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Searching for alternative danazol metabolites via Accurate-Mass by using Liquid Chromatography Quadrupole Time-of-Flight (LC-QTOF/MS)

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Abstract

Quadrupole Time-of-Flight (QTOF) systems providing high resolution and accurate mass capabilities have been used in the anti-doping 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 (AAS) 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 a loss of sensitivity. Nevertheless, several metabolic studies of AAS have been successfully carried out in the recent years by LC-MS systems, including free compounds, glucuronide- or sulfoconjugated AAS metabolites.
Danazol (17α-ethynyl-androst-4-en-17β-ol-(2,3-d)-isoxazole) is usually monitored by targeting its metabolites ethisterone (17α-ethynyl-androst-4-en-17β-ol-3-one) and 2α-hydroxymethylethisterone (17α-ethynyl-2α-hydroxymethyl- androst-4-en-17β-ol-3-one). In this work, alternative markers of danazol have been investigated by using a Liquid Chromatography Quadrupole Time-of-Flight (LC-QTOF/MS) system. An excretion study was conducted and all the samples collected were analyzed in positive and negative ionization modes after a simple sample preparation. Several alternative mono- and di-hydroxylated metabolites were detected in basic or acidic extractions and MS/MS fragment interpretation was tentatively performed. The detectability over time course using LC-QTOF/MS for each of the species by using excretion curves is also shown.

Introduction

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

Experimental

All solvents and reagents used were of analytical grade. Standards of danazol (17α-ethynyl-androst-4-en- 17β-ol-(2,3-d)-isoxazole), danazol M1 (ethisterone, 17α-ethynyl-androst-4-en-17β-ol-3-one) and danazol M2 (2α-hydroxymethylethisterone, 17α-ethynyl-2α-hydroxymethyl-androst-4-en-17β-ol-3-one) were acquired from Sigma-Aldrich (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 one 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.

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All samples were extracted following the scheme depicted in Figure 1 and analyzed by an LC-QTOF/MS system from Agilent Technologies, (Palo Alto, CA, USA). The instrument consisted of a 1290 Infinity HPLC coupled to a 6550 iFunnel QTOF mass analyser equipped with a dual AJS (Agilent Jet Stream) ESI source. The chromatographic conditions are described in Figure 1. Ionization was performed in positive and negative modes. Full scan mass spectral data were acquired from m/z 60 to 1100 at a rate of 3 spectra/sec while targeted MS/MS data was obtained from m/z 60 to 1100 at a rate of 1.5 spectra/sec 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 4000 V. The fragmentor voltage was set at 150 V. Nitrogen was used as desolvation and collision gas. Reference mass correction was used during the analyses to achieve the best mass accuracy, by means of an internal calibrant introduced throughout the run. The instrument was calibrated daily and operated with the MassHunter Workstation LC/MS Data Acquisition software version 05.01. The chromatograms were processed with the MassHunter Workstation Qualitative Analysis software version B.06.00 (both from Agilent Technologies, (Palo Alto, CA, USA)).

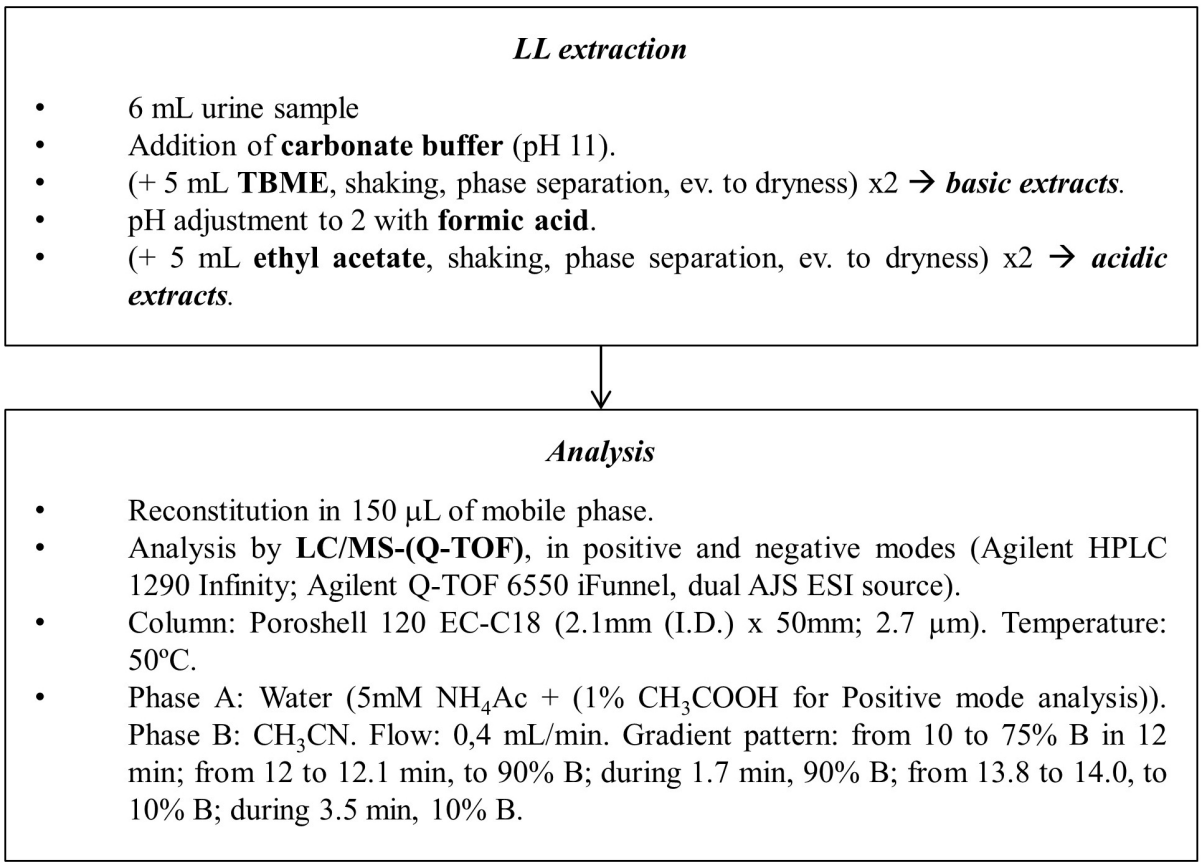

Figure 1: Sample preparation scheme

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Results and Discussion

Urine samples collected during the excretion study were prepared and analyzed as described above; more than 20 metabolites were detected in the full scan mass spectral data when comparing negative and post-administration samples. Figure 2 shows the main metabolites detected in terms of intensity of the signal. The structures are tentatively depicted on the basis of previously reported species [1-4] and common metabolic pathways for AAS in humans [5]. All main metabolites were found in their free form, although several sulphate and glucoconjugated species were detected as low signals in acid and basic extracts.

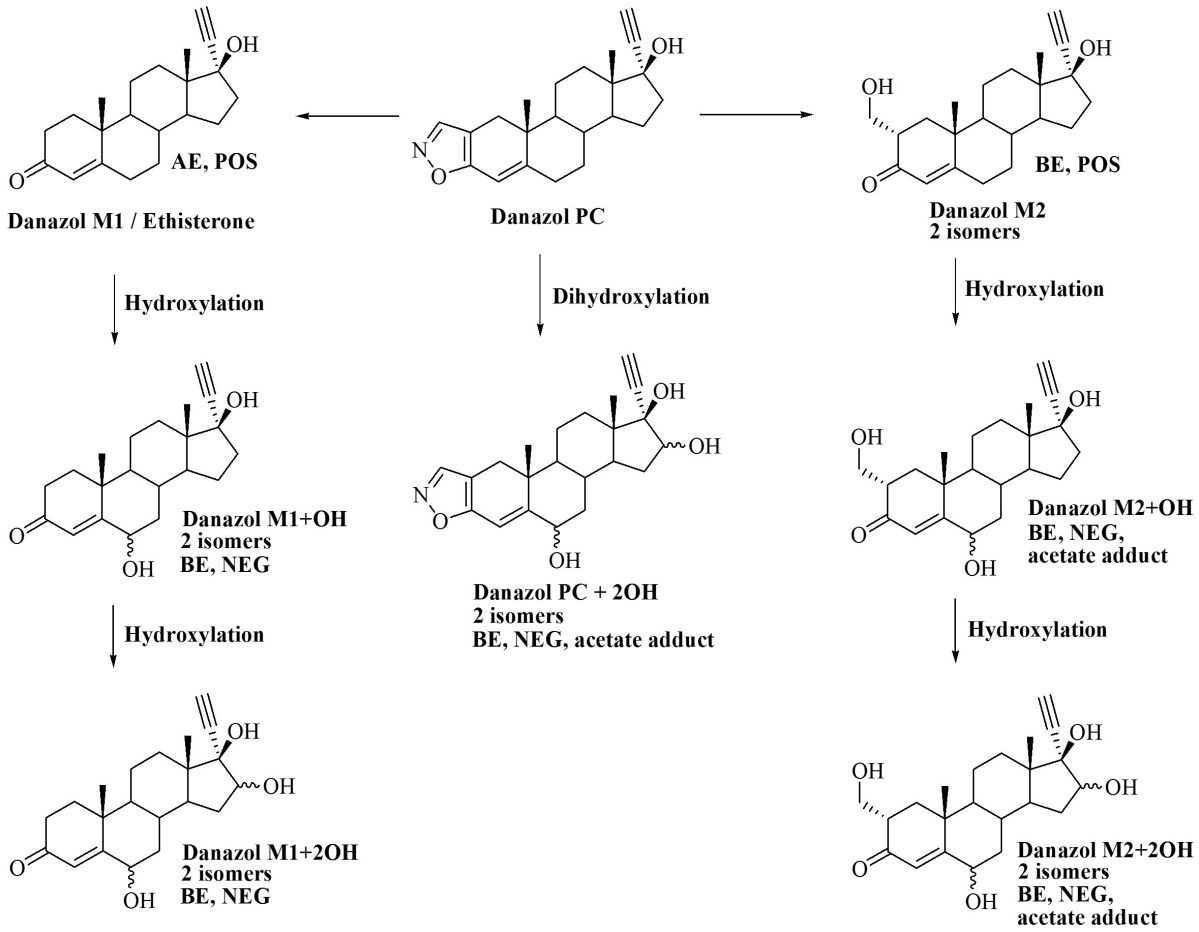

Figure 2: Proposed structures for the main metabolites detected. BE: metabolites found in samples from basic extraction. AE: metabolites found in samples from acidic extraction. NEG: metabolites found in negative polarity mode. POS: metabolites found in positive polarity mode

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

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MS/MS experiments were conducted in order to help in the elucidation of the structure of the hydro- and dihydroxylated metabolites. Figure 3 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', as well as the fragments identified by exact mass and the score calculated by the software.

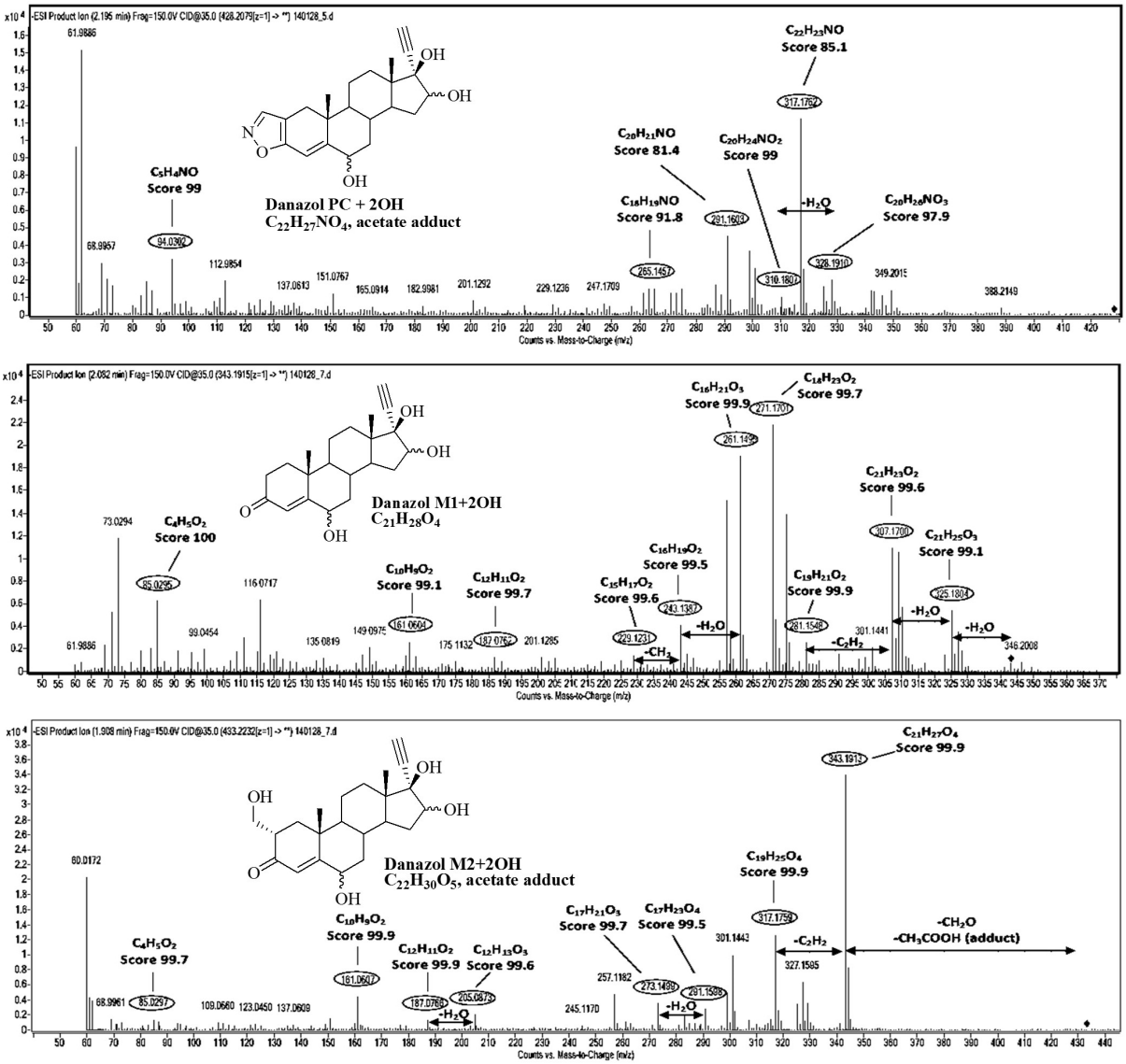

Figure 3: MS/MS spectra of 'danazol PC+2OH', 'danazol M1+2OH' and 'danazol M2+2OH' (collision energy 35 eV)

In terms of detectability over time, one of the isomers of the species 'danazol M2+2OH' probed to be the best marker in terms of retrospectivity for the detection of this steroid by using the LC-QTOF/MS system, since it could be detected up to 16 days after administration, as it is shown in Figure 4.

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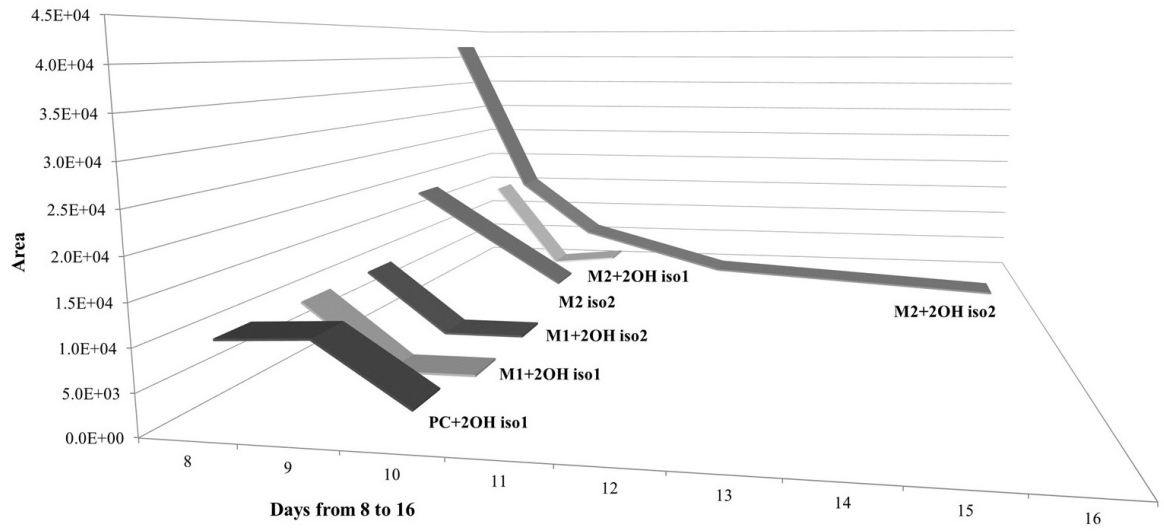

Figure 4: Detectability over time of the species 'danazol PC+2OH' (one of the isomers), 'danazol M1+2OH' (both isomers), danazol M2 (one of the isomers) and 'danazol M2+2OH' (both isomers) in the LC-QTOF/MS system.

Conclusions

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

References

[1] de Boer D, de Jong EG, Maes RAA. (1992) The detection of danazol and its significance in doping analysis. *J. Anal. Toxicol.* **16**, 14-18.
[2] Chrostowski K, Kwiatkowska D, Partyka E, Wójcikowska-Wójcik B. (1998) Danazol detection in doping analysis in urine. In: Schänzer W, Geyer H, Gotzmann A, Mareck-Elgelke U. (eds.) *Recent Advances in Doping Analysis (6)*, Köln, pp 53-64.
[3] Kim JY, Choi MH, Kim SJ, Kyong JB, Chung BC. (2001) GC-MS characterization of urinary metabolites and changes of ethisterone and testosterone profile after oral administration of danazol in equine. *J. Vet. Pharmacol. Therap.* **24**, 147-153.
[4] Montes de Oca Porto R, Rodríguez Fernández A, Martínez Brito D, Correa Vidal T, López Díaz A. (2006) Gas chromatography/mass spectrometry characterization of urinary metabolites of danazol after oral administration in human. *J. Chromatogr. B* **830**, 178-183.
[5] Fragkaki AG, Angelis YS, Tsantili-Kakoulidou A, Koupparis M, Georgakopoulos C. (2009) Schemes of metabolic patterns of anabolic androgenic steroids for the estimation of metabolites of designer steroids in human urine. *J. Steroid Biochem. Mol. Biol.* **115**, 44-61