Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (6)

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Danazol Detection in Doping Analysis in Urine

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1. Abstract

Danazol (17α-pregna-2-4-dien-20-yno[2,3,-D]isoxazol-17β-ol), structurally related to stanozolol, belongs to Class IV: Anabolic Agents group 1 anabolic-androgenic steroids. Detection and identification of the danazol can create same difficulties since the drug is excreted in urine in the form of metabolites exclusively. Metabolism of danazol and the presence of its main metabolites in urine [1, 4, 5]. We investigated the influence of long term application of danazol (by 21 days) and after one dose of the drug (200 mg) on changes of steroid profiles.

2. Material and method

We studied excretion of danazol in urine samples taken from the woman who applied danazol for medical purpose (2 tablets a' 200 mg/day for 21 days; Danazol, tab. 0,2 g Jelfa PL) and from a man volunteered after application of 1 tablet a' 200 mg. Three metabolites: ethisterone (17α-Hydroxypregn-4-en-20-yn-3-one), 2ε-hydroxymethylethisterone (17α-Ethinyl-17β-hydroxy-2α-hydroxymethyl-4-androsten-3-one) and 2-hydroxymethyldehydrethisterone (17α-Ethinyl-17β-hydroxy-2-hydroxymethyl-1,4-androstadiene-3-one) were found using the screening IV procedure for detection free and conjugated fraction of anabolic steroids in urine samples. We synthesised two substances to identification of danazol's metabolites: 2ε-hydroxymethylethisterone and 2-hydroxymethyldehydrethisterone.

2.1. Reagents and reference steroids

Testosterone (T) and Epitestosterone (Et) were purchase from Steraloids; methyltestosterone from Fluka; the mixture of deuterised internal standard solution: 17α-methylotestosterone, [16,6,17-^{2}H3]testosterone, [16,6,17-^{2}H3] epitestosterone, [2,2,4,4-^{2}H4] etiocholane and [2,2,4,4-^{2}H3]-11β-hydroxyandrosterone was obtained from Prof. W. Schänzer Cologne; β-glucuronidase ex e. coli (Boehringer Mannheim, Germany); dithioerithritol (Fluka, USA); diethylether A.R. (Labscan); methanol A.R. (POCH, Poland); MSTFA (Machery Nagel Germany); TMS-imidazole (Pierce, USA); TMS-J (Aldrich, USA); TMCS (Pierce, USA);

pregnandiol (Applied Science Laboratories); sodium sulfate anhydrous A.R. (POCH, Poland); potassium hydroxide A.R. (POCH, Poland); sodium dihydrogen phosphate (POCH, Poland); phosphoric acid 85% (Lachema); ethyl acetate (Chempur); phosphorus pentoxide (Aldrich, USA); potassium carbonate anhydrous (POCH, Poland); disodum hydrogen phosphate dodecahydrate (POCH, Poland) and XAD-2 resine (Serva, Germany); RIA kits (SEROZYME) for LH determination were supplied by Serono.

2.2. Procedure as for free steroids

Detection after extraction at pH 9.0, derivatisation and detection by scan

Sample preparation

PROCEDURE

(anabolic steroids: free steroid fraction)

	to 5 ml of urine
	+ 0,5 ml 5 M potassium hydroxide
	+2,0 ml diethyl ether (peroxide free)
	+10 µl solution of pregnandiol (10µg/ml) (IS)
	+ about 3 g Na_2SO_4 (anhydrous)
extraction	shake 20 min mechanical shaker
	centrifuge at 3000 rpm/min for 5 min
	freeze (temp - 30^{0}C)
	separate organic phase diethyl ether layer
	evaporation to dryness (vacuum rotary evaporator)
	+400 µl diethyl ether
	evaporation to dryness
	put to desiccator for 1 h above P_20_5
derivatisation	+50 µl MSTFA/TMCS/TMSImi. (100:5:2)
	heat 15 min at 60^{0}C
	inject 2 µl to GC/MS

Analytical parameters

GC/MS HP 5890

GC column 12 m H-1 0,2 mm ID, 0,33 µm film thickness

flow parameters	
-carrier gas:	helium
-flow:	0.9 ml/min
-head pressure:	15 psi
injector parameter	
- injector mode:	split 1:10
- injector volume:	2 µl
- injector temperature:	280^{0}C
oven temperature program	
-initial temperature:	150^{0}C
-initial time:	1 min
-rate:	35^{0}C/min to 290^{0}C
-final temperature:	290^{0}C
-final time:	5 min

MDS parameters

-ionisation mode:	EI, 70 eV
-acquisition mode:	Scan
-dwell time:	30 ms
-interference temperature:	290^0C

2.3. Procedure as for conjugated steroids

after enzymatic hydrolysis, extraction, trimethylsilylation and detection by scan; alternatively an extraction of the free and conjugated reaction with XAD-2 may be performed, followed by separation of the two fraction, treated and analysed as described above.

ample preparation

PROCEDURE

(anabolic steroids: conjugated steroid fraction)

	extraction 2 ml of urine are added to Amberlit XAD-2 columns (closed with a glass pearl, bed height ca. 2 cm washed with 2 ml of water)
	+ 10 µl methyltestosterone IS.
	elution 3 times with 1 ml of methanol
	evaporation to dryness (using vacuum rotary evaporator)
	+1 ml sodium phosphate buffer
	+ 50 µl β-glucuronidase (Escherichia coli K 12)
hydrolysis	60 min on 50^0C
	+ 0,2 ml 5% potassium carbonate
	+ 5 ml of diethyl ether
	shake for 10 min mechanically in laboratory shaker
	centrifuge 3000 rmp for 5 min
	freeze (below -30^0C)
	diethyl ether layer evaporate to dryness
	+400 µl diethyl ether and transfer to vial
	evaporate to dryness on 40^0C
	put to desiccator for 1 h above P_20_5
derivatisation	+50 µl MSTFA/TMIS (100:1)
	heat for 15 min on 60^0C
	inject 2 µl to GC/MS

Analytical parameters

GC/MS HP 5890

GC column 12 m HP-1 0,2 mm ID film thickness 0,33 µm

flow parameters

-carrier gas:	helium
-flow:	0,9 ml/min
-head pressure:	15 psi

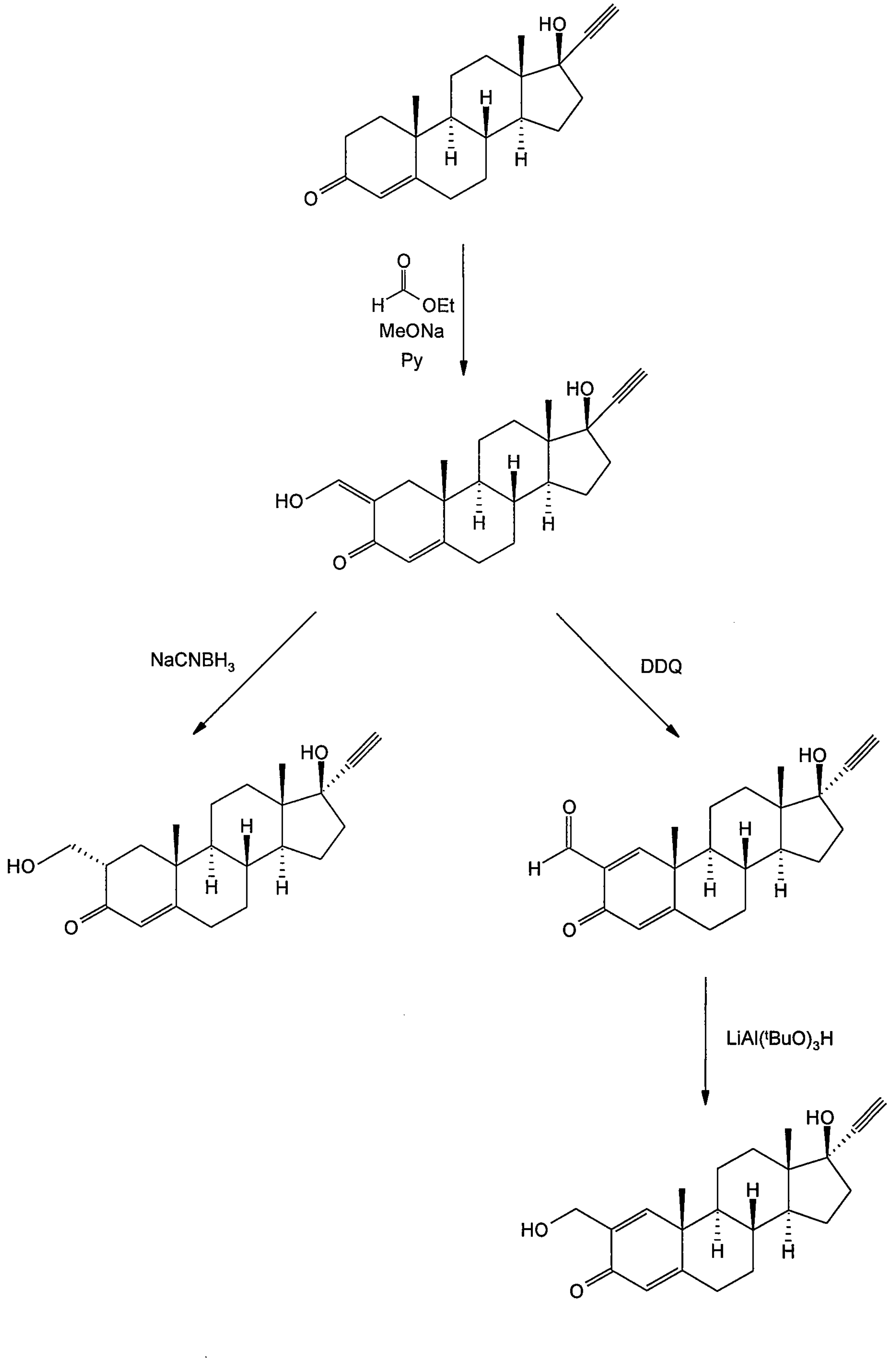
injector parameter

- injector mode:	split 1:10, automatic
- injector volume:	2 µl
- injector temperature:	280^0C

oven temperature program	
- initial temperature:	180^0C
- initial time:	1 min
- rate 1:	2^0C/min, 230^0C
- rate 2:	15^0C/min, 280^0C
- final temperature:	280^0C
- final time:	5 min
MDS parameters	
-ionisation mode:	EI, 70 eV
-acquisition mode:	Scan
-dwell time:	30 ms
-interference temperature:	290^0C

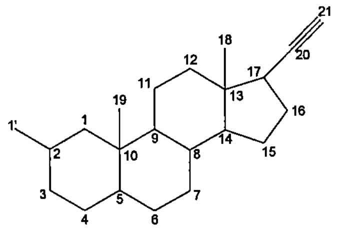
Identification of ethisterone was confirmed through synthetic metabolite obtained from Sigma. Two other metabolites were synthesised by ourselves accordingly to the following steps presented on the Fig. 1. The intermediate products of synthesis had been confirmed by NMR results.

Fig. 1. **SYNTHESIS OF DANAZOL METABOLITES:**
2ε-hydroxymethylethisterone, 2-hydroxymethyldehydroethisterone

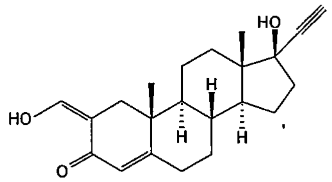


We confirmed metabolites on NMR.

NMR spectra of danazol metabolites

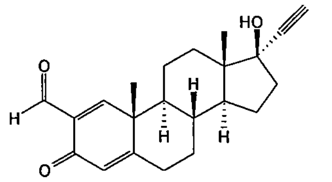


17α-Ethinyl-17β-hydroxy-2-hydroxymethylene-4-androsten-3-one



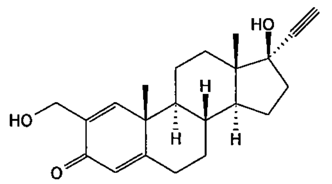
^{1}H NMR (200 MHz, $CDCl_3$) δ 7.38 (1H, s, 1'-H), 5.79 (1H, s, 4-H), 2.58 (1H, s, 21-H), 1.05 (3H, s, 19-H_3), 0.89 (3H, s, 18-H_3).
^{13}C NMR (50 MHz, $CDCl_3$) δ 189.1 (C-3), 170.1 (C-5), 165.1 (C-1'), 122.9 (C-4), 106.4 (C-2), 87.3 (C-17), 79.49 (C-20), 74.0 (C-21), 52.7 (C-9), 49.7 (C-14), 46.6 (C-13), 39.8 (C-10), 38.8 (C-16), 37.4 (C-1), 36.2 (C-8), 32.4 (C-12), 32.3 (C-6), 30.8 (C-7), 23.0 (C-15), 21.0 (C-11), 18.0 (C-19), 12.6 (C-18).

17α-Ethinyl-17β-hydroxy-2-carboxyaldehyde-1,4-androstadiene-3-one



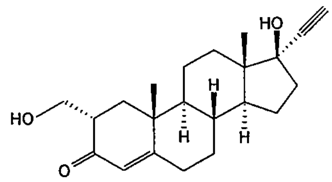
^{1}H NMR (200 MHz, $CDCl_3$) δ 10.19 (1H, s, 1'-H), 7.75 (1H, s, 1-H), 6.09 (1H, s, 4-H), 2.47 (1H, s, 21-H), 1.27 (3H, s, 19-H_3), 0.87 (3H, s, 18-H_3).
^{13}C NMR (50 MHz, $CDCl_3$) δ 190.4 (C-1'), 184.2 (C-3), 169.2 (C-5), 131.6 (C-2), 124.1 (C-4), 86.9 (C-17), 79.2 (C-20), 74.1 (C-21), 51.7 (C-9), 49.3 (C-14), 46.7 (C-13), 44.0 (C-10), 38.5 (C-16), 35.9 (C-8), 32.7 (C-12), 32.3 (C-6), 32.1 (C-7), 23.1 (C-15), 22.4 (C-11), 18.4 (C-19), 12.7 (C-18).

17α-Ethinyl-17β-hydroxy-2-hydroxymethyl-1,4-androstadiene-3-one



^{1}H NMR (200 MHz, $CDCl_3$) δ 7.02 (1H, s, 1-H), 6.08 (1H, s, 4-H), 4.38 (2H, bs, 1'-H_2), 3.22 (1H, bs, -OH), 2.53 (1H, s, 21-H), 1.24 (3H, s, 19-H_3), 0.92 (3H, s, 18-H_3).
^{13}C NMR (50 MHz, $CDCl_3$) δ 186.9 (C-3), 170.2 (C-5), 151.7 (C-1), 135.6 (C-2), 123.5 (C-4), 87.2 (C-17), 79.3 (C-20), 74.1 (C-21), 61.9 (C-1'), 52.0 (C-9), 49.4 (C-14), 46.9 (C-13), 43.5 (C-10), 38.7 (C-16), 36.0 (C-8), 33.1 (C-12), 32.5 (C-6), 32.3 (C-7), 23.2 (C-15), 22.6 (C-11), 18.7 (C-19), 12.8 (C-18).

17α-Ethinyl-17β-hydroxy-2α-hydroxymethy-4-androsten-3-one



^{1}H NMR (200 MHz, $CDCl_3$) δ 5.73 (1H, s, 4-H), 3,73 (2H, bm, 1'-H_2), 3.36 (1H, bs, -OH), 2.56 (1H, s, 21-H), 1.25 (3H, s, 19-H_3), 0.89 (3H, s, 18-H_3).
^{13}C NMR (50 MHz, $CDCl_3$) δ 202.6 (C-3), 171.9 (C-5), 123.4 (C-4), 87.3 (C-17), 79.3 (C-20), 73.9 (C-21), 63.5 (C-1'), 53.7 (C-9), 49.7 (C-14), 46.5 (C-13), 43.5 (C-2), 39.1 (C-10), 38.7 (C-16), 38.6 (C-1), 35.9 (C-8), 32.4 (C-6), 32.2 (C-7), 31.3 (C-12), 23.0 (C-15), 20.5 (C-11), 17.6 (C-19), 12.6 (C-18).

4. Results

4.1. Detection of danazol in urine samples

In Fig. 2 we demonstrated three metabolites in free fraction: ethisterone (m/z 369, 384, 279) 2ε-hydroxymethylethisterone, 2-hydroxymethyldehydroethisterone (m/z 471, 389, 486) in Fig. 3 and in conjugated fraction which were found: ethisterone (m/z 456, 301, 441) (in Fig. 4), 2ε-hydroxymethylethisterone and 2-hydroxymethyldehydroethisterone (m/z 558, 543, 418) (Fig. 5). A very good agreement has been found between ion spectra obtained from the synthesised metabolites and those obtained from the urine samples.

4.2. Influence of danazol on steroid profile in urine samples

Fig 6 presented ethisterone, one of the metabolites of danazol in urine excretion in urine after application of dose 200 mg. 2 x days during 21 days. In the same samples we analyses of steroid profiles as T/Et ratio, testosterone (T) and epitestosterone (Et) concentrations. We did not noticed any changes in these parameters. Fig 7 illustrated ethisterone excretion after application of the single dose 200 mg by the man (57 years old). There was rather low but lasting up to 70 h of ethisterone concentration in urine samples. Although same fluctuations of T and Et concentrations appeared, T/Et ratio has been on the same low level. Fig 8 we presented the influence of the ethisterone concentration on LH and testosterone (nmol/l)/ LH (IU/l) ratio in course of time of excretion of danazol in urine samples.

5. Conclusion

Detection and identification of danazol in urine samples by using screening procedure IV is simple and reliable method. Detection of all three metabolites of danazol in the urine sample by the comparison their spectra with the synthetics are necessary for confirmation procedure of doping with this drug. Presence of ethisterone alone is not enough for this purpose.

Our study presented that long term application (by 21 days) and after 200 mg (one tab) of danazol didn't have any effects on steroid profiling and T/Et ratios (which fluctuated from 0,36 to 1,04).

6. Reference

1. de Boer D., de Jong E.G., Maes A.A.: The Detection of Danazol and Its Significance in Doping Analysis. J. Analit. Toxicology. (1992) 16:14-18.

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5. Davison C., Banks W., Fritz A.: The Absorption, Distribution and Metabolic Fate of Danazol in Rate, Monkeys and Human Volonteetes. Arch. int. Pharmacodyn. (1976) 221, 294-310

Fig. 2.

Mass spectra of danazol metabolite - free fraction

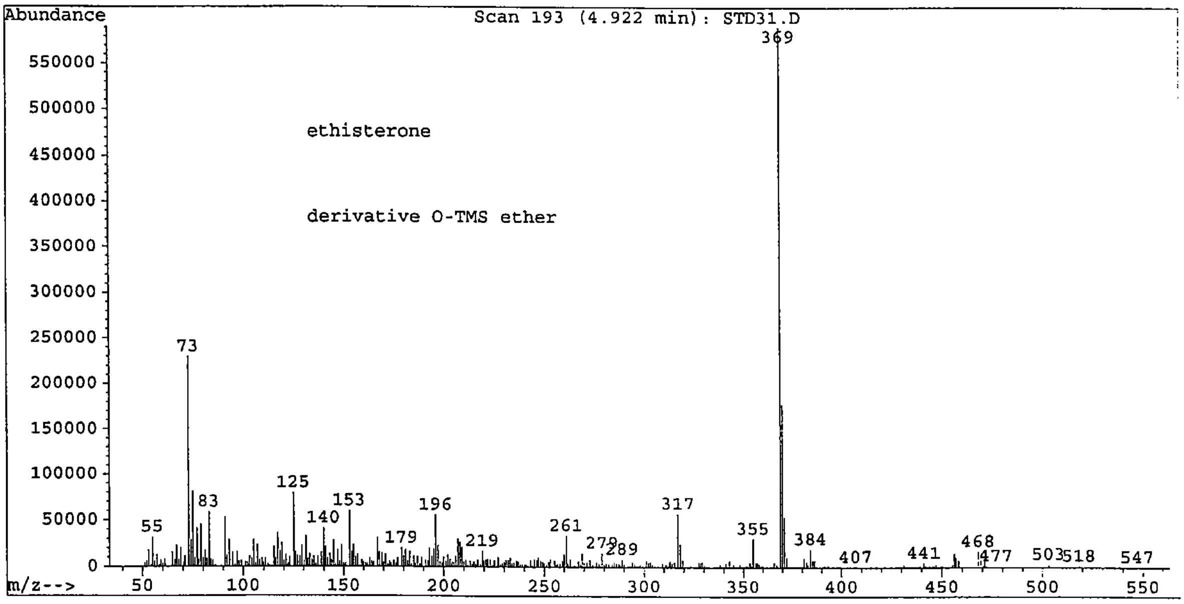


Fig. 3.

Mass spectra of danazol metabolite - free fraction

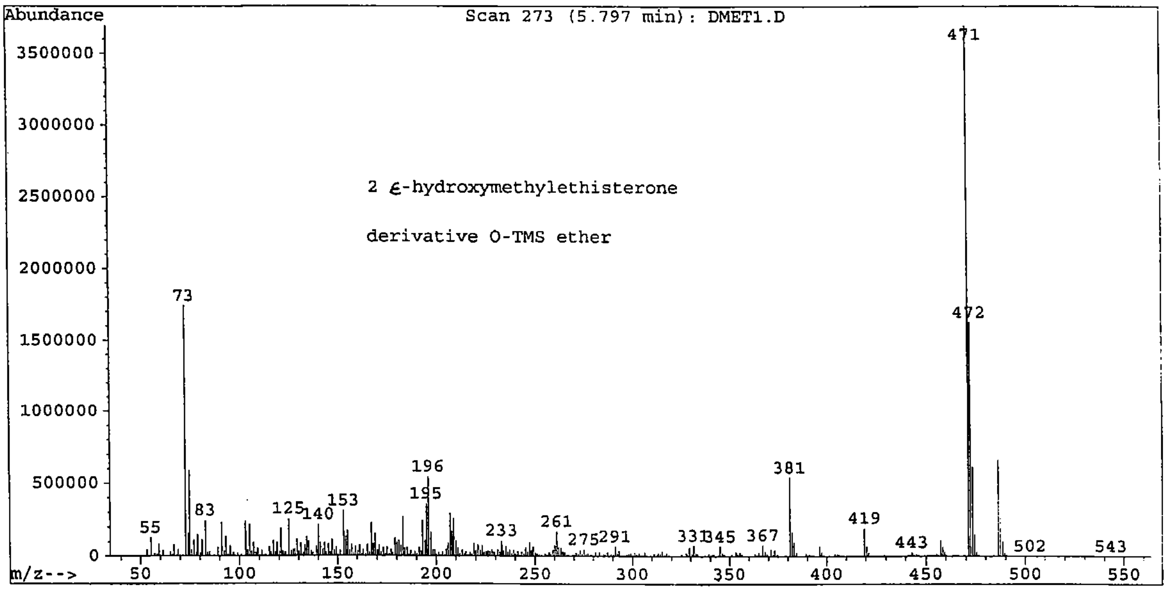


Fig. 4.

Mass spectra of danazol metabolite - conjugated fraction

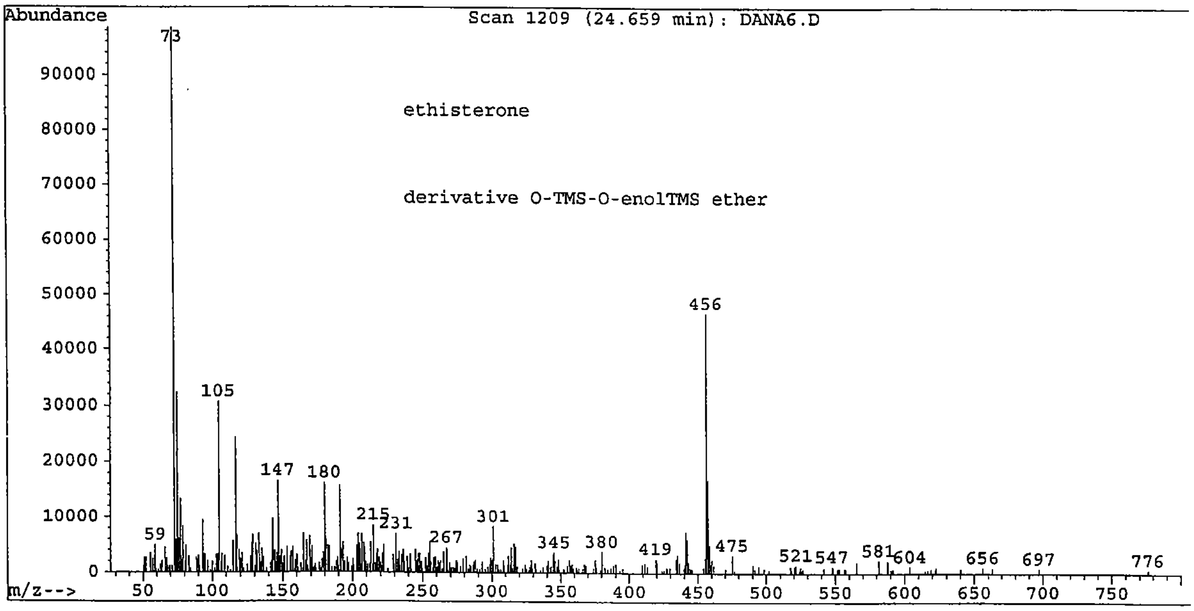


Fig. 5.

Mass spectra of danazol metabolite - conjugated fraction

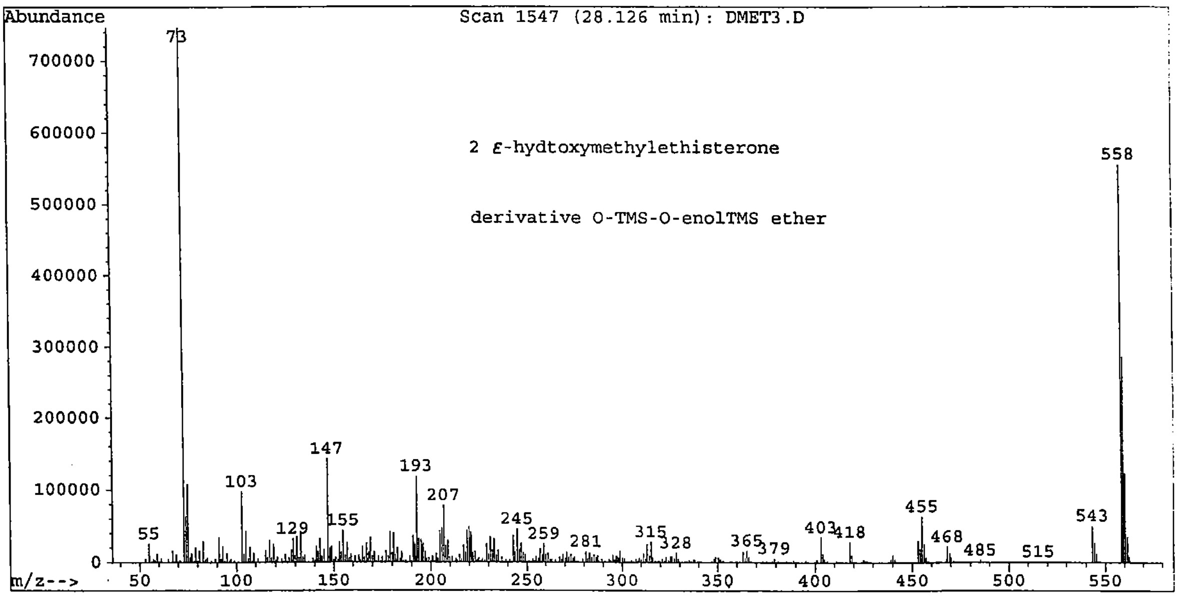


Fig. 6

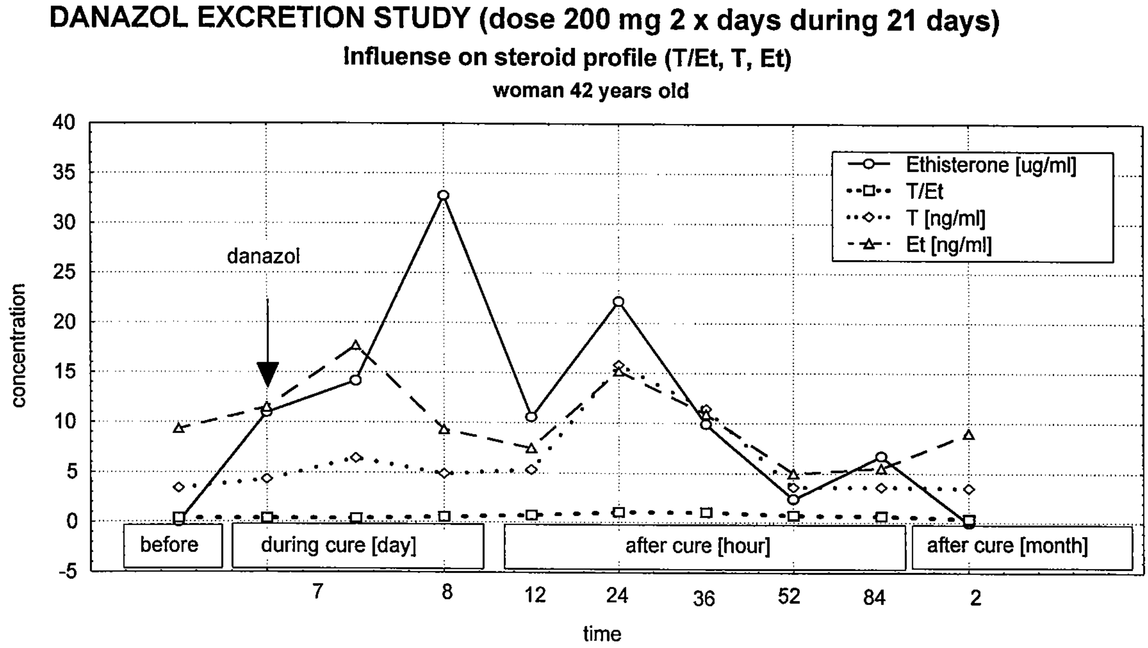

DANAZOL EXCRETION STUDY (dose 200 mg 2 x days during 21 days)
Influense on steroid profile (T/Et, T, Et)
woman 42 years old
Ethisterone [ug/ml]
T/Et
T [ng/ml]
Et [ng/ml]
danazol
concentration
40
35
30
25
20
15
10
5
0
-5
before
during cure [day]
after cure [hour]
after cure [month]
7
8
12
24
36
52
84
2
time

Fig. 7

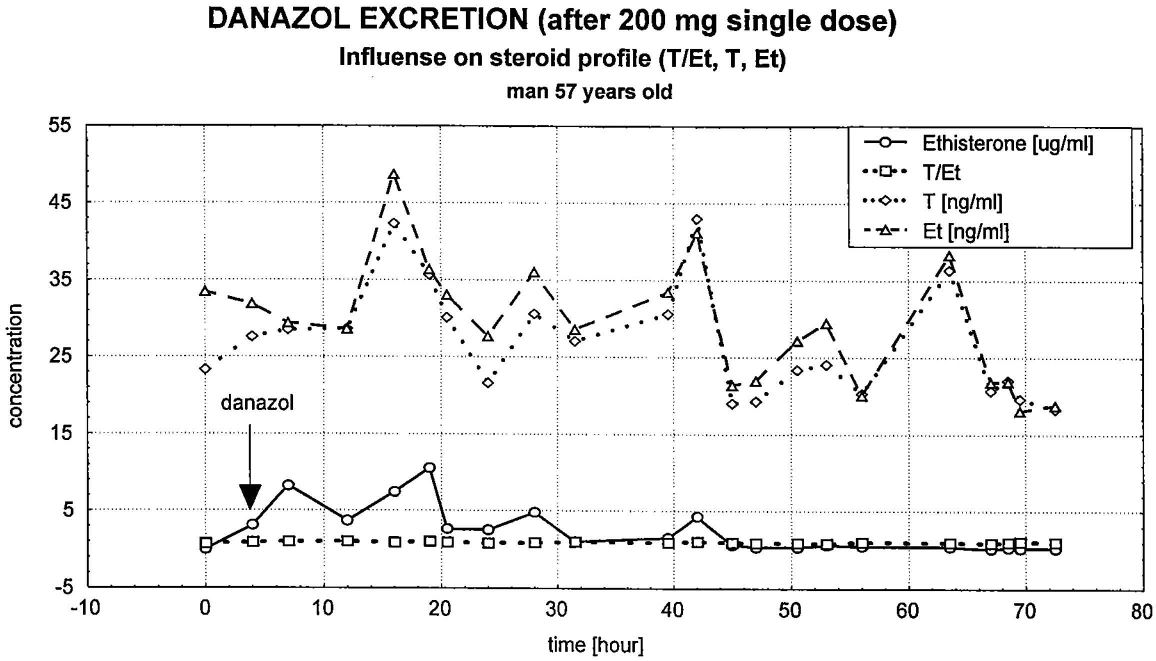
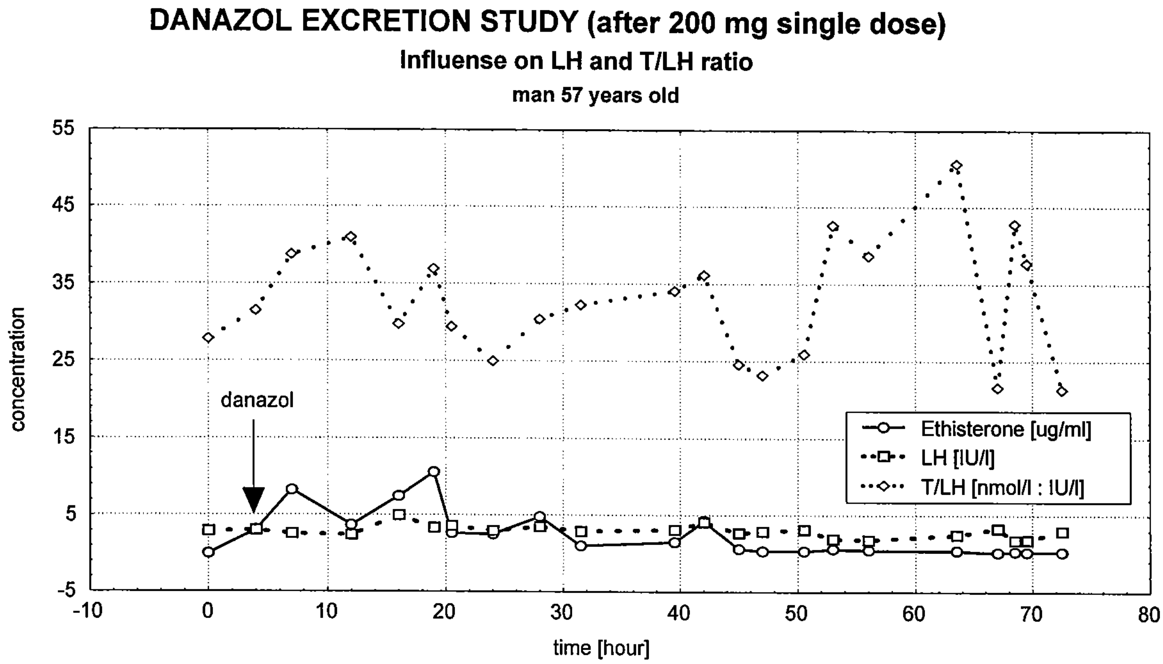

DANAZOL EXCRETION (after 200 mg single dose)
Influense on steroid profile (T/Et, T, Et)
man 57 years old
Ethisterone [ug/ml]
T/Et
T [ng/ml]
Et [ng/ml]
danazol
concentration
55
45
35
25
15
5
-5
-10
0
10
20
30
40
50
60
70
80
time [hour]

Fig. 8



DANAZOL EXCRETION STUDY (after 200 mg single dose)
Influense on LH and T/LH ratio
man 57 years old
concentration
55
45
35
25
15
5
-5
-10
0
10
20
30
40
50
60
70
80
time [hour]
danazol
Ethisterone [ug/ml]
LH [IU/l]
T/LH [nmol/l : IU/l]