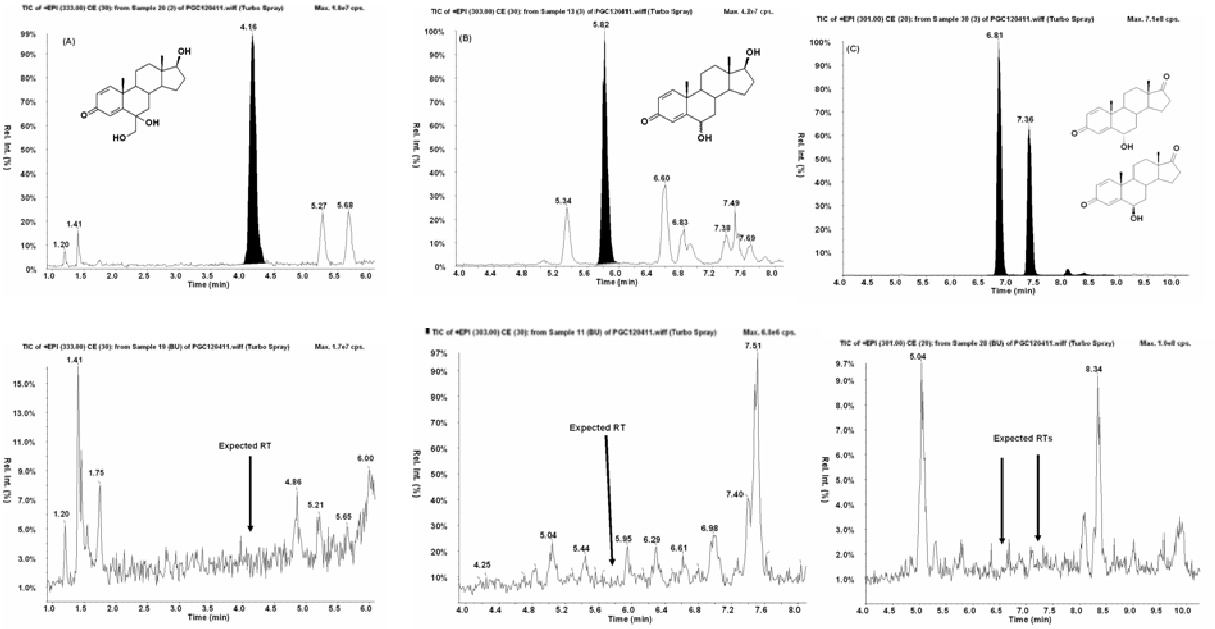

Figure 2: Total ion chromatogram of enhanced product ions of *m/z* 333 (A), *m/z* 303 (B) and *m/z* 301 (C) from excretion urine samples and blank urine samples (down).

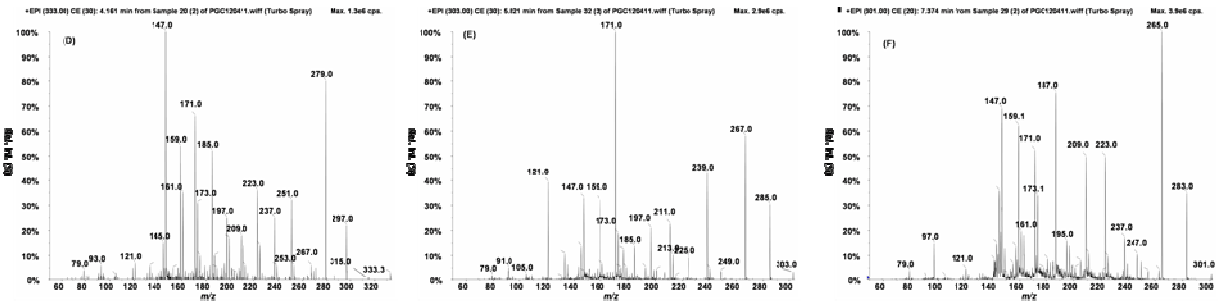

Figure 3: Electrospray (ESI) product ion spectra of *m/z* 333 (D), *m/z* 303 (E) and *m/z* 301 (F).

Table 1: Accurate masses and mass deviations of proposed exemestane metabolites obtained by LC-QTOFMS.

metabolite	calc.mass (*m/z*)	theor. Mass (*m/z*)	error (ppm)
M1	333.2060	333.2065	1.5
M2	303.1957	303.1960	0.99
6α/βhydroxyexmestane	301.1798	301.1804	1.99

Discussion

The total ion chromatogram (TIC) of the enhanced product ion *m/z* 333 showed an intense signal at the retention time (RT) of 4.16 min. Observing its 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 (M1) **(Fig 2A)**. Its electrospray (ESI) product ion spectrum shows a characteristic fragment ion *m/z* 267 arised from neutral loss of formaldehyde (-30 Da) **[5]** from fragment ion *m/z* 297 $[M\text{-}2.H_2O]^+$, suggesting a C6-hydroxymethyl group **(Fig 3 - D)**.Another suspect signal related to *m/z* 303 was observed at 5.82 min **(Fig 2B)**. Its ESI product ion spectrum is very similar as the one 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 **[6]** with the fragment ions observed in ESI product ion spectrum of compound 6ξ-17β-dihydroxyandrosta-1,4-dien-3-one (M2) **(Fig 3E)**. Two closely eluting signals (at 6.81 min and 7.37 min) were observed at *m/z* 301 **(Fig 2C)**. The ESI product ion spectra of these compounds showed identical fragment ions **(Fig 3F)**, and the fragmentation pattern is similar to androsta-1,4,6-triene-3,17-dione **[7]**. 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. According to the data achived in accurate mass experiments, differences between the theoretical and experimentally detected masses were acceptable (errors below 2 ppm – **table 1**), allowing the elemental composition assignment.

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