

Searching for Alternative Danazol Metabolites Through Accurate Mass with LC/MS Q-TOF

Application Note

Doping Control

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Abstract

Quadrupole time-of-flight (Q-TOF) systems providing high resolution and accurate mass capabilities have been used in the antidoping field for the last decade. This technology is one of the most powerful tools to investigate new doping agents and markers. LC, coupled to an ESI-TOF, has been particularly efficient in determining low- and high-molecular doping-related species in biological matrices.

Anabolic androgenic steroids (AASs) are usually analyzed by GC techniques with electron impact (EI) ionization, mainly due to the poor proton affinity of most of them, which limits the ionization process by LC systems and results in loss of sensitivity. Nevertheless, several metabolic studies of AASs have been successfully carried out in the recent years by LC/MS systems, including free compounds, glucuronide- or sulfo-conjugated AAS metabolites.

Danazol (*17 α -ethynyl-androst-4-en-17 β -ol-(2,3-d)-isoxazole*) is usually monitored by targeting its metabolites ethisterone (*17 α -ethynyl-androst-4-en-17 β -ol-3-one*) and *2 α -hydroxymethylethisterone* (*17 α -ethynyl-2 α -hydroxymethyl-androst-4-en-17 β -ol-3-one*). We investigated alternative markers of danazol with an LC/MS Q-TOF system. An excretion study was conducted and all the samples collected were analyzed in positive and negative ionization modes after simple sample preparation. Several alternative mono- and di-hydroxylated metabolites were detected in basic or acidic extractions. MS/MS fragment interpretation was tentatively performed. Detectability over time using LC/MS Q-TOF for each of the species was also assessed by using excretion curves.



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Introduction

The accurate-mass capability provided by LC/MS quadrupole time-of-flight (Q-TOF) systems has attracted the attention of doping control research in recent years. LC, coupled to an ESI source, has been successfully explored for metabolic studies of doping-related species in biological matrices, even for poorly ionizable anabolic androgenic steroids. In this work, alternative markers of the synthetic steroid danazol were investigated in human urine using a LC/MS Q-TOF system. Urine samples were collected over a month after the administration of a single dose of danazol to a male volunteer. The LC/MS Q-TOF analyses were carried out in positive and negative polarity modes after simple sample preparation.

Experimental

All solvents and reagents were of analytical grade. Standards of danazol (17 α -ethynyl-androst-4-en-17 β -ol-(2,3-d)-isoxazole), danazol M1 (ethisterone, 17 α -ethynyl-androst-4-en-17 β -ol-3-one), and danazol M2 (2 α -hydroxymethylethisterone, 17 α -ethynyl-2 α -hydroxymethyl-androst-4-en-17 β -ol-3-one), were acquired from Sigma-Aldrich, Corp. (St. Louis, MO, USA), Steraloids (Newport, RI, USA), and NMI (Pymble, Australia), respectively. Urine samples were obtained from an excretion study following the administration of a single dose of 100 mg of danazol to a healthy male volunteer (Caucasian, 39 years, 60 kg). The samples were collected before and up to 30 days after administration, and stored at -25 °C until analysis.

Liquid-liquid extraction

To extract danazol metabolites, use 6 mL urine samples. For basic extracts, add carbonate buffer (pH 11) (+ 5 mL TBME, shake, evaporate to dryness) \times 2. For acidic extracts, adjust to pH 2 with formic acid (+ 5 mL ethyl acetate, shake, evaporate to dryness) \times 2. Reconstitute in 150 μ L mobile phase.

Conditions

Column:	Agilent Poroshell 120 EC-C18, 2.1 \times 50 mm, 2.7 μ m (p/n 699775-932)
Mobile phase:	A) water (5 mM NH ₄ Ac + (1% CH ₃ COOH for positive mode analysis)) B) CH ₃ CN
Gradient:	10 to 75% B in 12 min, to 90% B in 12.1 min, hold for 1.7 min, 13.8 to 14.0 to 10% B, hold 3.5 min
Flow rate:	0.4 mL/min
Temperature:	50 °C

All samples were extracted and analyzed on an LC/MS Q-TOF system from Agilent Technologies, Inc. (Palo Alto, CA, USA). The instrument consisted of an Agilent 1290 Infinity LC coupled to an Agilent 6550 Accurate-Mass Q-TOF LC/MS with iFunnel technology, equipped with a dual Agilent Jet Stream (AJS) ESI source. Ionization was performed in positive and negative modes. Full-scan mass spectral data were acquired from m/z 60 to 1,100 at a rate of 3 spectra/s, while targeted MS/MS data was obtained from m/z 60 to 1,100 at a rate of 1.5 spectra/s, and collision energy of 35 eV. The drying gas flow and temperature were set at 12 L/min and 250 °C, respectively, and the nebulizer gas pressure was set at 40 psi. The applied capillary voltage was 4,000 V. The fragmentor voltage was 150 V. Nitrogen was used as desolvation and collision gas. Reference mass correction was used during the analyses to achieve the best mass accuracy. The instrument was calibrated daily, and operated with Agilent MassHunter Workstation LC/MS Data Acquisition Software version B.05.01. The chromatograms were processed with Agilent MassHunter Workstation Qualitative Analysis Software version B.06.00.

Results and Discussion

Urine samples collected during the excretion study were prepared and analyzed as described. More than 20 metabolites were detected in the full-scan mass spectral data when comparing negative and post-administration samples. Figure 1 shows the main metabolites detected in terms of intensity of signal. The structures are tentatively depicted based on previously reported species [1-4] and common metabolic pathways for anabolic androgenic steroids (AASs) in humans [5]. All main metabolites were found in their free form, although several sulphate and glucoconjugated species were detected as low signals in acidic and basic extracts.

The parent compound (danazol PC) was not detected in any of the urine samples, in accordance with previous work [1-4]. Conversely, the most common markers of danazol consumption, ethisterone (danazol M1) and 2 α -hydroxymethylethisterone (danazol M2), were detected in positive mode. Their identification was assessed accordingly by analysis of the reference material. All other main metabolites were detected in negative mode, and were hydroxylated and dihydroxylated derivatives of danazol PC, M1, and M2.

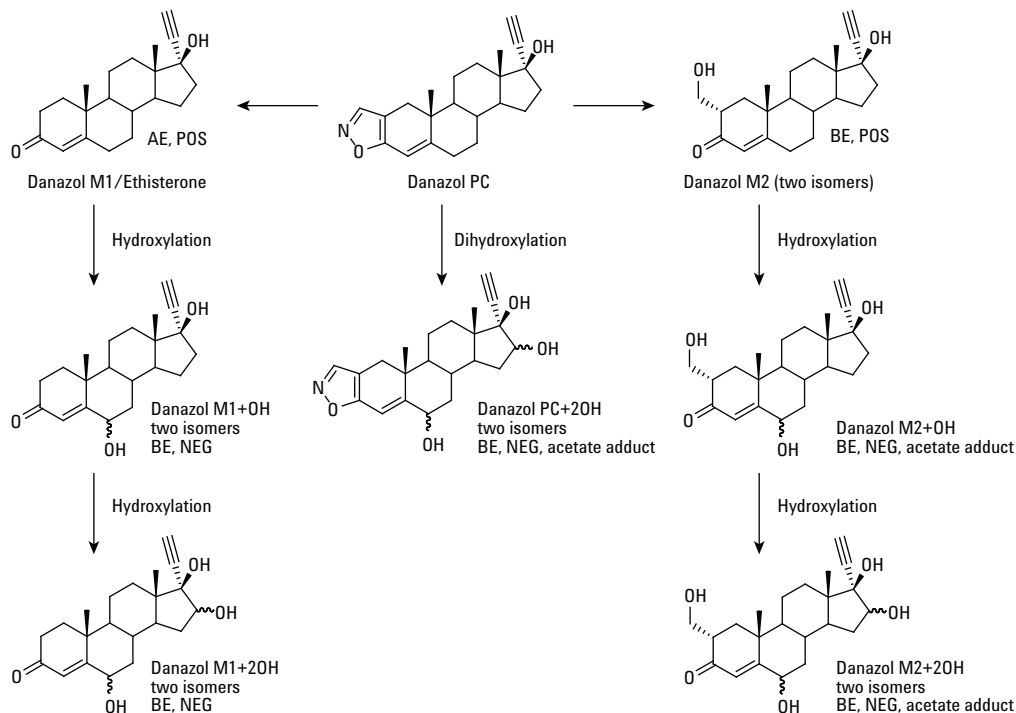


Figure 1. Proposed structures for the main metabolites detected. BE = metabolites in samples from basic extraction, AE = metabolites in samples from acidic extraction, NEG = metabolites in negative polarity mode, POS = metabolites in positive polarity mode.

MS/MS experiments were conducted to help in the elucidation of the structure of the hydro- and dihydroxylated metabolites. Figure 2 depicts the MS/MS spectra of one of the two isomers of the detected species danazol PC+2OH, danazol M1+2OH, and danazol M2+2OH. The figure also shows fragments identified by exact mass and the score calculated by the software.

In terms of detectability over time, one of the isomers of the species danazol M2+2OH proved to be the best marker in terms of retrospectivity for the detection of this steroid by LC/MS Q-TOF, since it could be detected up to 16 days after administration (Figure 3).

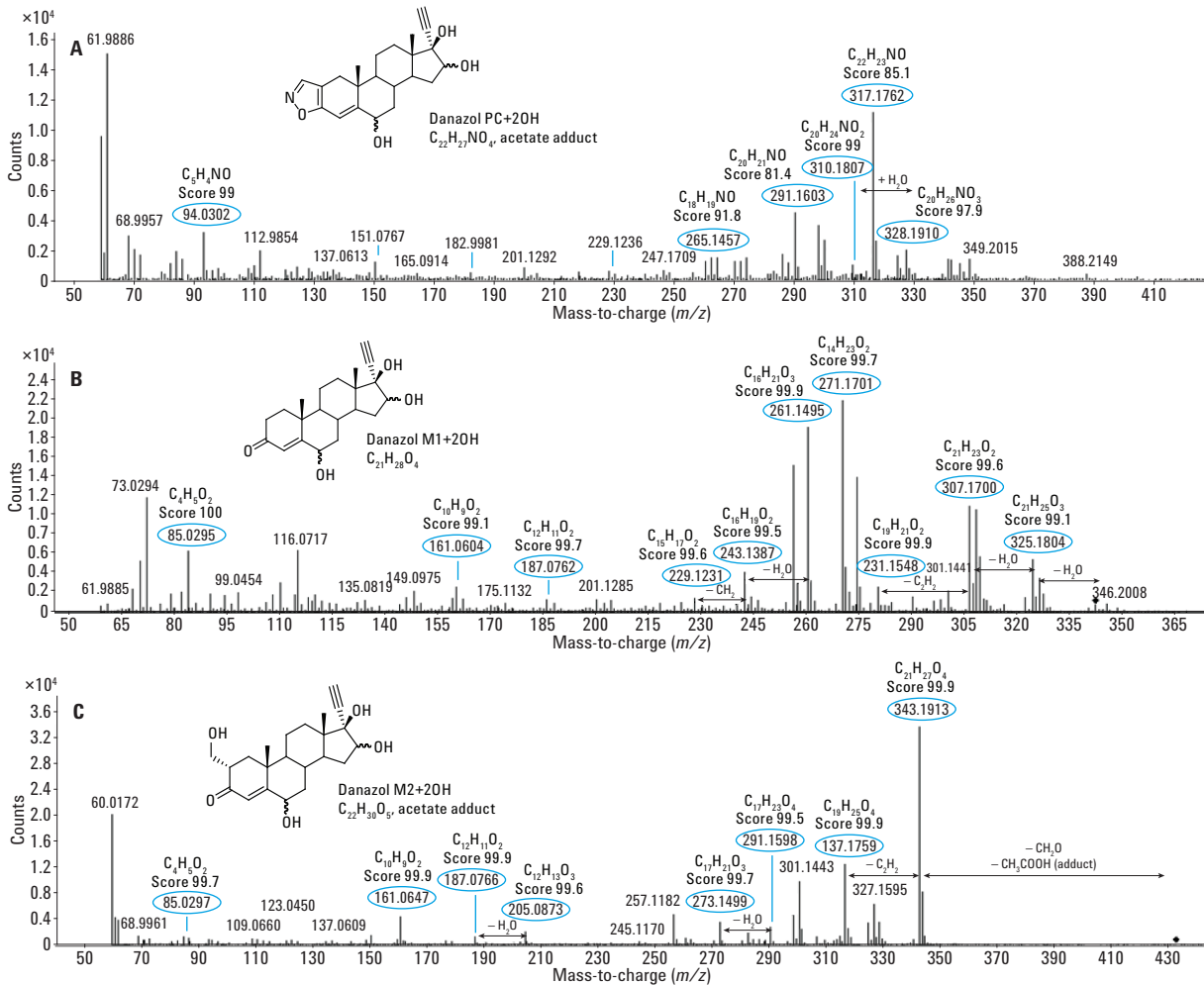


Figure 2. MS/MS spectra of danazol PC+2OH, danazol M1+2OH, and danazol M2+2OH (collision energy 35 eV).

Conclusions

The metabolism of the AAS danazol was investigated by using accurate-mass analysis in a Q-TOF system. A triple bond on the C17 prevents phase II conjugation to the neighboring hydroxyl group and, therefore, free urinary metabolites were mainly expected. Accordingly, metabolites mainly excreted as nonconjugated forms were detected, four of which, danazol PC+2OH (two isomers) and danazol M1+2OH (two isomers), have never been reported until now, to the best of our knowledge. Six of them could be detected one week after administration, and one of them (one of the two observed isomers of danazol M2+2OH) could be detected 16 days after consumption.

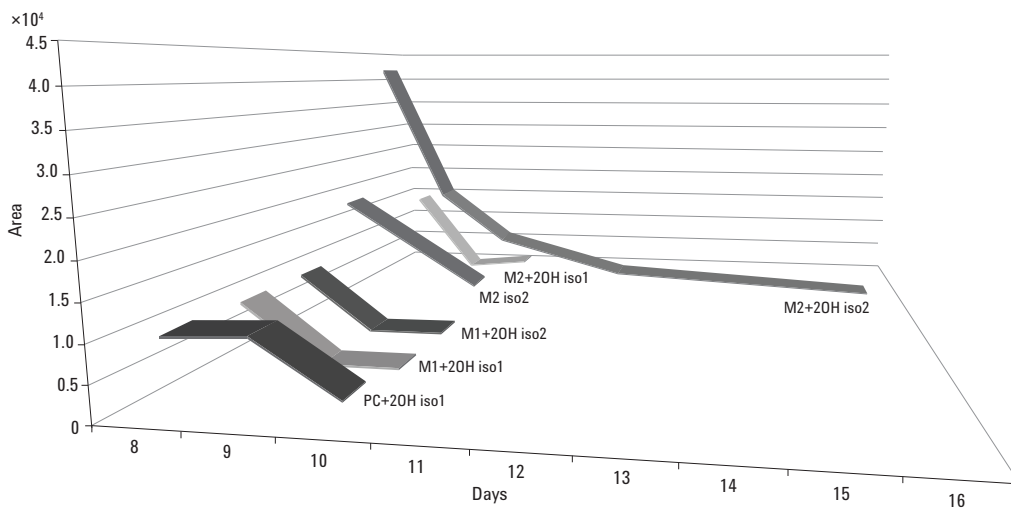


Figure 3. Detectability over time of the species danazol PC+2OH (one of the isomers), danazol M1+2OH (both isomers), danazol M2 (one of the isomers), and danazol M2+2OH (both isomers) in the LC/MS Q-TOF system.

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