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Journal of Chromatography B, 830 (2006) 178–183
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Short communication

Gas chromatography/mass spectrometry characterization of urinary metabolites of danazol after oral administration in human

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Received 9 March 2005; accepted 22 October 2005
Available online 8 November 2005

Abstract

Danazol (17α-pregna-2,4-dien-20-yno [2,3-d]-isoxazol-17β-ol), is a synthetic derivative of ethisterone, structurally related to stanozolol. For this reason its use as doping agent has been investigated. Danazol (Runch®) (200 mg) were orally administered to two healthy male volunteers. Urine samples were colleted up to 1-week post-dose. Four new metabolites have been identified in addition to the five previously reported. We propose the monitorization of 6β-hydroxy-2-hydroxymethyl-1,2-dehydroethisterone and 6β,16ξ-dihydroxy-2ξ-hydroxymethyl-ethisterone by free fraction analysis. In a same way, we proposed to detect the principal isomer of a mono-hydroxylated metabolite of 6β-hydroxy-2ξ-hydroxymethylethisterone in the conjugated fraction. We conclude that new metabolites can be included for the detection of danazol abuse since the main metabolite ethisterone is excreted relatively fast in urine.
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Keywords: Danazol; Ethisterone; Doping control

1. Introduction

Since 1976, the use of anabolic androgenic steroids (AAS) and related compounds has been banned by the International Olympic Committee (IOC). In spite of their ban, AAS are still misused by athletes to improve performance [1,2].

Danazol (17α-pregna-2,4-dien-20-yno[2,3-d]-isoxazol-17β-ol) belongs to Class VI: Anabolic Agent of the list of forbidden substances in sports by the IOC and World Antidoping Agency (WADA) [3]. 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 [4,5]. It has been also reported that danazol, as attenuated androgen, may have some efficacy in increasing the platelet count of patients with myelodysplastic syndromes [6].

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 has been reported [7]. Danazol is extensively biotransformed and the main described metabolites in human urine are ethisterone, 2-hydroxymethylethisterone and 2-hydroxymethyl-1,2-dehydro-ethisterone [8].

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, long-term detection of the abuse of this substance was investigated.

2. Experimental

2.1. Urine samples and drug administration

A single 200 mg dose of Danazol (1 capsule, Runch®) 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-day post-dose and stored at 4 °C until analysis. The

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doi:10.1016/j.jchromb.2005.10.032

Sports Medicine Institute Ethics Committee approved the excretion study protocol.

2.2. Chemicals

All reagents and solvents used were of analytical quality and obtained from Aldrich (Milwaukee, USA), β-glucuronidase enzyme of *Helix pomatia* was obtained from Sigma–Aldrich (Steinheim, Germany), solid phase extraction columns Detectabuse™ (Divinylbenzene/Styrene copolymer, 15 mL) were obtained from Biochemical Diagnostics (New York, USA). The methyltestosterone (internal standard) was purchase from Research Plus (Bayonne, New York, USA), the *N*-Metil-*N*-trimethylsilyltri-fluoroacetamide (MSTFA) and the ammonium iodide (NH_4I) were obtained from Sigma–Aldrich (Steinheim, Germany), mercaptoethanol and dithioerythritol were obtained from Fluka (Busch, Switzerland). The *N*-methyl-*N*-trimethylsilyl heptafluorobutyramide (MSHFBA), *N*-methyl-bis-heptafluorobutyramide (MBHFBA) and trimethylsilyl Imidazole (TSIM) were obtained from Macherey-Nagel (Duren, Germany).

2.3. 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).

2.3.1. Procedure IVa

The samples were prepared for two variants. Internal standard (methyltestosterone, 500 ng) is added to 2.5 mL of urine and it is extracted as follow: to each urine sample is added 200 mg of solid buffer of sodium carbonate/bicarbonate 2:1 (p:p) and 1 mL of saturate sodium chloride solution. The extraction was carried out using two different solvents. In the variant 1, we used 6 mL of ethyl acetate on a shaker during 30 min and in the variant 2, we used 6 mL of diethyl ether. The samples were centrifuged for 5 min at 1232 × *g* and the organic phase was separated and evaporated to dryness under nitrogen stream at 40 °C. Before derivatization the samples were dried in a desiccator for 1 h. The dried residue was derivatized as follows:

Variant 1: the residue was 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 residue was derivatized with 60 μl of MSTFA:NH_4I:dithioerythritol (1000:2:6, v/w/v), and then incubated for 30 min at 80 °C.

2.3.2. Procedures IVb and IVc

After addition of the internal standard (500 ng of methyltestosterone) 2.5 mL of urine were concentrate on a Detectabuse™ column, previously conditioned with 2 mL methanol and 2 mL deionized water. The column was washed with 2 mL of deionized water and the analytes were then eluted with 2 mL of methanol. After evaporation of the methanol under a stream of nitrogen at 40 °C, the residue was taken up in 1 mL of sodium acetate buffer (pH 5.2, 0.2 M) and then hydrolysed with 25 μL of β-Glucuronidase from *Helix pomatia* at 55 °C for 1 h. The hydrolysate was cooled to room temperature and 250 μL of potassium carbonate 5% solution was added. The mixture was extracted with 5 mL of *tert*-butyl methyl ether on a shaker for 20 min. The extracted sample was centrifuged for 10 min at 1232 × *g* and the discarded organic phase was then evaporated to dryness under nitrogen stream at 40 °C. Before derivatization the samples were dried in a desiccator for 1 h. The dried residue was derivatized with 50 μL of MSTFA-NH_4I-Mercaptoethanol (1000:2:6, v/w/v), and then incubated for 30 min at 60 °C.

2.4. Instrumentation

2.4.1. Procedure IVa

The analysis was carried out using a Hewlett-Packard 6890 gas chromatograph (Palo Alto, CA, USA) coupled with a 5973 quadrupole mass spectrometer detection system (GC/MS). Gas Chromatography separation was achieved on an Ultra 1 capillary column (J & W Scientific, USA), length 17 m, inside diameter 0.20 mm and film thickness 0.11 μm; operated with helium at a flow rate of 1 mL/min and temperature programming of 176 °C for 1 min, ramped at 30 °C/min to 300 °C held for 3 min. Run time was 8.13 min. Injections of 3 μL were effected at 280 °C in the split mode (split ratio 1:10 and split flow 9 mL/min). The transfer line was heated at 280 °C and the ion source temperature was 230 °C. The acquisition mode was Full Scan. The *m*/*z* range was from 45 to 800 amu and the mass spectrometer was operated in the electron impact mode at 70 eV.

2.4.2. Procedure IVb

The analysis were carried out using a Hewlett-Packard 6890 gas chromatography (Palo Alto, CA, USA) coupled with a 5973 quadrupole mass spectrometer detection system. Gas Chromatography separation was achieved on a capillary column Ultra-1, length 17 m, inside diameter 0.20 mm and film thickness 0.11 μm; operated with helium at a flow rate of 1 mL/min and temperature programming of 178 °C ramped at 3 °C/min to 235 °C, ramped 40 °C/min to 310, then 310 °C held for 3 min. Run time was 24 min. Injections of 3 μL were effected at 280 °C in the split mode (split ratio 1:10 and split flow 12.0 mL/min). The transfer line was heated at 280 °C and the ion source temperature was 230 °C. The acquisition mode was Full Scan. The *m*/*z* range was from 45 to 800 amu and the mass spectrometer was operated in the electron impact mode at 70 eV.

2.4.3. Procedure IVc

The analyses were carrying out using a Polaris Q Ion Trap mass detector (Finnigan Inc., San Jose City, CA, USA) equipped with a Trace GC gas chromatograph. GC separation was achieved on an Ultra 1 capillary column, length 20 m, inside diameter 0.20 mm and film thickness 0.11 μm; oper-

Table 1
Names and characteristics ions of nine metabolites of danazol found in this study

Metabolites	Names	*m/z*	Procedures		
			IVa	IVb	IVc
M1	Ethisterone	456, 441, 301	X	X	X
M2	2ξ-Hydroxymethylethisterone	558, 543, 468	X	X	X
M3	6β-Hydroxyethisterone	544, 529, 389		X	X
M4	2-Hydroxymethyl-1,2-dehydroethisterone	556, 541, 446	X	X	X
M5	6β-Hydroxy-2ξ-hydroxymethylethisterone	646, 631, 543		X	X
M6	6β-Hydroxy-2-hydroxymethyl-1,2-dehydroethisterone	572, 557, 482	X		
M7	6β,16ξ-Dihydroxy-2ξ-hydroxymethylethisterone	662, 647, 243	X		
M8	Mono-hydroxylated M5	734, 719, 631	X	X	X
M9	Mono-hydroxylated M5	734, 719, 631	X	X	X

ated with helium at a flow rate of 1 mL/min and temperature programming of 181 °C for 1 min, ramped at 10 °C/min to 210 °C, then to 310 °C (20 °C/min) held for 2 min. Run time was 10.90 min. Injections of 3 μL were effected at 280 °C in the split mode (split ratio 1:20 and split flow 20 mL/min). The transfer line was heated at 300 °C and the ion source temperature was 225 °C. The acquisition was in Tandem MS/MS mode (GC/MS/MS).

2.5. Excretion profile

The excretion profile of all metabolites was obtained by area ratio between the main ion of each metabolite (underlined ions in Table 1) and ion *m/z* 446 corresponding to internal standard. The mean density of the samples analyzed was 1.021 with a minimum of 1.014 and maximum of 1.029. Keeping it in mind no correction was necessary. The figures show the data obtained for the most representative volunteer because of both urine analyses were similar.

3. Results and discussion

In this study, it was possible to identify nine 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

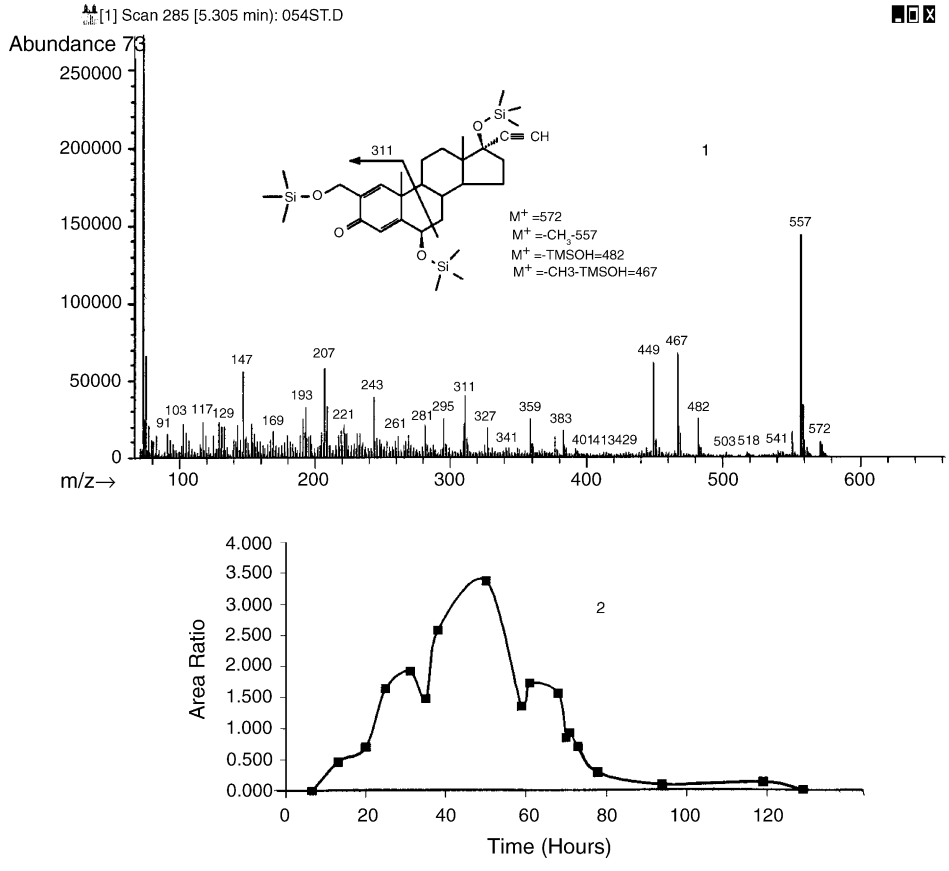

Fig. 1. (1) Mass spectrum, structure and fragmentation proposed for metabolite M6. (2) Excretion profile of M6.

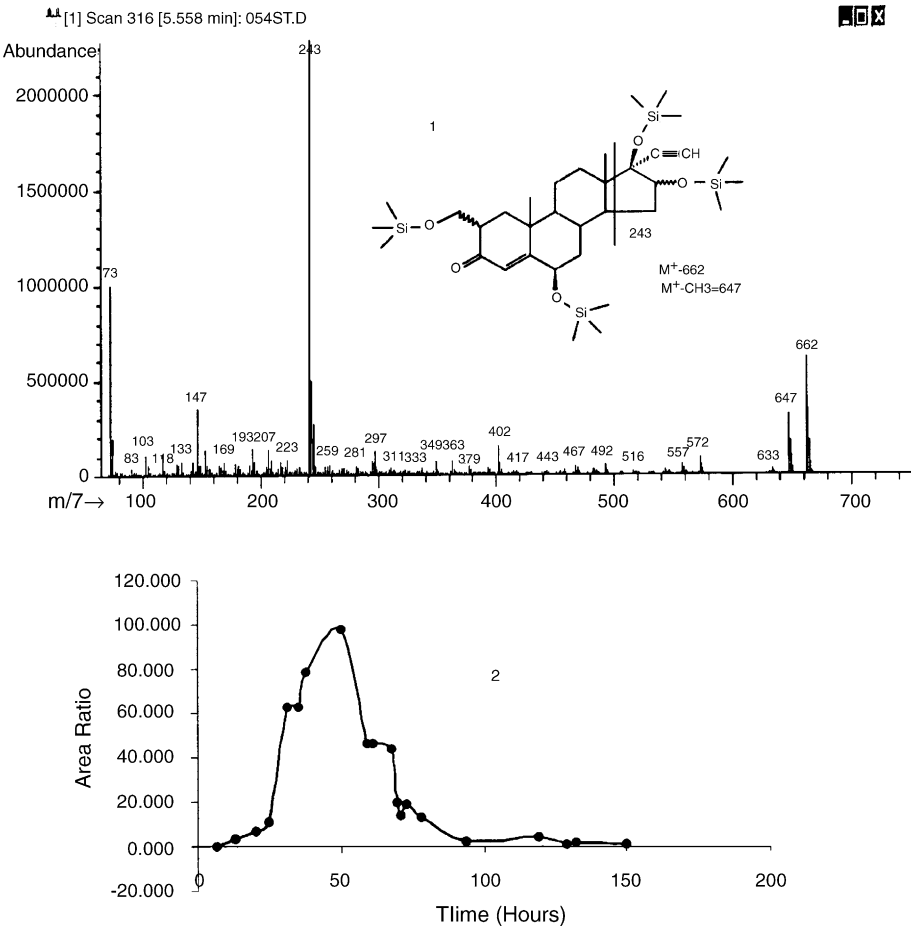

Fig. 2. (1) Mass spectrum, structure and fragmentation proposed for metabolite M7. (2) Excretion profile of M7.

by Chrostowski et al. [9] and Davinson et al. [10] (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 [11]. We found also 6β-hydroxyethisterone and 6β-hydroxy-2-hydroxymethylethisterone that were reported in horse urine [12].

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 $m/z = 572$, 557, 467 and 311 was found. We propose as being 6β-hydroxy-2ξ-hydroxymethyl-1,2-dehydroethisterone, a hydroxylated metabolite of 2-hydroxymethyl-1,2-dehydroethisterone proposed by Davinson et al. [10]. We proposed it as 6β-hydroxylated metabolite because of previous reports about steroids metabolism [13]. The m/z 572 corresponds to molecular ion, being the m/z 467 the loss of $-CH_3-$TMSOH. The fragmentation proposed for m/z 311 corresponds to rupture of the B ring (Fig. 1). This metabolite can be detected up to 100-h post-dose. We found also a metabolite with $m/z = 662$ (molecular ion), m/z 647 (methyl loss from molecular ion) y m/z 243 and we proposed it as 6β,16ξ-dihydroxy-2ξ-hydroxymethyl-ethisterone, which is a 16-hydroxylated metabolite of 6β-hydroxy-2ξ-hydroxymethyl-ethisterone. We proposed it as 16-hydroxylated metabolite because of 16α and 16β hydroxylation as the principal metabolism reaction in D ring in human [13]. Also 16α and 16β hydroxy metabolites have been described for stanozolol, related substance to danazol [14]. The fragmentation proposed for m/z 243 corresponds to rupture of the D ring (Fig. 2). This metabolite can be detected up to 130-h post-dose. For this reason this metabolite can be used for detection of danazol by the procedure IVa.

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 two 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 in human, although they are already described in equine urine [12]. They could be

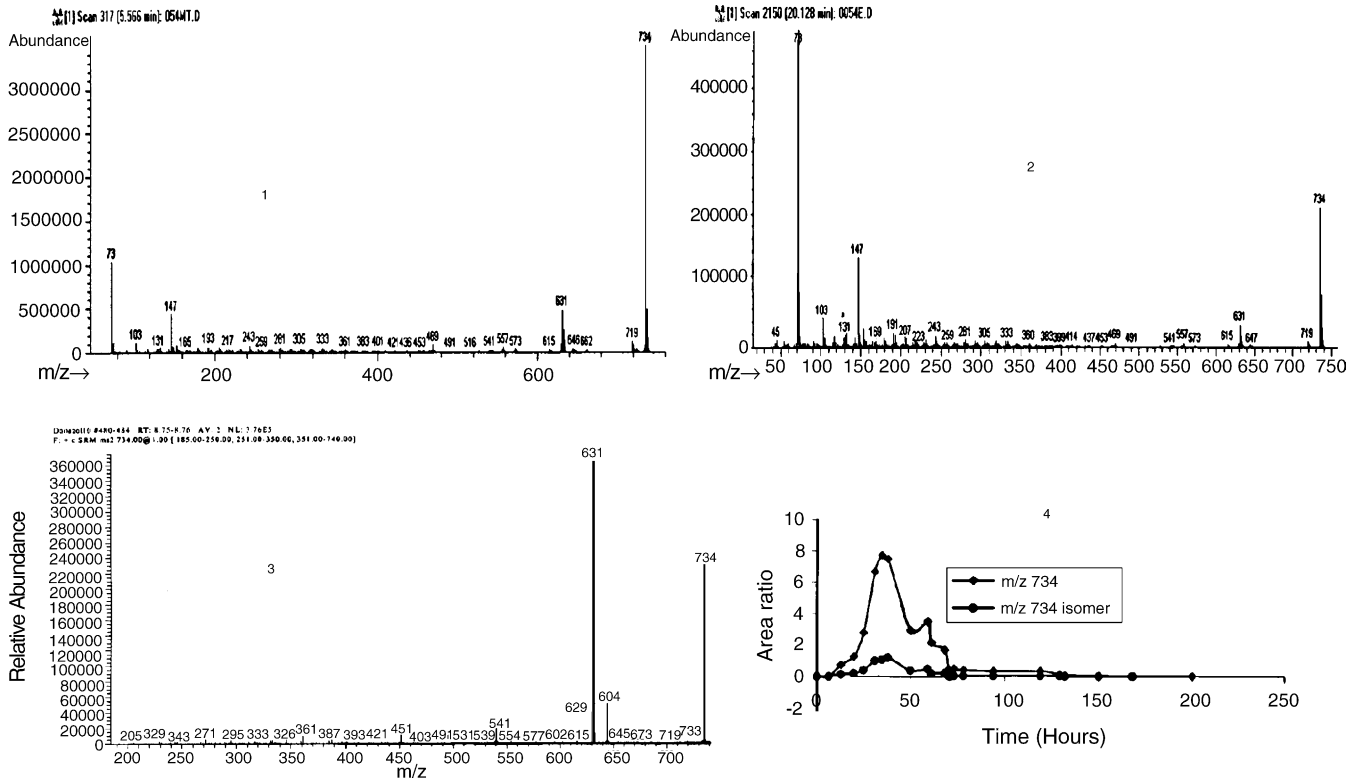

Fig. 3. Mass spectra of the metabolites *m/z* 734 (isomers) obtained for the procedure (1) IVa (variant 2); (2) IVb; (3) IVc; (4) is the excretion profile of these metabolites obtained by procedure IVb.

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 correspond to the loss of OTMS–CH_2–OTMS. In the spectrum it is possible to see the loss of five OTMS (*m/z* = 631, 541, 451, 361, 271) suggesting the presence of 5-hydroxyl group. It happens because of during derivatization procedure the 3-keto group is converted in TMS-enol ether derivative. For this reason we see the loss of 5 OTMS instead of 4 OTMS. The mass spectra and the excretion profile of these metabolites with *m/z* 734 are shown in Fig. 3.

Also, the data obtained suggest that M4 is the first metabolite excreted while M1 and M2 can be detected in human urine until 50 and 170 h, respectively, after oral administration of 200 mg of danazol. M5 was found near to 150 h although it could be detected easily until 70 h, meanwhile M3 is excreted in small quantities and it can be detected near to 70 h. The excretion profile of these minor danazol metabolites is shown in Fig. 4.

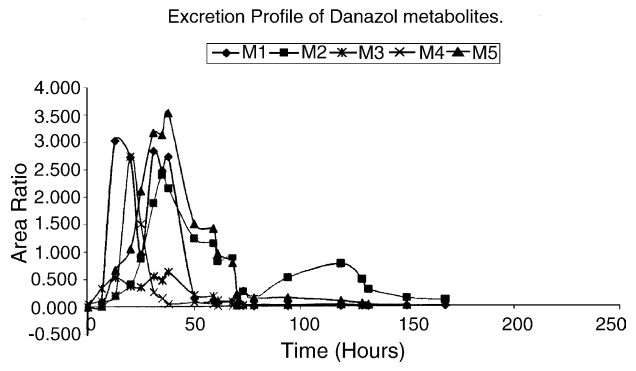

Fig. 4. Excretion profile of other danazol metabolites.

4. Conclusions

In this study, it was possible to identify nine 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 that is monitored for doping control disappeared relatively fast from urine; therefore monitoring additional metabolites is recommended. For the procedure IVa we proposed two new metabolites, 6β-hydroxy-2-hydroxymethyl-1,2-dehydroethisterone and 6β,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. These metabolites are new human metabolites but already described in equine urine. The principal metabolite with *m/z* 734 can be detected in urine 200 h 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.

Acknowledgement

We gratefully acknowledge Dr. X. de la Torre for useful discussion and comments during manuscript preparation.

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