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Detection of 19-norandrosterone after ingestion of the oral contraceptives norethisterone acetate and lynestrenol and ethylestrenol

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1. Introduction

Norethisterone and lynestrenol are oral contraceptives that are frequently used by female athletes.

Previous studies (1, 2) have shown that the use of norethisterone can lead to the detection of 19-norandrosterone. However, these results were purely qualitative and the WADA technical document on 19-norandrosterone findings states that "the Laboratory will determine whether it is reasonable that the 19-norandrosterone was excreted in the amount measured consequent to the intake of norethisterone, by verifying that the major isomer of glucuroconjugated tetrahydronorethisterone is present. The Laboratory will in such a case add the following phrase to the report "could be compatible with a norethisterone treatment" "(3).

This sentence is somewhat ambiguous, since it states "the amount 19-norandrosterone excreted", which means that one needs to have quantitative data, but on the other hand it also states that you need to verify the presence of tetrahydronorethisterone, which is a qualitative criterion.

Lynestrenol has been identified as a prodrug of norethisterone (4) and hence could theoretically also lead to the detection of 19-norandrosterone.

Two years ago, our laboratory provided a pooled excretion urine sample after the administration of norethisterone that was sent to all WAADS members as part of a WAADS PT.

The feed-back from the PT indicated that quantitative information on 19-norandrosterone detection after use of norethisterone in therapeutic doses is missing.

Previously, Ward et al. (5) had investigated the metabolism ethylestrenol in the marmoset monkey and classified the drug as a prodrug of norethandrolone. 17α-ethyl-5α-estrane-3α,17β-diol and 17α-ethyl-5β-estrane-3α,17β-diol were identified as metabolites. This study revealed that the metabolic pathways of ethylestrenol and lynestrenol were similar. Therefore urine samples after the ingestion of ethylestrenol were also analyzed.

2. Materials and methods

Reagents

Norethisteron acetate (Primolut-Nor) was from Schering (Machelen, Belgium) and lynestrenol (Orgametril) was from Organon Europe B.V. (Oss, Nederland). Six pills from each drug were tested for the presence of nandrolone or is precursors using a previously described method (5).

17-hydroxy-19-nor-5α,17α-pregn-20-yn-3-one (5α-NET); 19-nor-5α,17α-pregn-20-yn-3α,17-diol (3α,5α-tetrahydronorethisterone, 3α,5α-NET); 19-nor-5α,17α-pregn-20-yn-3β,17-diol (3β,5α -NET) were synthesized at the Institute of Organic Chemistry and Biochemistry (Academy of Sciences of the Czech Republic). 17α-ethyl-5α-estrane-3α,17β-diol (5α-estrane); 17α-ethyl-5β-estrane-3α,17β-diol (5β-estrane), 19-norandrosterone and 19-norethiocholanolone were purchased from NMI (Pymble, Australia). Ethylestrenol and 17α–methyltestosterone were a gift from Organon (Oss, The Netherlands).

The enzymatic preparation β-glucuronidase type K12 from *E. coli* containing 4.23 KU/25 ml/47 mg β-glucuronidase activity.

N-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Karl Bücher Chem. Fabrik (Germany).

All other chemicals were of analytical grade.

Administration

10 mg norethisteron, 5 mg lynestrenol acetate and 10 mg ethylestrenol were administered orally to 1 male volunteer in two separate studies. Each time, urine was collected quantitatively at 2, 3, 4, 6, 9 and 12 h after administration. Additional samples were collected 24, 30, 36, 48, 72and 96 h after administration. All urine samples were stored at -20°C awaiting analysis

Extraction

To 5 ml urine 1 ml phosphate buffer (pH=7,0), 50 µl internal standard (methyltestosterone, 2 µg/ml) and 50 µl β-glucuronidase were added. Hydrolysis was performed for 15 h at 42°C. After hydrolysis, the samples were made alkaline by addition of ± 300 mg K_2CO_3/$NaHCO_3$ (2/1) and extracted by rolling 20 min with 5 ml n-pentane. The organic layer was separated and evaporated under OFN. The dried residues were derivatised for 20 min at 80°C with MSTFA/NH_4I/ethanethiol.

GC-MS analysis

An Agilent 6890 GC with an Agilent 5973 MSD was employed for all analyses. Chromatographic separation was performed by using a capillary column (HP-Ultra 1; column length 17 m × 0.2 mm, df= 0.11µm film) from J&W Scientific (Agilent Technologies, USA). The column temperature was programmed as follows: the initial temperature was 120°C, 70°C/min → 190°C (1 min), 4°C/min → 218°C (0 min), 2°C/min → 225°C (0 min), 40°C/min → 270°C (0 min).

Helium was used as carrier gas at a flow rate 0.9 ml/min. The injector temperature was maintained at 250 °C, and the injection volume was 0.5 µl in the splitless mode. Electron energy was set at 70 eV. Mass spectra were obtained in the scan mode (*m/z* 50–600). Selected ion monitoring (SIM) mode was employed for quantification. Three diagnostic ions were monitored for each analyte.

3. Results and discussion

Analysis of the pills, using a previously published method (6), did not reveal the presence of nandrolone or its precursors.

The quantitative method was linear in the range of 5-100 ng/ml for all analytes, except 19-norandrosterone and 19-noretiocholanolone (linearity in the range 1-100 ng/ml).

3α,5α-tetrahydronorethisterone was identified as the major urinary metabolite after administration of norethisterone and lynestrenol. After administration of primolut-nor ® and orgametril ®, 3α,5α-NET was detected for 36 and 72 h and maximum concentrations were 215 ng/ml and 14 ng/ml, respectively.

The excretion profiles of 19-norandrosterone after administration of 10 mg norethisterone acetate is shown in Fig. 1. The maximum urinary concentration of 47 ng/ml was already reached 2 h post administration and the concentration exceeded the threshold level for up to 30 h post administration. After the administration of lynestrenol the maximum attained concentration was 4,5 ng/ml (Fig.2). This concentration was reached after 3 h and was the only sample exceeding the threshold level. 19-norandrosterone remained however detectable for the first 12 h.

Although the study is based on a limited number of subjects, it is clear that a single administration of norethisterone or lynestrenol can lead to 19-norandrosterone levels exceeding 2 ng/ml. 3α,5α-NET was detected always simultaneously with 19-norandrosterone, but the current results indicate that 19-norandrosterone and 3α,5α-NET concentrations can vary significantly. Hence, the statement criteria by WADA should be considered qualitatively until a significant number of studies have established a quantitative correlation. Although the limited number of test subjects does not allow to draw definitive conclusions, the ratio of 19-norandrosterone to 19-noretiocholanolone might offer opportunities to discriminate between the sole use of oral contraceptives and their combination with nandrolone (or prohormones). Indeed, in this experiment only trace amounts of 19-noretiocholanolone were detected.

According to Ward et al. de-ethylation of ethylestrenol and norethandrolone was not detected in a previous study and 17α-ethyl-5α-estrane-3α,17β-diol and 17α-ethyl-5β-estrane-3α,17β-diol were identified as the major metabolites of norethandrolone and ethylestrenol (5). Our results confirmed that 17α-ethyl-5α-estrane-3α,17β-diol and 17α-ethyl-5β-estrane-3α,17β-diol are the major metabolites of ethylestrenol (Fig. 3). However, 19-norandrosterone was also detected between 2 and 9 h post administration which proves that de-ethylation is a minor metabolic pathway. Maximum urinary concentrations of 19-norandrosterone reached up to 4,3 ng/ml and slightly exceeded the threshold at 3 and 6 h post administration (Fig.4). Hence, concurrent detection of 19-norandrosterone and the two major metabolites of norethandrolone/ethylestrenol should not be regarded as proof of co-administration.

4. Conclusion

The metabolic pathways of lynestrenol/norethisterone and ethylestrenol/norethandrolone are similar. De ethylation/de-ethynylation is a minor metabolic pathway and leads to the detection of 19-norandrosterone as a minor metabolite.

Intake of the oral contraceptives lynestrenol and norethisterone leads to the simultaneous detection of 19-norandrosterone and 3α,5α-tetrahydronorethisterone. The current legislation which demands that samples of female athletes are analyzed for the presence of 3α,5α-tetrahydronorethisterone before claiming an adverse analytical finding for 19-norandrosterone provides sufficient safety to avoid false positive results, since 19-norandrosterone is always detected concurrent with 3α,5α-tetrahydronorethisterone.

5. List of Figures

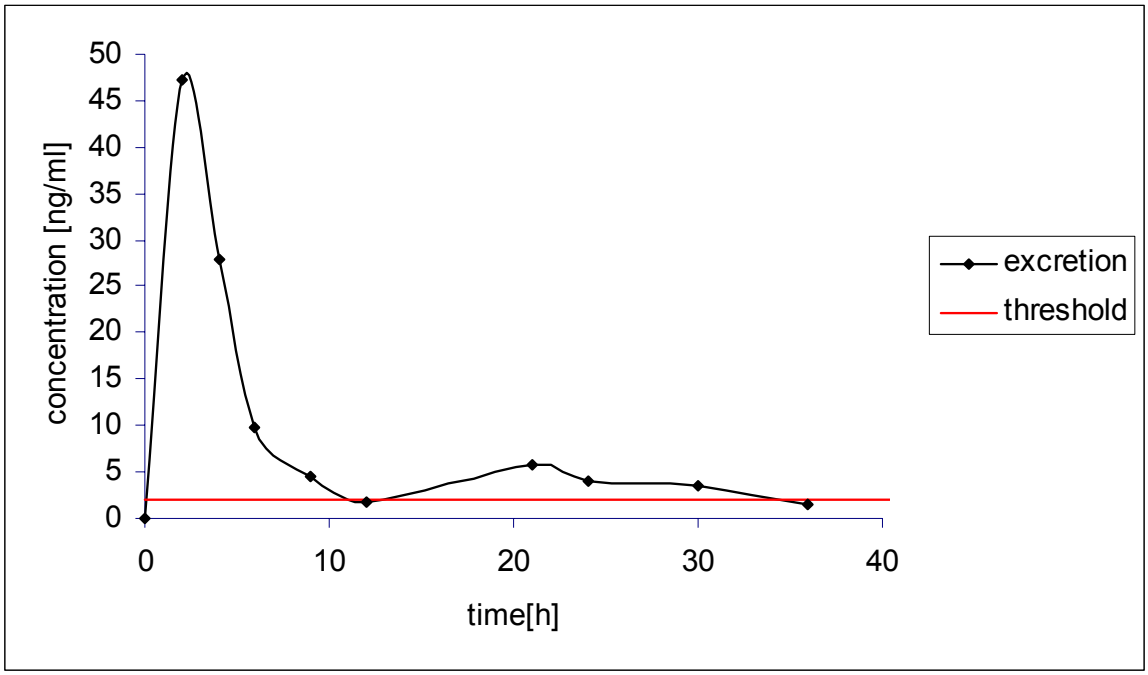

Fig. 1. Excretion profile of 19-norandrosterone after a single oral administration of 10 mg norethisterone acetate (Primolut-Nor ®)

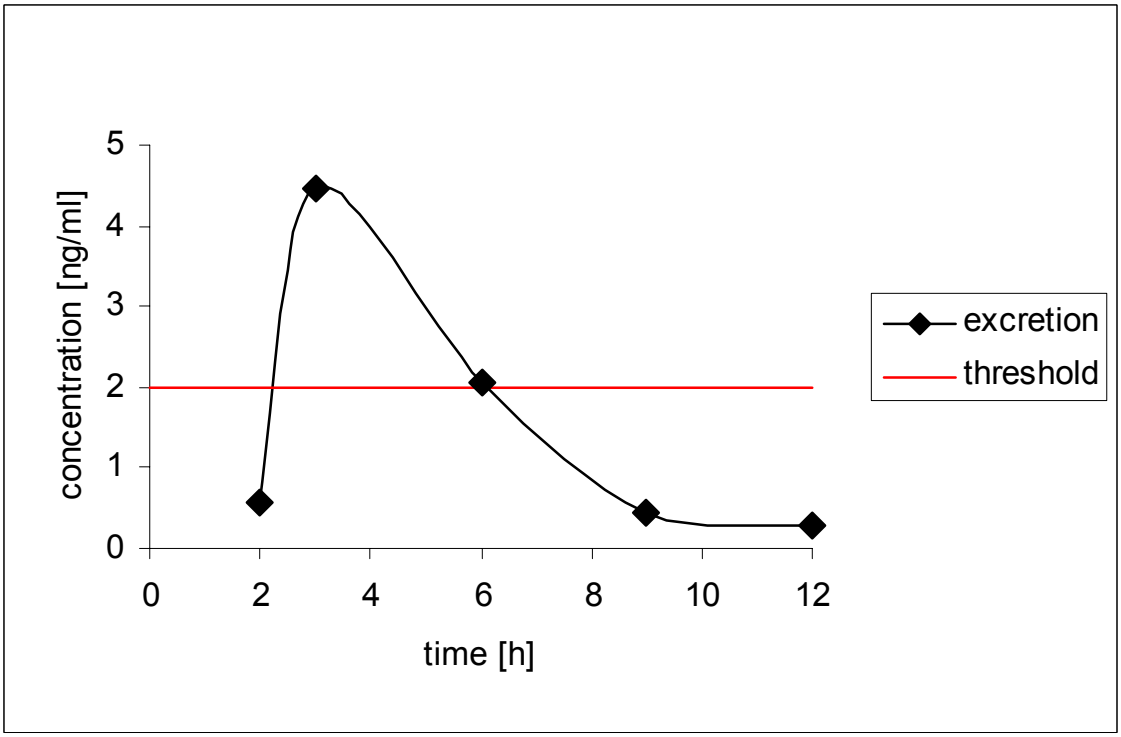

Fig. 2. Excretion profile of 19-norandrosterone after a single oral administration of 5 mg lynestrenol (Orgametril ®)

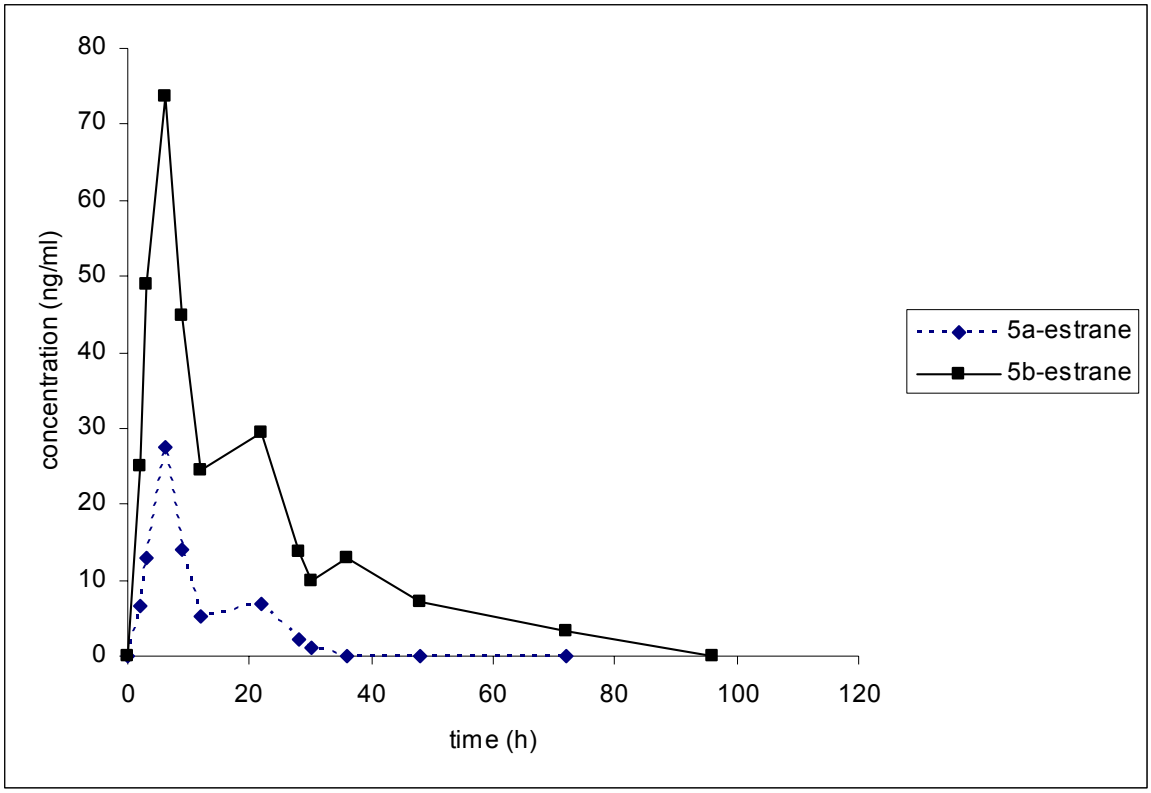

Fig. 3. Excretion profile of 17α-ethyl-5α-estrane-3α,17β-diol (5a-estrane) and 17α-ethyl-5β-estrane-3α,17β-diol (5b-estrane) after a single oral administration of 10 mg ethylestrenol

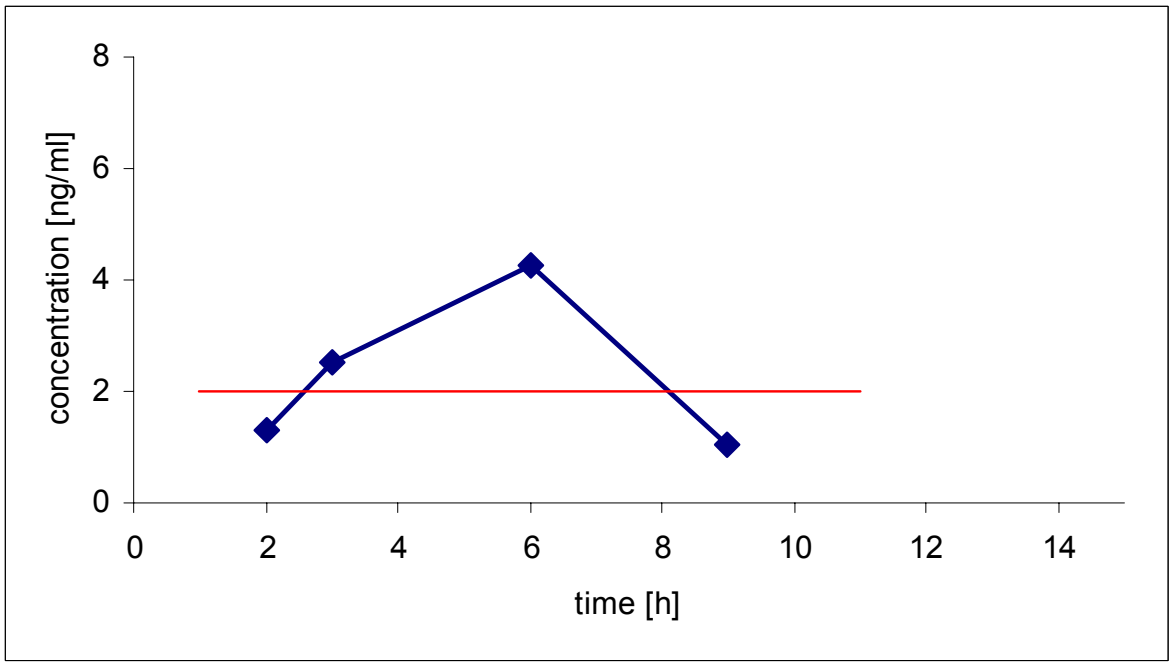

Fig. 4. Excretion profile of 19-norandrosterone after a single oral administration of 10 mg ethylestrenol

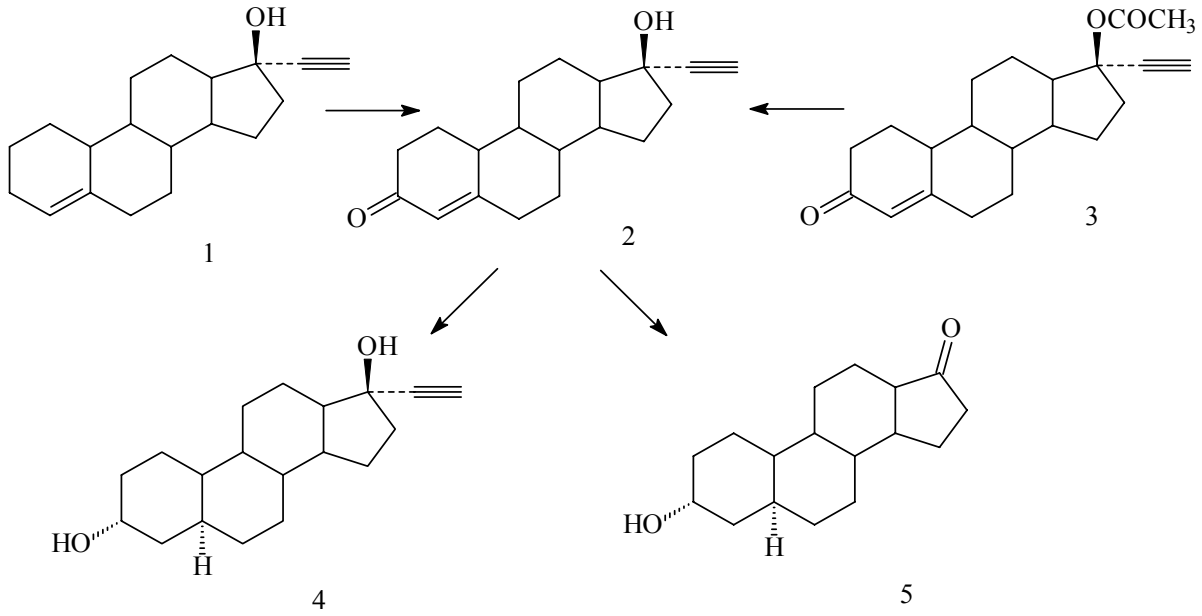

Fig. 5. Metabolism of lynestrenol (1) and norethisterone acetate (3), prodrugs of norethisterone (2) to 3α,5α-tetrahydronorethisterone (major urinary metabolite, 4) and 19-norandrosterone (minor urinary metabolite, 5).

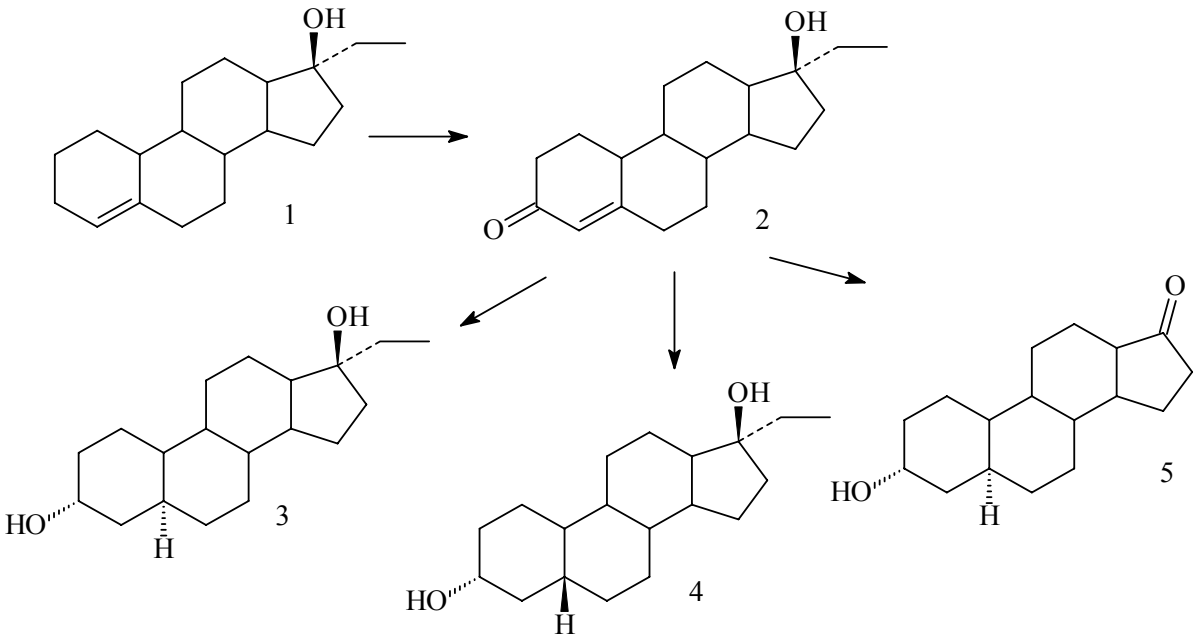

Fig. 6. Metabolism of ethylestrenol (1) to norethandrolone (2), 17α-ethyl-5α-estrane-3α,17β-diol (3), 17α-ethyl-5β-estrane-3α,17β-diol (4) and 19-norandrosterone (minor urinary metabolite, 5).

6. References

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