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Identification of urinary metabolites of exemestane oxidized in the exomethylene group by GC-MS

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Introduction

Exemestane (6-methylenandrosta-1,4-diene-3,17-dione) is a specific steroidal aromatase inhibitor. This class of compounds is often misused to avoid some side effects caused by anabolic steroids and to increase testosterone plasmatic concentration [1]. This drug is extensively metabolized, but 17β-hydroxy-6-methylene-androstadienone (17β-OH-exemestane) is the only metabolite identified. The metabolism of exemestane in men is not clearly understood. The 6-exomethylene group undergoes oxidation reactions generating several metabolites not yet described [2]. Buzzetti *et al*. have synthesized potential exemestane metabolites oxidized in the 6-exomethylene group [3]. The aim at this work was to evaluate new exemestane metabolites oxidized in C6 exomethylene group, using Gas Chromatography coupled to Mass Spectrometry (GC-MS) as the technique of choice.

Materials and Methods

Excretion study urine samples

An excretion study was performed with four healthy male volunteers (age: 20-30 years, weight: 75-80 Kg). One exemestane tablet (25 mg) was orally administered after a meal. Urine samples were collected for 14 days and stored at -20^{o} C.

Sample preparation

Excretion urine samples were prepared according to the method described by Schanzer and Donike [4], but two alternative derivatizations were employed: MSTFA/NH_4I/2-mercaptoethanol (1000:2:6 v/w/v, 60^{o}C/20 min), and methyloxime-pyridine (8:100 w/v, 60^{o}C/30 min), with pyridine removal under nitrogen flow at 40^{o}C followed by an addition of MSTFA/TMS-imidazole (100:2 v/v, 60^{o}C/20 min).

GC-MS procedure

GC conditions: carrier gas was helium at 1 mL/min constant flow mode; the column used was 100% methylpolysiloxane, 17 m x 0.2 mm x 0.11 μm; injector temperature was 280°C; 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 (70 eV); temperatures of ion source, 220°C; quadrupole, 150°C; transfer line, 280°C. All mass spectra were obtained in full scan mode; mass range, 50-750 Da.

Results and Discussion

In this study two new exemestane metabolites oxidized in C6 exomethylene were detected: 6ξ-hydroxy-6ξ-hydroxymethylandrosta-1,4-diene-3,17-dione (metabolite 1) and 6ξ-hydroxyandrosta-1,4-diene-3,17-dione (metabolite 2 - 6-hydroxyandrostadienedione). Two metabolite signals were observed in all urine fractions which were not observed in blank urines from the same subjects. Metabolite 1 (retention time 19.10 min) could be generated by epoxide hydrolase, probably derived from an epoxide intermediate. Observing its electron impact mass spectrum it is possible to notice three diagnostic fragment ions (**Figure 1-spectrum A)**. Mass spectrum from metabolite 2 (retention time 16.55 min) does not provide good information because it has few significant fragment ions **(Figure 1-spectrum B)** as expected for 1,4 dien-3-one steroid *O*-TMS derivatives containing a C6-hydroxyl group, such as the C6-hydroxy metabolites of dehydrochloromethyltestosterone, boldenone and methandienone. Their *tris O*-TMS mass spectra usually show an $[M-15]^+$ fragment ion as the most abundant fragment. B ring characteristic fragments and molecular ions show low abundances, due to the presence of C6-hydroxy groups [5]. Furthermore, Schanzer and Donike have already reported the formation of a C6-hydroxylated metabolite from boldenone (another 1,4-diene steroid), with a identical mass spectrum [6]. All these mass spectral characteristics are consistent with structure of metabolite 2 proposed in this work. C6 hydroxylation could not occur in just one step because this site is hindered by an exomethylene group. Hence, this metabolite is probably originated from metabolite 1, which is further oxidized at the primary hydroxyl group, leading to a carboxylic acid. Then, the metabolite 2 would be formed by decarboxylation of the carboxylic intermediate, this hypothesis is in accordance to Masse *et al*. that have reported the decarboxylation of β-keto acid intermediate from formebolone [7].

Opfermann *et al*. have already reported exemestane and 17β-OH-exemestane artifacts when the enolization reaction occurs when the analagous derivatization mixture

MSTFA/NH_4I/ethanethiol is employed [8]. Thus, the samples were submitted to an alternative derivatization process, which employs methyloxime and MSTFA/TMS-imidazole. The proposed structures for the two metabolites described in this study were confirmed by mass spectra interpretation of their MO-TMS derivatives. The fragment ions obtained with this derivatization were very diagnostic for both metabolites MO-TMS derivatives (**Figure 1- spectra C and D, respectively**). Thus, it is possible to affirm that the signals detected in all urine samples come from exemestane metabolites and are not derivatization artifacts formed when the mixture MSTFA/NH_4I/2-mercaptoethanol is used.

Conclusions

Two new exemestane metabolites oxidized in the C6-exomethylene group were observed in all urine samples fractions: 6ξ-hydroxy-6ξ-hydroxymethylandrosta-1,4-diene-3,17-dione (metabolite 1) and 6ξ-hydroxyandrosta-1,4-diene-3,17-dione (metabolite 2). The GC-MS technique using alternative derivatization reactions was a helpful tool to elucidate structures of new exemestane metabolites.

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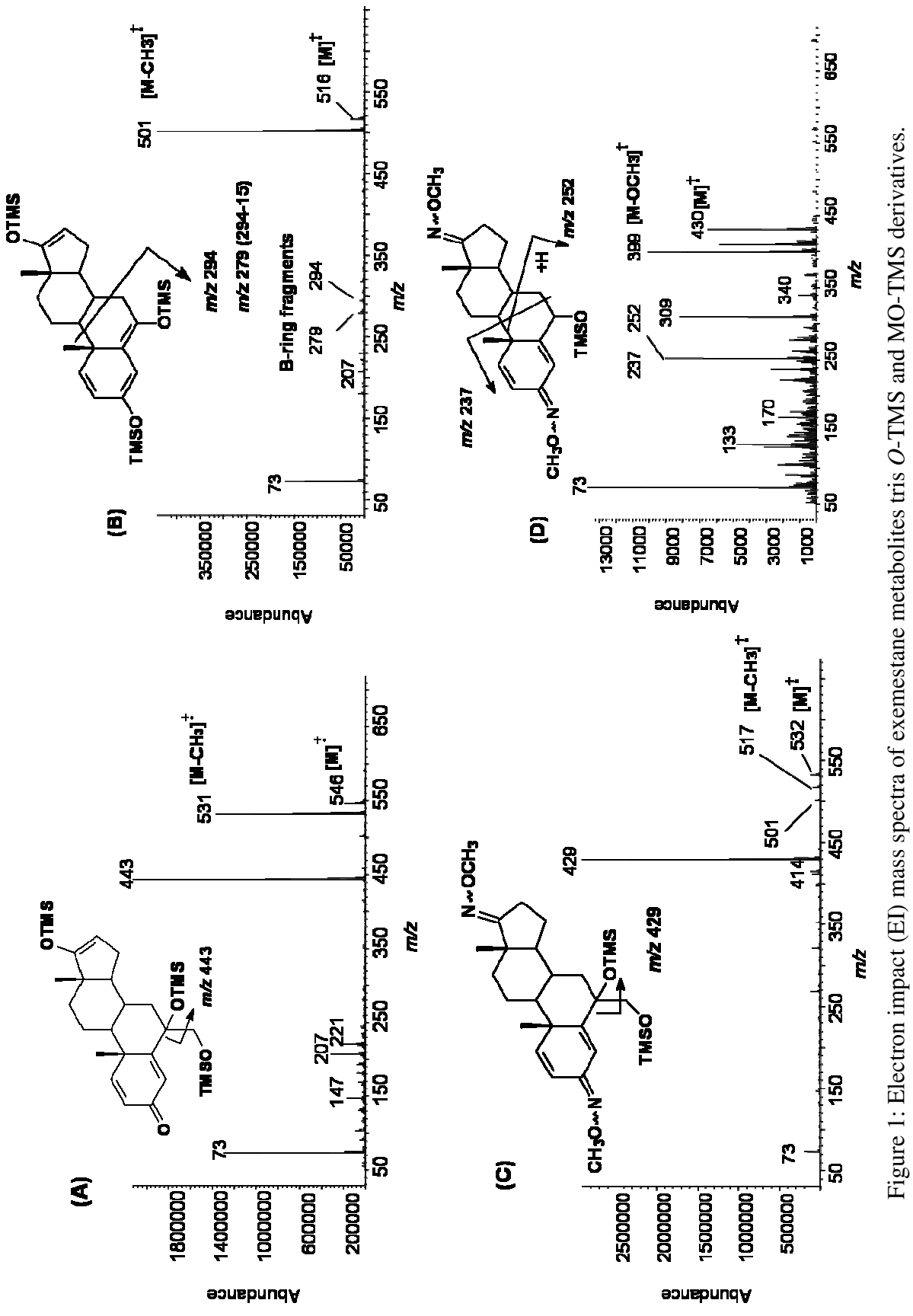

Figure 1: Electron impact (EI) mass spectra of exemestane metabolites tris *O*-TMS and MO-TMS derivatives.