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W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck
(Editors)

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R. MONTES DE OCA, A. RODRIGUEZ, D. MARTINEZ, T. CORREA:
Excretion Study of Danazol. New Metabolites Proposed for Doping Control
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Montes de Oca, R.[1] Rodríguez,A.[1]Martínez, D.[1] Correa,T.[1]

Excretion study of Danazol. New metabolites proposed for doping control.

[1]Antidoping Laboratory, La Habana, Cuba.

1. INTRODUCTION.

Danazol (17 α-pregna-2,4-dien-20-ino(2,3-d)isoxazol-17β-ol) belongs to Class VI: Anabolic Agent of the list of forbidden substances in sports by the International Olympic Committee(IOC) and World Antidoping Agency (WADA)[1]. Danazol is a synthetic derivative of ethisterone (ethinyltestosterone) that may be used in a wide variety of medical states, mainly for the treatment of endometriosis, which is a common gynecologic disorder in women with pelvic pain and women with infertility[2, 3]. Danazol is structurally related to stanozolol (17α-methyl-17β-hydroxy-5α-androst-2-enol[3,2C] pyrazole) and for this reason its use as doping agent is a real possibility, in fact some anabolic effect attributed to it have been reported, although its weak androgenic effect is known[4]. Danazol is extensively biotransformed and the main described metabolites in human urine are ethisterone, 2-hydroxymethylethisterone and 2-hydroxymethyl-1,2-dehydro-ethisterone. The aim of this work was to carry out an excretion study of danazol in human urine with the purpose to detect and to identify some of not previously reported metabolites after a single 200 mg oral administration dose of danazol to healthy volunteers. Also we investigated a long term detection of the abuse of this substance.

2. MATERIALS AND METHODS.

2.1 Urine samples and drug administration.

A single 200 mg dose of Danazol (1capsule, Runch[R]) was orally administered to two healthy male volunteers (20 and 40 years, 75 and 80 kg). Urine samples were collected before and up to 8 days post dose and stored at 4 °C until analysis.

2.2 Urine samples preparation.

The analysis were carried out using the internal laboratory standard operating procedures (SOP) Detection and Confirmation of anabolic agents excreted in free and conjugated form (Procedures IVa, IVb respectively) and High sensitive detection of anabolic agents excreted in free and conjugated form by means of Mass Tandem Spectrometry (MS/MS)(Procedure IVc).

Procedures IVb and IVc were carried out by standard procedures for doping control. Procedure IVa was carried out using two variants in samples preparation.

Variant 1: The extraction was carried out with ethyl acetate and derivatized with 40 µl of MSHFBA/TSIM (20 min at 80°C). After the first step of derivatization 10 µL of MBHFBA was added. (20 min at 80°C).

Variant 2: The extraction was carried out with diethyl ether. The residue was derivatized with 60 µl of MSTFA: NH_4I: dithioerithritol (30 min at 80 °C).

2.3 Instrumentation.

The analysis were carried out using a Hewlett-Packard 6890 gas chromatograph (Palo Alto, CA, USA) coupled with a 5973 quadrupole mass spectrometer detection system. The procedure IVc was carried out using Polaris Q Ion Trap mass detector (Finnigan, CA, USA) equipped with a Trace GC gas chromatograph. The columns used were Hewlett Packard (Agilent) HP-1 for procedures IVa, IVb and Supelco (Belafonte, USA) DB-1 for GC/MS/MS.

3. RESULTS AND DISCUSSION

In this study, it was possible to identify eight urinary metabolites of danazol including the already reported main metabolites such as ethisterone, 2-hydroxymethyl ethisterone (stereoisomers) and 2-hydroxymethyl-1,2-dehydroethisterone as reported by Chrostowski et al and de Boer et al [5,6](See Table 1). By the use of the GC/MS and GC/MS/MS methods after TMS derivatization, danazol metabolites were first detected in the chromatograms. In the Total Ion Chromatogram (TIC) many peaks were found, which were not observed in negative control urine specimens used from the same subjects. The structures assigned to these peaks were based on the fragmentation patterns observed, and also from the data previously reported for danazol metabolism[7]. We found also 6β- hydroxyethisterone and 6β- hydroxy-2-hydroxymethyl-ethisterone that were reported in horse urine [8].

In the normal procedure for analyzing excreted free steroids (Procedure IVa), the detection of danazol is carried out by monitoring the main ions of metabolite 1 (ethisterone). In our study we obtained that some metabolites were detected in higher concentration and for a longer period of time using variant 1 procedure. A peak showing a spectrum with m/z= 572, m/z 557, m/z 467and m/z 311 was found. We propose as being 2-hydroxymethyl-6β-hydroxy-1,2-dehydroethisterone, a hydroxylated metabolite of 2-hydroxymethyl-dehydroethisterone proposed by De Boer [6]. The fragmentation proposed for m/z 311 corresponds to rupture of the B ring (see figure 1). This metabolite can be detected up to 100 hours post-dose (Figure 1). We found also a metabolite m/z= 662, m/z 647 y m/z 243 and we propose as 6β, 6ξ-

dihydroxy-2ξ-hydroxymethyl-ethisterone, which is a 16-hydroxylated metabolite of 6β-hydroxy-2-hydroxymethyl-ethisterone. The fragmentation proposed for m/z 243 corresponds to rupture of the D ring (see figure 2). This metabolite can be detected up to 130 hours post-dose. For this reason we think that this metabolite can be used for detection of danazol by the procedure IVa (Figure 2).

In the TIC two peaks with m/z 734 were found. The presence of these peaks in different retention times and the mass spectra suggests the existence of 2 metabolites (isomers) (mono-hydroxylated M5). The two isomers with m/z 734 were found by the procedures IVa (variant 2), IVb, and IVc. The mass spectra suggest that the ion with m/z 631 present in the 3 spectra is formed by the loss of m/z 103 [- CH_2OTMS] from the 2-position of the molecule (2-Hydroxymethyl steroid with M^{+}=734). The structure and the proposal fragmentation pattern of these metabolites have not been reported and they could be characterized from the MS/MS spectrum basically by the loss of the fragment m/z 103 to produce a peak base m/z 631 that could be result only from the incorporation of hydroxyl group in the rest of the molecule. The ion with m/z=541 corresponds to the loss of OTMS-CH_2-OTMS. In the spectrum it is possible to see the loss of 5 OTMS (m/z=631, m/z=541, m/z=451,m/z=361,m/z=271) suggesting the presence of 5-hydroxyl group. The mass spectrum and the excretion profile of the metabolite with m/z 734 are shown in Figure 3.

4. CONCLUSIONS.

In this study it was possible to identify eight urinary metabolites of danazol after oral administration of 200 mg. The main metabolites in human urine and others minor metabolites were detected. Ethisterone, the main metabolite monitored for doping control, disappeared relatively fast from urine; therefore we recommended monitoring additional metabolites. For the procedure IVa we proposed two new metabolites, 2-hydroxymethyl-6β-hydroxy-1,2-dehydroethisterone and 6ξ,16ξ-dihydroxy-2ξ-hydroxymethyl-ethisterone. The last one presented a longer-term elimination compared to the rest of all metabolites. In the TIC were found some prominent peaks with m/z 734 and they have a long time of elimination. The possible metabolites with m/z 734 must be a mono-hydroxylated M5 with a hydroxyl group in different positions but their structure have not yet elucidated. The metabolites with m/z 734 can be detected in urine 200 hours post-administration. We conclude that the new metabolites can be included for the detection of danazol abuse because of the main metabolite ethisterone is excreted relatively fast in urine.

Metabolites	Names	m/z
M1	Ethisterone	456,441,301
M2	2-Hydroxymethylethisterone	558,543,468
M3	6β-Hydroxyethisterone	544,529,389
M4	2-Hydroxymethyl-1,2-dehydroethisterone	556,541,446
M5	6β-Hydroxy-2-hydroxymethylethisterone	646,631,543
M6	6β-Hydroxy-2-hydroxymethyl-1,2-dehydroethisterone	572,557,482
M7	6β, 16ξ-Dihydroxy- 2ξ-hydroxymethyl-ethisterone	662,647,243
M8	Mono-Hydroxylated M5	734,719,631

Table 1. Characteristics ions of 8 metabolites of Danazol.

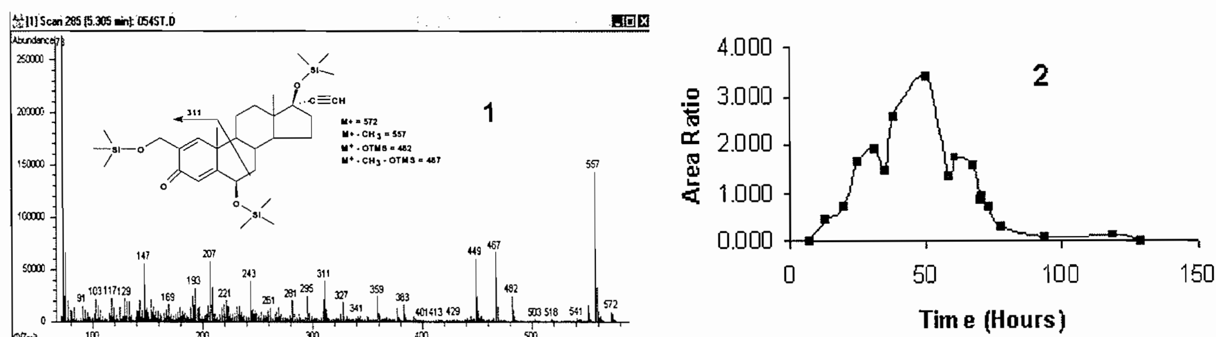


Fig.1.(1) Mass spectrum, structure and fragmentation proposed for metabolite M6. (2) Excretion profile of M6.

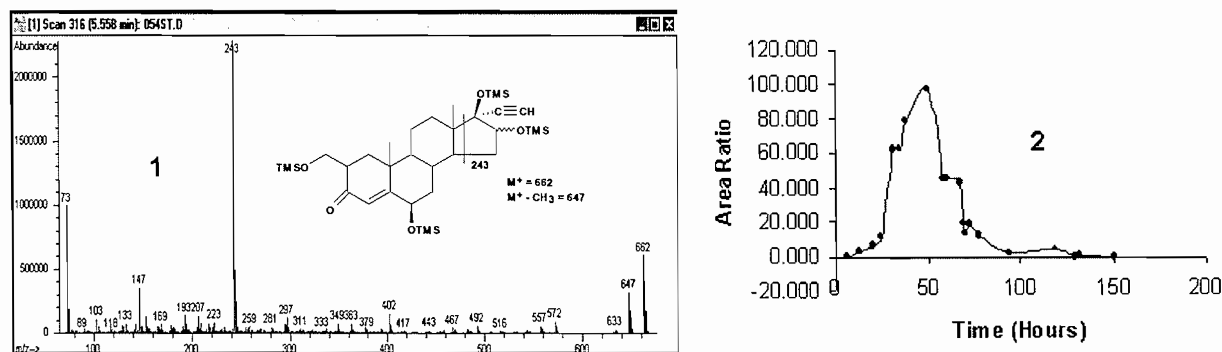
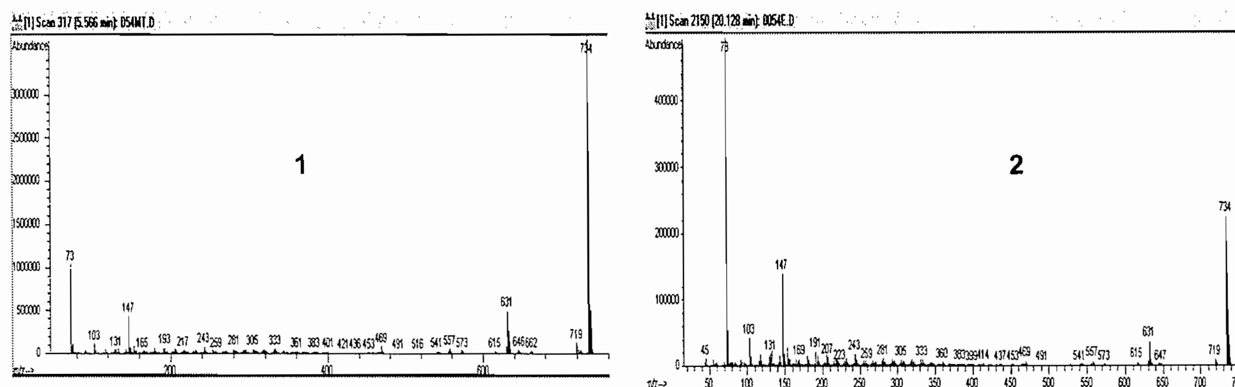


Fig.2.(1) Mass spectrum, structure and fragmentation proposed for metabolite M7. (2) Excretion profile of M7.



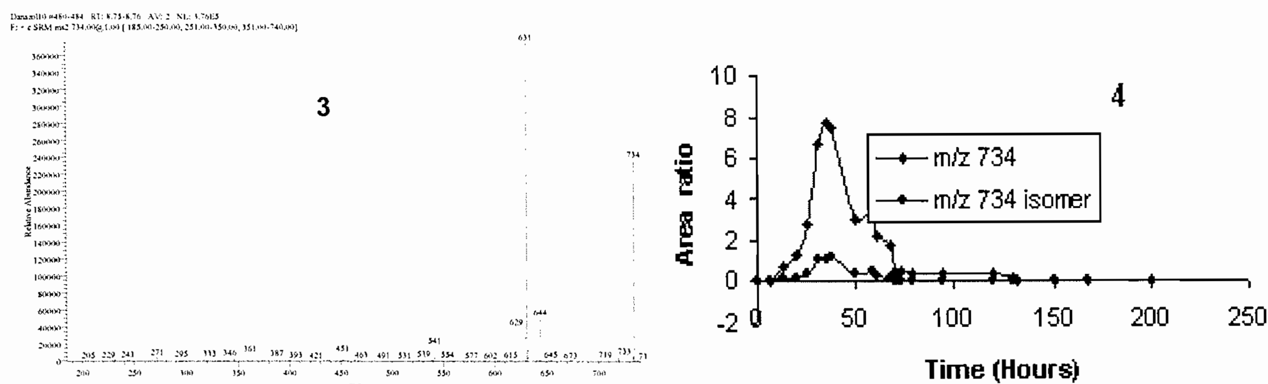


Fig 3. Mass spectra of the metabolites m/z 734(isomers) obtained for the procedure (1)IVa (Variant 2), (2) IVb, (3) IVc. (4) Is the excretion profile of these metabolites.

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