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Synthesis of 17-dihydroexemestane as a reference compound in doping control

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Abstract

17-Dihydroexemestane – the main metabolite of exemestane – was synthesized for doping analytical purposes. The synthesized metabolite was purified and characterized, and LC-MS/MS and LC/TOFMS methods for the characterization of the exemestane metabolite in urine were developed. Based on the excretion study of a single male volunteer, 17-dihydroexemestane was found to be the main metabolite in human urine, emphasizing the importance of its synthesis and availability as a reference material with respect to doping analysis. The metabolite synthesized within this project will be available without charge to all WADA-accredited anti-doping laboratories.

Introduction

Exemestane (Aromasin®) (**1**) belongs to a group of drugs known as aromatase inhibitors and is used as an early-phase treatment for postmenopausal women with estrogen-receptor positive breast cancer. It acts as an antagonist for the aromatase enzyme which is responsible for converting androgens to estrogens. Exemestane binds permanently to the active site of the aromatase enzyme and thus blocks it [1]. In sports the abuse of exemestane stems from the fact that it can be used to counteract the adverse effects of an extensive use of anabolic androgenic steroids and to increase testosterone concentration by affecting biosynthesis of testosterone [2]. As a part of this WADA-funded research project (Grant 07C04JY/2007) we synthesized 17-dihydroexemestane (**2**) for doping analytical purposes. The synthesized metabolite was purified and characterized, and LC-MS/MS and LC/TOFMS methods for the characterization of the exemestane metabolite in urine were developed. The synthesized and

fully characterized 17-dihydroexemestane enables the reliable and legally defensible confirmation analysis of exemestane, and could also be used in quality assurance and in the development of new analytical methods.

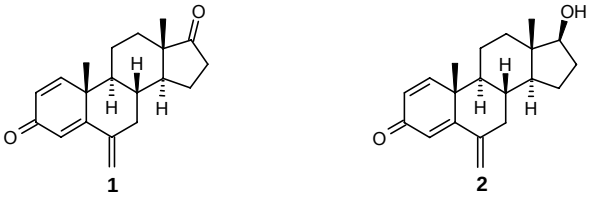
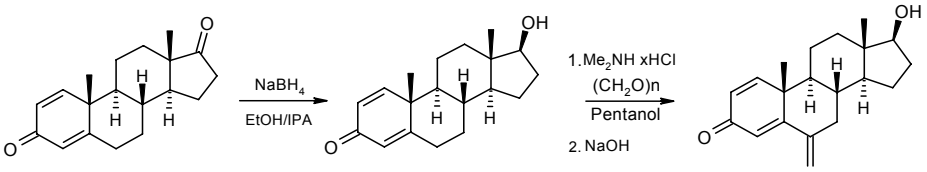


Figure 1. Exemestane (**1**) and 17-dihydroexemestane (**2**)

Materials and Methods

Synthesis of 17-dihydroexemestane

17-Dihydroexemestane was synthesized according to Scheme 1. The carbonyl group in carbon 17 of androsta-1,4-diene-3,17-dione was converted to hydroxyl group by sodium borohydride reduction. 1,4-Androstadien-17β-ol-3-one was first treated with dimethylamine and paraformaldehyde in pentanol and subsequently with sodium hydroxide solution to give 17-dihydroexemestane [3].



Scheme 1. Synthesis of 17-dihydroexemestane

Sample pretreatment

To 4 mL of urine 1.5 mL of sodium-potassium phosphate buffer (0.8 M, pH 7) and 50 µL of β-glucuronidase (*E. coli*) were added. The samples were vortexed and incubated at 50 °C for one hour. The samples were then cooled to room temperature and 1 mL of buffer (20% of K_2CO_3-$KHCO_3$), 10 mL of diethyl ether and 4 g of anhydrous sodium sulfate were added. The samples were vortexed again and centrifuged (2000 g, 5 min). The ethereal phase was separated and evaporated to dryness in a water bath at 50-60 °C under nitrogen atmosphere. The sample was reconstituted in the mobile phase.

LC-MS/MS-analysis

The LC system was a Surveyor from ThermoFinnigan and chromatographic separation was performed using a Zorbax Eclipse XDB-C18 (2.1 × 50 mm, 3.5 µm) column from Agilent,

with a guard column of the same packing materials (2.1 × 12.5 mm, 5 µm), also from Agilent. The mobile phase consisted of a 2.5 mM ammonium acetate buffer with 0.1% formic acid (A) and 2.5 mM ammonium acetate with 0.1% formic acid in 90% methanol (B). The gradient started at 50% B and was increased to 100% in a linear fashion during 5 minutes, followed by an equilibration step of two minutes. The temperature of the column compartment was set to 25 °C. Detection was carried out by a TSQuantum triple quadrupole from ThermoFinnigan. The examination of the analytes' mass spectrometric properties was conducted with the synthesized metabolites using an electrospray (ESI) interface in the positive ion mode. The MS- and MS/MS-spectra were measured and five ion transitions were selected for the MRM-analysis. Argon was used as the collision gas at a pressure of 1 mTorr. Nitrogen was used both as sheath and auxiliary gas (49 and 5 arbitrary units). The spray voltage was 4000 V and the capillary temperature 270 °C.

LC-TOF-MS analysis

The LC system was a 1200 Series Rapid Resolution LC from Agilent. Chromatographic separation was carried out on an Agilent Zorbax Eclipse Plus C18 column (2.1 × 50 mm; 1.8 µm) including an in-line frit. The mobile phase contained a 2.5 mM ammonium formate buffer with 0.1% formic acid (A) and 2.5 mM ammonium formate buffer with 0.1% formic acid in 90% acetonitrile (B). Gradient elution was applied starting from 10% B, the proportion of B was held at 10% for 1 minute and then linearly increased to 40% in 2 min, to 70% in 1 min, to 90% in 2 min and held at 90% for 0.5 min and then back to 10% in 0.5 min. The total run time, with 1 minute post run time, was 8 minutes at a flow rate of 0.4 mL/min. The column oven temperature was set to 40 °C. Detection was based on time-of-flight mass spectrometry (TOF-MS) using a Bruker microTOF instrument with positive ion mode ESI. The mass range was *m/z* 50-600. External calibration with sodium formate clusters was used for mass range calibration. Nitrogen was used as dry and nebulizer gas, at 8 L/min and 1.6 bar, respectively.

Results and discussion

Three aliquots of drug-free urine were analyzed to check the selectivity of the assay. The biological background was low with no interfering peaks at the retention time of the metabolite. The metabolite could be detected with LC-MS/MS at a concentration level of 50 ng/mL with a signal-to-noise ratio higher than 150. Mass accuracy in LC-TOFMS analysis

was 4 mDa. Examples of the chromatograms of 17-dihydroexemestane are presented in Figure 1.

In the current project a method for the synthesis of 17-dihydroexemestane, the main metabolite of the aromatase inhibitor exemestane, was developed. The characterization of the metabolite was carried out by LC-MS/MS and LC/TOFMS, comparing the synthesized metabolite with an authentic urine sample. The synthesized and fully characterized 17-dihydroexemestane enables the reliable and legally defensible confirmation analysis of exemestane in doping control, and could also be applied for quality assurance and the development of new analytical methods.

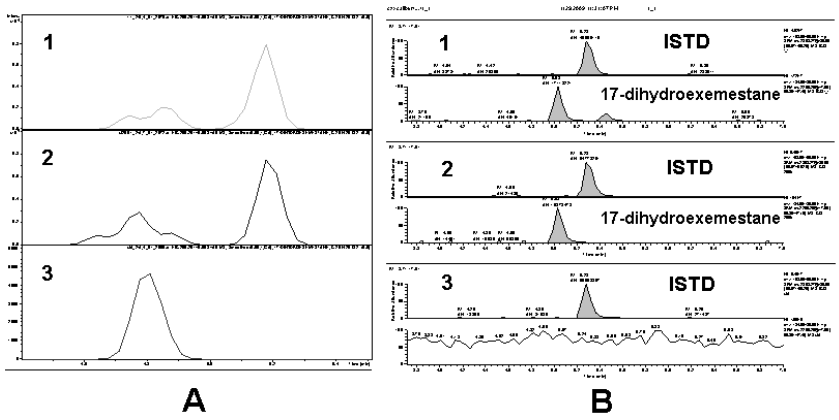


Figure 1. (A) LC-TOF-MS extracted ion chromatogram (EIC) for the exemestane metabolite (*m/z* 299. 2006) and (B) LC-MS/MS chromatograms (MRM, transition 299.20 → 135.10, 16 V for the exemestane metabolite shown, methyltestosterone as the internal standard). 1= an authentic patient urine sample collected after seven hours of oral administration of AROMASIN® 25 mg (Pharmacia Italia S.p.A., Ascoli Piceno, Italia) 2= the synthesized exemestane metabolite standard (c 250 ng/mL) 3= blank urine reference

References

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