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Danazol Detection in Doping Analysis in Urine
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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), 2α-hydroxyethisterone (17α-Ethinyl-17β-hydroxy-2α-hydroxymethyl-4-androsten-3-one) and 2-hydroxymethyldehydrothisterone (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: 2α-hydroxymethylthisterone and 2-hydroxymethyldehydrothisterone.

2.1. Reagents and reference steroids

Testosterone (T) and Epitesterone (Et) were purchase from Steraloids; methyltestosterone from Fluka; the mixture of deuterised internal standard solution: 17α-methyltestosterone, [16,6,17-2H3]testosterone, [16,6,17-2H3] epitesterone, [2,2,4,4-2H4] etiocholane and [2,2,4,4-2H3]-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); disodium hydrogen phosphate dodecahydrate (POCH, Poland) and XAD-2 resin (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 Na₂SO₄ (anhydrous)

extraction

shake 20 min mechanical shaker
centrifuge at 3000 rpm/min for 5 min
freeze (temp - 30°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 P₂O₅

derivatisation

+50 µl MSTFA/TMCS/TMSI. (100:5:2)
heat 15 min at 60°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°C
oven temperature program
-initial temperature: 150°C
-initial time: 1 min
-rate: 35°C/min to 290°C
-final temperature: 290°C
-final time: 5 min

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MDS parameters
-ionisation mode: EI, 70 eV
-acquisition mode: Scan
-dwell time: 30 ms
-interference temperature: 290°C

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)
eextraction 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°C
+ 0,2 ml 5% potassium carbonate
+ 5 ml of diethyl ether
shake for 10 min mechanically in laboratory shaker
centrifuge 3000 rpm for 5 min
freeze (below -30°C)
diethyl ether layer evaporate to dryness
+400 μl diethyl ether and transfer to vial
evaporate to dryness on 40°C
put to desiccator for 1 h above P2O5

derivatisation
+50 μl MSTFA/TMIS (100:1)
heat for 15 min on 60°C
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°C
oven temperature program
- initial temperature: 180°C
- initial time: 1 min
- rate 1: 2°C/min, 230°C
- rate 2: 15°C/min, 280°C
- final temperature: 280°C
- final time: 5 min

MDS parameters
-ionisation mode: EI, 70 eV
-acquisition mode: Scan
-dwell time: 30 ms
-interference temperature: 290°C

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:
2α-hydroxymethylethisterone, 2-hydroxyethyldehydroethisterone

\[ \text{Reactions and conversions shown in the diagram} \]

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We confirmed metabolites on NMR.

**NMR spectra of danazol metabolites**

**17α-Ethyl-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-Ethyl-17β-hydroxy-2-carboxyaldehyde-1,4-androstanediene-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-Ethyl-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-Ethyl-17β-hydroxy-2-α-hydroxymethyl-4-androstene-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) 2α-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), 2α-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


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

![Mass spectrum image](image-url)

Fig. 5.

Mass spectra of danazol metabolite - conjugated fraction

![Mass spectrum image](image-url)
Fig. 6

DANAZOL EXCRETION STUDY (dose 200 mg 2 x days during 21 days)
Influence on steroid profile (T/Et, T, Et)
woman 42 years old

Fig. 7

DANAZOL EXCRETION (after 200 mg single dose)
Influence on steroid profile (T/Et, T, Et)
man 57 years old
Fig. 8

DANAZOL EXCRETION STUDY (after 200 mg single dose)
Influence on LH and T/LH ratio
man 57 years old

danazol

- Ethisterone [ug/ml]
- LH [IU/l]
- T/LH [nmol/l : IU/l]

concentration

-10 0 10 20 30 40 50 60 70 80

time [hour]