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METABOLISM OF METANDIENONE IN MAN: IDENTIFICATION AND SYNTHESIS OF CONJUGATED EXCRETED URINARY METABOLITES, DETERMINATION OF EXCRETION RATES AND GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC IDENTIFICATION OF BIS-HYDROXYLATED METABOLITES

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Summary—After oral administration of metandienone (17α-methyl-androsta-1,4-dien-17β-ol-3-one) to male volunteers conjugated metabolites are isolated from urine via XAD-2-adsorption, enzymatic hydrolysis and preparative high-performance liquid chromatography (HPLC). Four conjugated metabolites are identified by gas chromatography–mass spectrometry (GC/MS) with electron impact (EI)-ionization after derivatization with *N*-methyl-*N*-trimethyl-silyl-trifluoroacetamide/trimethylsilyl-imidazole (MSTFA/TMS-Imi) and comparison with synthesized reference compounds: 17α-methyl-5β-androst-1-en-17β-ol-3-one (II), 17α-methyl-5β-androst-1-ene-3α,17β-diol (III), 17β-methyl-5β-androst-1-ene-3α,17α-diol (IV) and 17α-methyl-5β-androstane-3α,17β-diol (V).

After administration of 40 mg of metandienone four bis-hydroxy-metabolites—6β,12-dihydroxy-metandienone (IX), 6β,16β-dihydroxy-metandienone (X), 6β,16α-dihydroxy-metandienone (XI) and 6β,16β-dihydroxy-17-epimetandienone (XII)—were detected in the unconjugated fraction.

The metabolites III, IV and V are excreted in a comparable amount to the unconjugated excreted metabolites 17-epimetandienone (VI), 6β-hydroxy-metandienone (VII) and 6β-hydroxy-17-epimetandienone (VIII). Whereas the unconjugated excreted metabolites show maximum excretion rates between 4 and 12 h after administration the conjugated metabolites III, IV and V are excreted with maximum rates between 12 and 34 h.

INTRODUCTION

Metandienone (Fig. 1, I) is an anabolic steroid which was first synthesized in 1955 by Vischer *et al.* [1] by microbiological dehydrogenation of 17α-methyltestosterone. Meystre *et al.* [2] published in 1956 the dehydrogenation of 17α-methyltestosterone with selenium dioxide.

Metandienone is widely misused in sports. Its detection is performed by GC/MS-screening for the urinary excreted metabolite 6β-hydroxy-metandienone (VII).

This metabolite was first identified by Rongone and Segaloff in 1963 [3]. They also found an isomer of metandienone but could not confirm its structure. This isomer was identified as 17-epimetandienone (VI) and synthesized by Macdonald *et al.* [4, 5] in 1971.

Dürbeck *et al.* [6, 7] investigated the metabolism of the orally applied drug in man by GC/MS in 1979 and detected three metabolites and two artifacts in the unconjugated urine fraction: 17-epimetandienone (VI), 6β-hydroxy-metandienone (VII), 6β-hydroxy-17-epimetandienone (VIII), 17α-methyl-androsta-1,4,6-trien-17β-ol-3-one and 17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one. No conjugated excreted metabolite was found after hydrolysis of the urine with β-glucuronidase [5, 7].

Two conjugated metabolites were detected in metabolic studies of metandienone in rats by Steele and Schlesinger in 1979 [8]: 17α-methyl-5β-androstane-3α,17β-diol (V) and 17α-methyl-5α-androstane-3β,17β-diol. V is also the main metabolite in the human metabolism of 17α-methyltestosterone [9–11].

In this study four conjugated excreted metabolites are isolated from urine after oral

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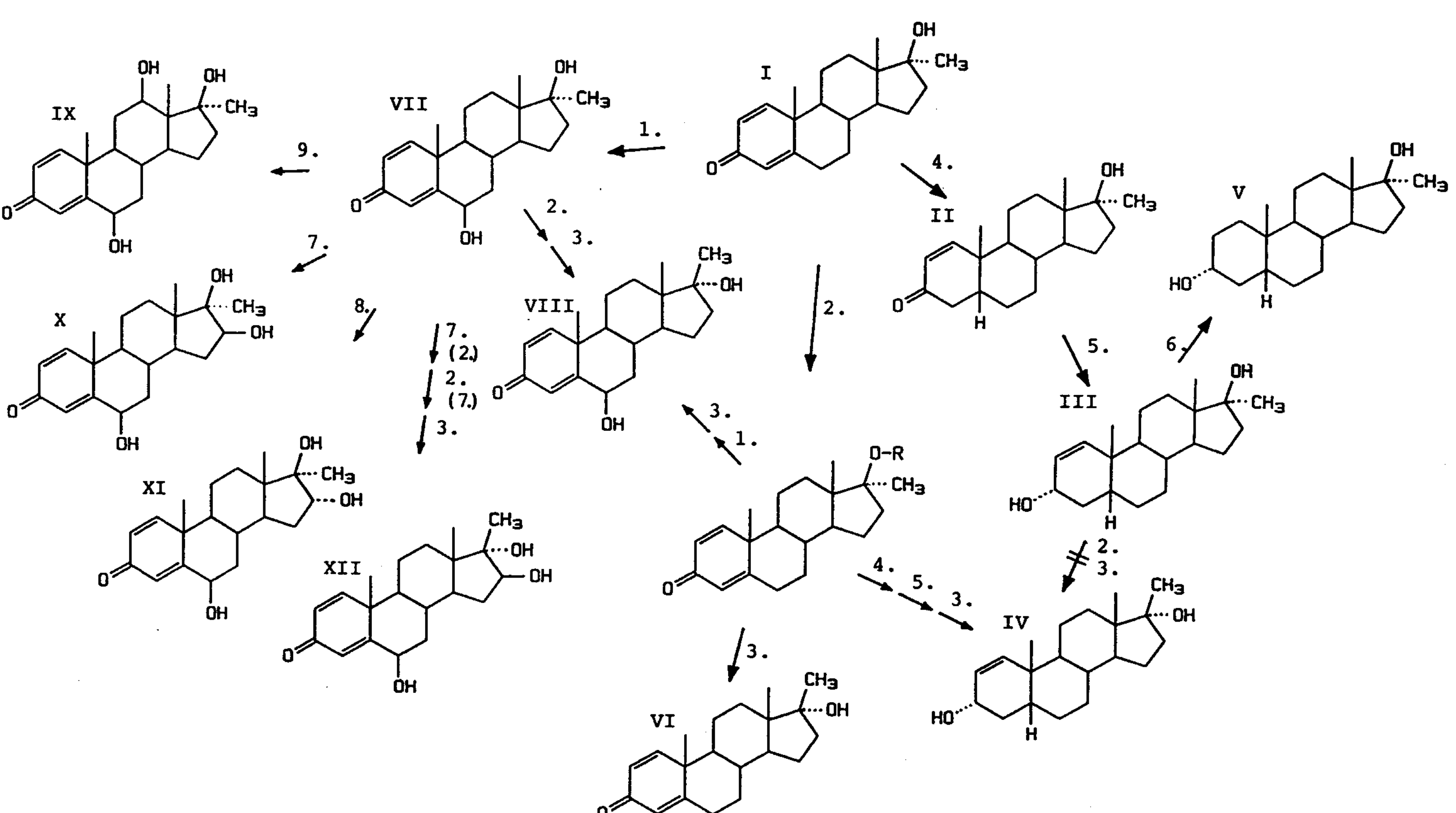

Fig. 1. Metabolism of metandienone. I Metandienone, II 17α-Methyl-5β-androst-1-en-17β-ol-3-one. III 17α-Methyl-5β-androst-1-ene-3α,17β-diol. IV 17β-Methyl-5β-androst-1-ene-3α,17α-diol. V 17α-Methyl-5β-androstane-3α,17β-diol. VI 17-Epimetandienone. VII 6β-Hydroxy-metandienone. VIII 6β-Hydroxy-17-epimetandienone. IX 6β,12-Dihydroxy-metandienone. X 6β,16β-Dihydroxy-metandienone. XI 6β,16α-Dihydroxy-metandienone. XII 6β,16β-Dihydroxy-17-epimetandienone. 1. 6β-Hydroxylation. 2. 17-Conjugation (sulfate ?). 3. Urinary inversion. 4. 5β-Reduction. 5. 3α-Dehydrogenation. 6. 1,2-Reduction. 7. 16β-Hydroxylation. 8. 16α-Hydroxylation. 9. 12-Hydroxylation.

application of metandienone and characterized by GC/MS. All metabolites are identified by comparison of the GC-properties and the mass spectra with synthesized reference compounds. Furthermore excretion profiles of these conjugated metabolites and of unconjugated metabolites are presented.

EXPERIMENTAL

Steroids and reagents

Metandienone, testosterone, 17-epitestosterone, and androsterone, etiocholanolone, androst-5-en-3β-ol-17-one, 5α-androstane-3α,-11β-diol-17-one and 5β-androstane-3α,11β-diol-17-one were purchased from Sigma, Deisendorf; cortisol, cortisone, 19-nortestosterone, 5α-androstane-3,17-dione and androst-4-en-17β-ol-3,17-dione from Serva, Heidelberg; 6β-hydroxy-metandienone and fluoxymesterone were gifts from Ciba-Geigy; 4-chloro-dehydromethyltestosterone was a gift from Jenapharm, Jena; and 4-chloro-testosterone from Farmitalia, Carlo Erba. Epimetandienone, 6β-hydroxy-epimetandienone and 17α-methyl-androsta-1,4,6-trien-17β-ol-3-one were synthesized in our laboratory (publication in preparation).

Metabolism studies

Metandienone was administered to male volunteers orally: (a) 75 kg, 35 yr, 24 mg, (b) 62 kg, 30 yr, 12 mg, (c) 75 kg, 36 yr, 40 mg.

10 mg of synthesized 17α-methyl-5β-androst-1-ene-3α,17β-diol was administered to a male person (75 kg, 36 yr).

After application the urine was collected in fractions as produced and stored at 4°C.

Isolation of steroids for GC/MS-screening

Conjugated and unconjugated metabolites are isolated as described by Donike *et al.* [12]. The conjugated metabolites are hydrolyzed either with 25 μl of β-glucuronidase from *Escherichia coli* (Boehringer, Mannheim) in 1 ml of 0.2 M sodium phosphate buffer pH 7.0 for 1 h at 50°C or with 50 μl of arylsulfatase/β-glucuronidase from *Helix pomatia* (Serva, Heidelberg) in 1 ml of 0.2 M sodium actate buffer pH 5.2 for 3 h at 50°C.

Isolation of conjugated metabolites

Five 20 ml portions of urine are passed through 5 XAD-2 columns (pasteur pipette with 5 mm i.d., closed with a glass pearl, 3 cm bed height). Each column is washed with 10 ml of water and eluted with 2 ml of methanol. The eluates are evaporated to dryness and each residue is dissolved in 1 ml of 0.2 M phosphate buffer pH 7. The unconjugated steroids are extracted twice with 5 ml of diethyl ether. The ether is completely removed from the aqueous phases. Each residue is hydrolyzed with 50 μl of β-glucuronidase from *E. coli* for 2 h at 50°C. After hydrolysis about 100 mg of potassium carbonate are added and the steroids are extracted twice with 5 ml of diethyl ether. The ether layers are combined, evaporated to dryness *in vacuo* and the residue is dissolved in 100 μl of methanol. The methanolic solution is injected on the HPLC-column. The fractions are collected at 0.5 min intervals from 10 to 30 min and evaporated to dryness *in vacuo*. HPLC-condition: HPLC 1084 Hewlett–Packard, solvents: (A) water (B) acetonitrile/water 90:10 (v/v), flow: 6 ml/min gradient: starting with 30% of B and linear increase in 25 min to 80% of B, column: Nucleosil (R) RP-18, 7 μm, 25 × 1 cm (Macherey-Nagel, Düren).

Derivatization for GC/MS-analysis

Trimethylsilylation. (a) The dry residue is dissolved in 50 μl of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide/trimethylsilylimidazole (MSTFA/TMS-Imi) 100:2 (v/v) and heated for 10 min at 60°C [12]. (b) To obtain enol-TMS-ethers the sample is derivatized with 50 μl of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide/trimethyliodosilane (MSTFA/TMIS) 100:0.2 (v/v) containing 0.2% of 1,4-dithioerythreitol and heated for 10 min at 60°C [12].

GC/MS-determination

Instrument. (A) GC/MS Hewlett–Packard (GC 5890/MS 5970), electron impact ionization with 70 eV, column: fused silica capillary column crosslinked 5% phenyl methyl silicone (SE-54), 17 m, i.d. 0.2 mm, film thickness 0.33 μm; carrier gas: helium 1 ml/min, split 1:10, temperature program: initial temperature 200°C, program rate 20°C/min, final temperature 300°C, injector temperature 300°C, interface temperature 300°C.

(B) GC/MS Hewlett–Packard 5996B, EI-ionization at 70 eV, column: fused silica capillary column crosslinked methyl silicone (OV-1), 17 m, i.d. 0.2 mm, film thickness 0.11 μm, carrier gas: hydrogen 1.5 ml/min, split

1:10, temperature program: initial temperature 180°C, program rate 4°C/min.

The presented mass spectra are not normalized.

Nuclear magnetic resonance spectroscopy

NMR-data were registered by Dr C. G. B. Frischkorn, KFA-Jülich. 90 MHz spectra were run using a Varian EM 390.

Synthesis of reference compounds

17α-Methyl-5β-androst-1-en-17β-ol-3-one (II). 2 g (6.6 mmol) of metandienone are reduced with hydrogen in 60 ml of methanol + 1 ml of 6 N aqueous sodium hydroxide using 10 mg of 10% palladium on charcoal (Aldrich, Steinheim) as catalyst. The reaction was stopped when 60 ml of hydrogen have been used. The mixture is diluted with 200 ml of bidistilled water and extracted twice with 500 ml of diethyl ether. The combined ether layers are dried over sodium sulfate and evaporated to dryness. The reaction yields 13% of 17α-methyl-androst-4-en-17β-ol-3-one, 20% of 17α-methyl-5β-androst-1-en-17β-ol-3-one (II), 18% of 17α-methyl-5β-androstan-17β-ol-3-one and 49% of unchanged metandienone. II is isolated by chromatography on silica gel 60 (Merck, 35–70 mesh ASTM, bed 1 × 50 cm) with *n*-pentane/ethyl acetate 70:30 (v/v) and recrystallized four times from acetone/*n*-heptane. About 190 mg (9.5% of the theory) of pure compound are obtained, m.p. 176–177°C. [^{1}H]NMR ($CDCl_3$): d = 0.90 (s,3H,18-H3), d = 1.21 (s,3H), d = 1.24 (s,3H). EI-spectrum of bis-TMS-II see Fig. 2.

17α-Methyl-5β-androst-1-en-3α,17β-diol (III). 100 mg (0.33 mmol) of 17α-methyl-5β-androst-1-en-17β-ol-3-one (II) are dissolved in 50 ml of diethyl ether, 45 mg of lithium aluminium hydride are added and the mixture is stirred for 1 h at ambient temperature. 100 ml of water are added to the reaction mixture and the reduced steroid is extracted with 250 ml of diethyl ether. The ether layer is dried over sodium sulfate and evaporated to dryness *in vacuo*. The crude product is first recrystallized from 10 ml of ethyl acetate + 2 ml of benzene and then from *n*-heptane/ethyl acetate 1:1 (v/v) to obtain 60 mg of pure compound, m.p. 173–175°C. [^{1}H]NMR ($CDCl_3/CD_3OD$ 1:1, v/v): d = 0.86 (s,3H,18-H3), d = 1.06 (s,3H,19-H3), d = 1.21 (s,3H,20-H3). EI-spectrum of bis-TMS-III see Fig. 4.

17α-Methyl-5β-androstane-3α,17β-diol (V). 500 mg (1.72 mmol) of etiocholanolone (Sigma, Deisenhofen) are dissolved in 50 ml of diethyl ether and dropped within 30 min to a Grignard mixture prepared from 300 mg (13 mmol) of magnesium and 0.8 ml (13 mmol) of methyl iodide in 10 ml of diethyl ether. After stirring for

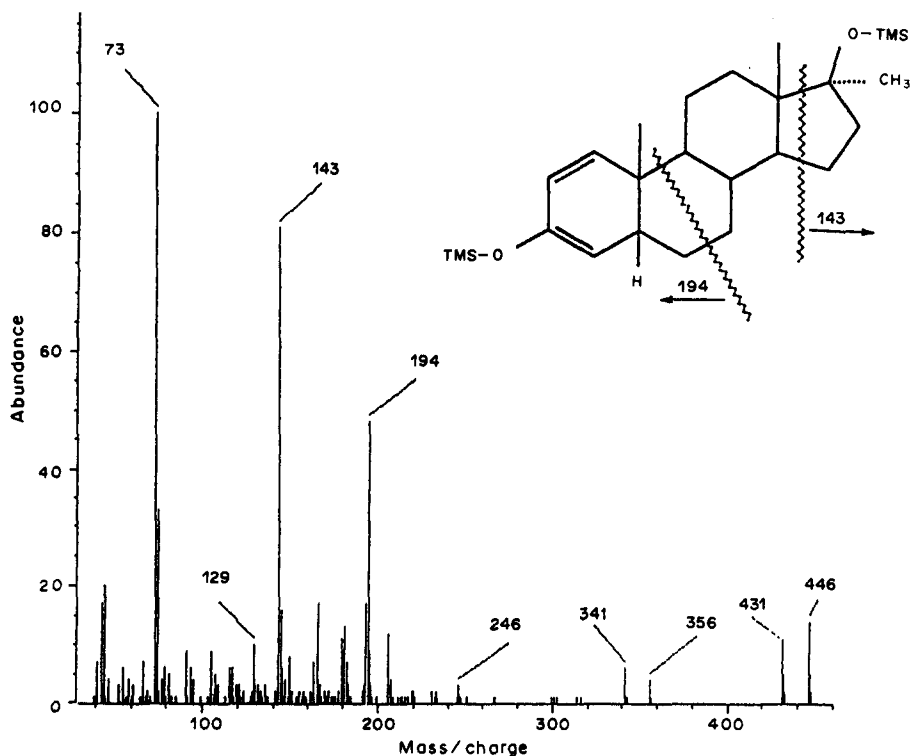

Fig. 2. EI-spectrum of 17α-methyl-5β-androst-1-en-17β-ol-3-one,bis-TMS (II).

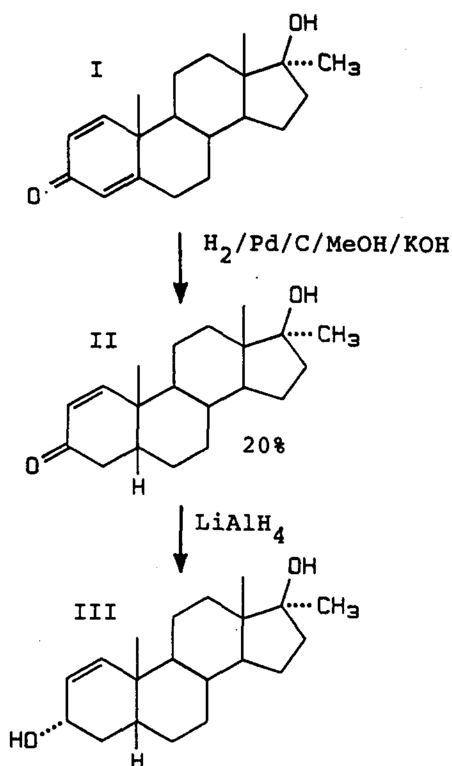

Fig. 3. Synthesis of 17α-methyl-5β-androst-1-en-17β-ol-3-one (II) and 17α-methyl-5β-androst-1-ene-3α,17β-diol (III).

2 h 70% of the etiocholanolone has reacted to the 17α-methyl-derivative (GC/FID-, GC/MS-control). The reaction mixture is poured on 200 ml of ice, stirred for 30 min and then extracted with 500 ml of diethyl ether. The 17α-methyl-5β-androstane-3α,17β-diol is separated from etiocholanolone on silica gel (Merck, 35–70 mesh ASTM, bed 1 × 50 cm) using iso-octane/ethyl acetate 70:30 (v/v) to obtain 170 mg (33% of the theory) of pure substance, m.p. 165–166°C (Lit [8]: 164–166°C). EI-spectrum of bis-TMS-V see Fig. 8.

17β-Methyl-5β-androst-1-ene-3α,17α-diol (IV)

This metabolite was synthesized in μg-amount and characterized by GC/MS. Two different reaction schemes were used.

(A). 1. 3α-Acetoxy-17α-methyl-5β-androst-1-en-17β-ol (XV): 5 mg of 17α-methyl-5β-androst-1-ene-3α,17β-diol (III) are dissolved in 1 ml of pyridine/acetic anhydride 10:1 (v:v) and heated for 1 h at 60°C. The mixture is evaporated to dryness and an aliquot is analysed by GC/MS. About 90% of XV is obtained. EI-spectrum of TMS-XV: 418 (0.3), 403 (3), 343 (2), 286 (3), 268 (6), 229 (6), 143 (100), 130 (15), 73 (25), 43 (19).

2. 3α-Acetoxy-17β-methyl-5β-androst-1-en-17α-ol (XVII): The dry residue of 1. is dissolved in 0.5 ml of dimethyl-formamide and 50 mg of sulfur trioxide pyridine complex are added. After 2 h at ambient temperature 5 ml of 2% potassium carbonate solution are added. After

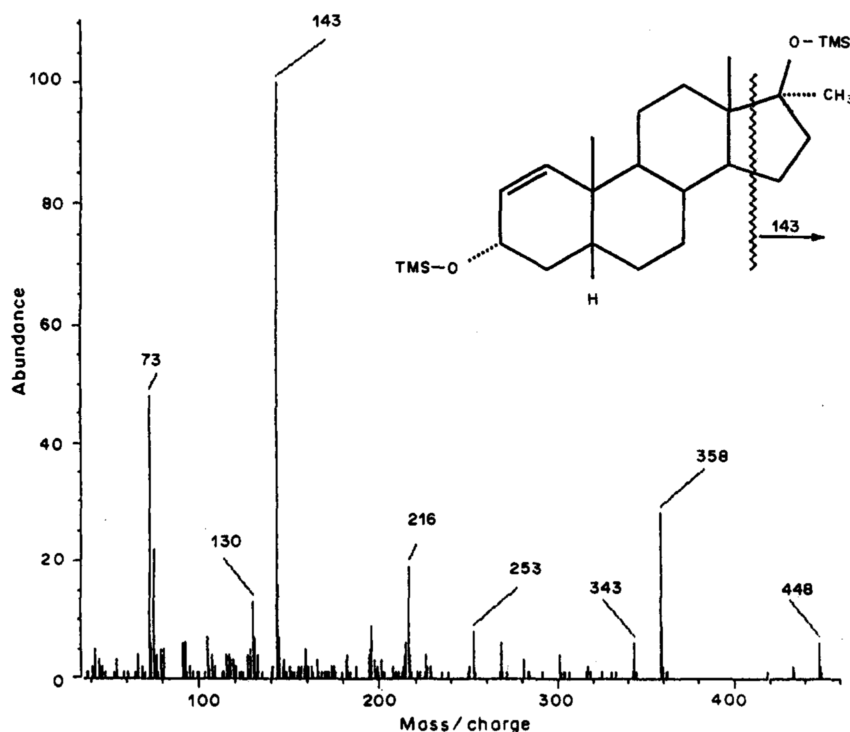

Fig. 4. EI-spectrum of 17α-methyl-5β-androst-1-ene-3α,17β-diol,bis-TMS (III).

a further 2 h at ambient temperature the reaction mixture is extracted with 5 ml of diethyl ether. 0.5 ml of the ether layer are evaporated to dryness, derivatized with MSTFA/TMS-Imi and analyzed by GC/MS. The mixture contains about 60% of dehydration products (mainly the 3β-acetoxy-18-nor-17.17-dimethyl-5β-androst-1,13-diene, 47%) and about 35% of 3α-acetoxy-17β-methyl-5β-androst-1-en-17α-ol (XVII). EI-spectrum of TMS-XVII: 418 (0.3), 403 (4), 343 (2), 286 (3), 268 (7), 229 (5), 143 (100), 130 (17), 73 (47), 43 (44).

3. 17β-methyl-5β-androst-1-ene-3α,17α-diol (IV): The ether extract of 2. (4.5 ml) is evaporated to dryness, dissolved in 1 ml of 6 N KOH in MeOH and heated for 1 h at 60°C. The methanolic phase is concentrated by evaporation to about 50 μl, diluted with 1 ml of water and extracted with 5 ml of diethyl ether. The ether layer is dried over sodium sulfate and evaporated to dryness (2 mg of residue, containing 35% of 17β-methyl-5β-androst-1-ene-3α,17α-diol (IV)). EI-spectrum of bis-TMS-IV see Fig. 5.

(B). 1. 17β-Methyl-5β-androst-1-en-17α-ol-3-one (XIX): 5 mg of 17α-methyl-5β-androst-1-en-17β-ol-3-one (II) are dissolved in 0.5 ml of dimethylformamide and 50 mg of sulfur trioxide pyridine complex are added and after 1 h at ambient temperature about 5 ml of water. The reaction mixture is extracted after further 2 h with 5 ml of diethylether. 0.5 ml of the ether layer is evaporated to dryness and analyzed by GC/MS after derivatization with MSTFA/TMIS. The reaction yields about 60% of dehydration products (four isomers with 18-nor-17,17-dimethyl-5β-androst-1,13-dien-3-one as the main dehydration product) and about 34% of 17β-methyl-5β-androst-1-en-17α-ol-3-one (XIX). EI-spectrum of bis-TMS-XIX: 446 (11), 431 (7), 356 (5), 341 (8), 194 (44), 143 (49), 73 (100).

2. 17β-Methyl-5β-androst-1-ene-3α,17α-diol (IV): The ether extract of 1. (4.5 ml) is evaporated to dryness, dissolved in 2 ml of absolute diethyl ether, 50 mg of lithium aluminium hydride are added and after 1 h at ambient temperature 1 ml of water. The reaction products are extracted with 5 ml of diethyl ether. 1 ml of the ether layer is evaporated to dryness and analyzed after derivatization with MSTFA/TMIS by GC/MS. The reaction mixture contains about 35% of 17β-methyl-5β-androst-1-ene-3α,17α-diol (IV) (EI-spectrumn of bis-TMS-IV see Fig. 5), 3% of 17β-methyl-5β-androst-1-ene-3β,17β-diol and about 60% of dehydration products.

17α-Methyl-5β-androstane-3,12,17β-triol

(1) 17α-Methyl-5β-androstane-11α,17β-diol-3-one. 1.1 g (3.44 mmol) of 11α-hydroxy-

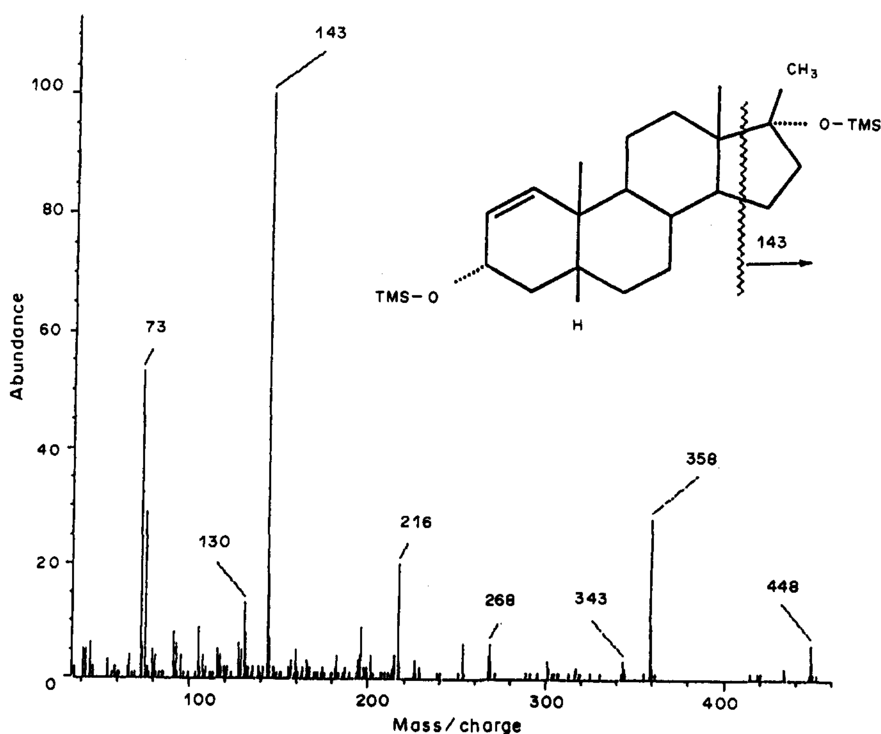

Fig. 5. EI-spectrum of 17β-methyl-5β-androst-1-ene-3α,17α-diol,bis-TMS (IV).

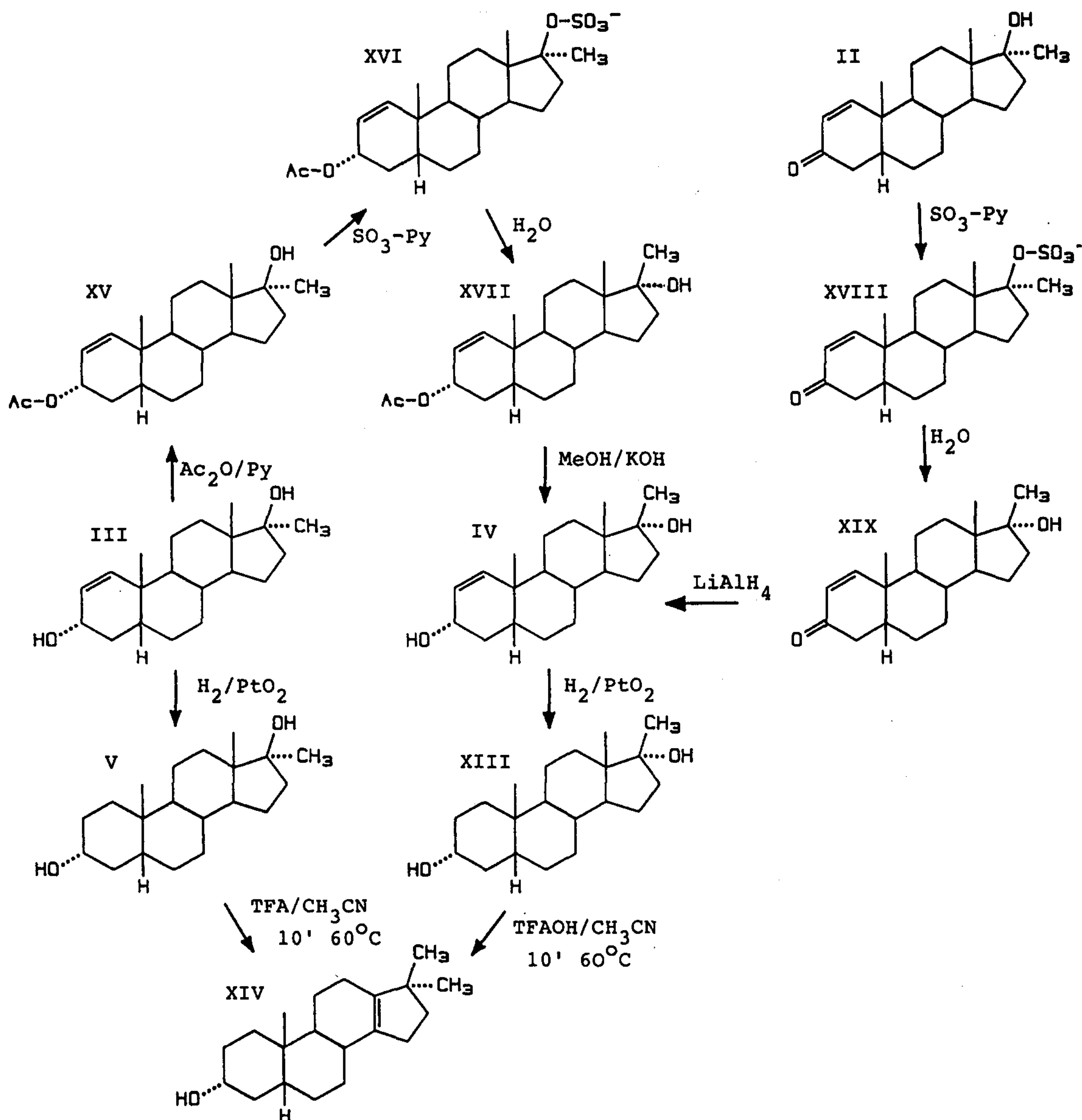

Fig. 6. Synthesis of 17β-methyl-5β-androst-1-ene-3α,17α-diol (IV).

methyltestosterone (Sigma) in 20 ml of methanol are reduced with hydrogen using 13 mg of palladium on charcoal (10%) as catalyst within 3 h at ambient temperature yielding 76% of 17α-methyl-5β-androstane-11α,17β-diol-3-one (EI-spectrum of the Tris-TMS-derivative: 536 (1.2), 446 (2), 431 (2), 356 (6), 341 (7), 304 (10), 214 (63), 143 (47), 73 (100)) and 24% of 17α-methyl-5α-androstane-11α,17β-diol-3-one (EI-spectrum of the Tris-TMS-derivative: 536 (1), 446 (21), 431 (17), 356 (5), 341 (8), 194 (29), 181 (25), 143 (63), 73 (100)).

The mixture is filtered and the filtrate is evaporated to dryness.

(2) 17α-Methyl-5β-androst-9(11)-en-17β-ol-3-one. The dry residue of the first reaction (about 1.12 g of crude product) is dissolved in 20 ml of pyridine containing 1.2 g of *p*-toluenesulfonyl chloride (7.5 mmol) and stirred for 24 h at ambient temperature. 100 ml of water are added and the reaction products are extracted with 400 ml of diethyl ether. The ether layer is evaporated to dryness. The residue is dissolved in 60 ml of 5% (w/v) methanolic potassium hydroxide and refluxed for 5 h. The methanolic solution is evaporated to a volume of 10 ml, diluted with 100 ml of water and extracted with 400 ml of diethyl ether. The ether layer is dried over sodium sulfate, filtered and evaporated to dryness yielding 1.1 g of crude product.

The GC/MS-analysis shows one main product (55%): 17α-methyl-5β-androst-9(11)-

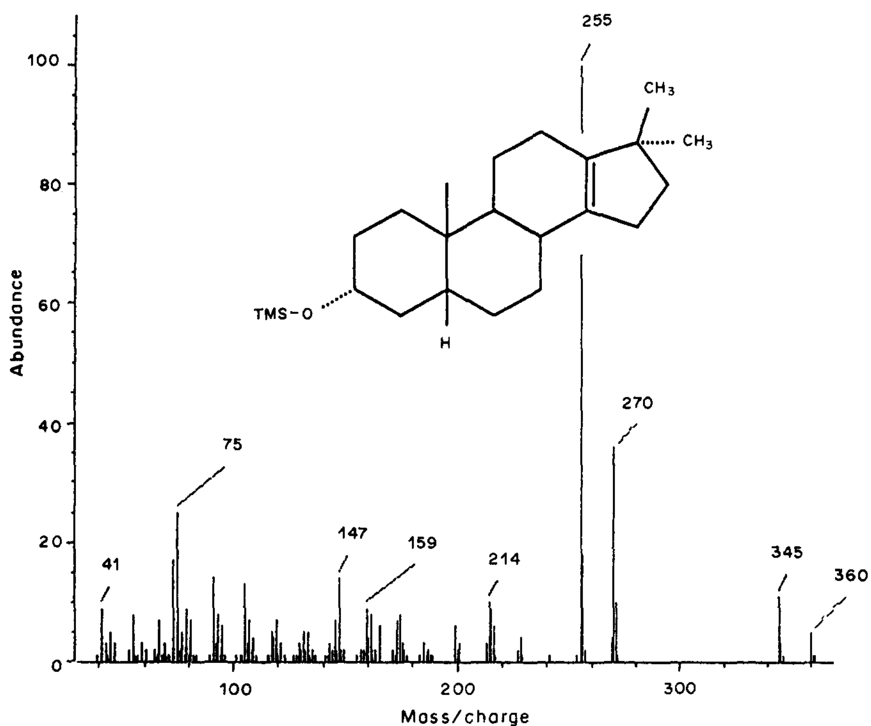

Fig. 7. EI-spectrum of 17,17-dimethyl-18-nor-5β-androst-13-en-3α-ol,TMS (XIV).

en-17β-ol-3-one. EI-spectrum of the bis-TMS-derivative: 446 (5), 431 (5), 356 (6), 341 (16), 251 (12), 214 (34), 143 (58), 73 (100).

The mixture is purified over silica gel 60 (Merck, 35–70 mesh ASTM, bed 50 × 1 cm) with *n*-pentane/ethyl acetate 75:25 (v/v). The

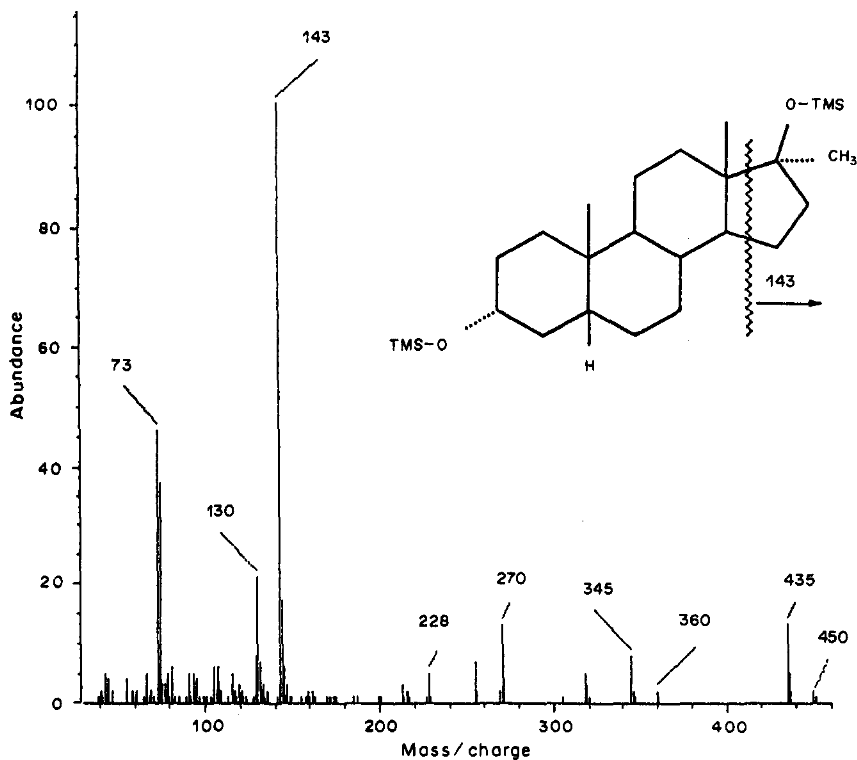

Fig. 8. EI-spectrum of 17α-methyl-5β-androstane-3α,17β-diol,bis-TMS (V).

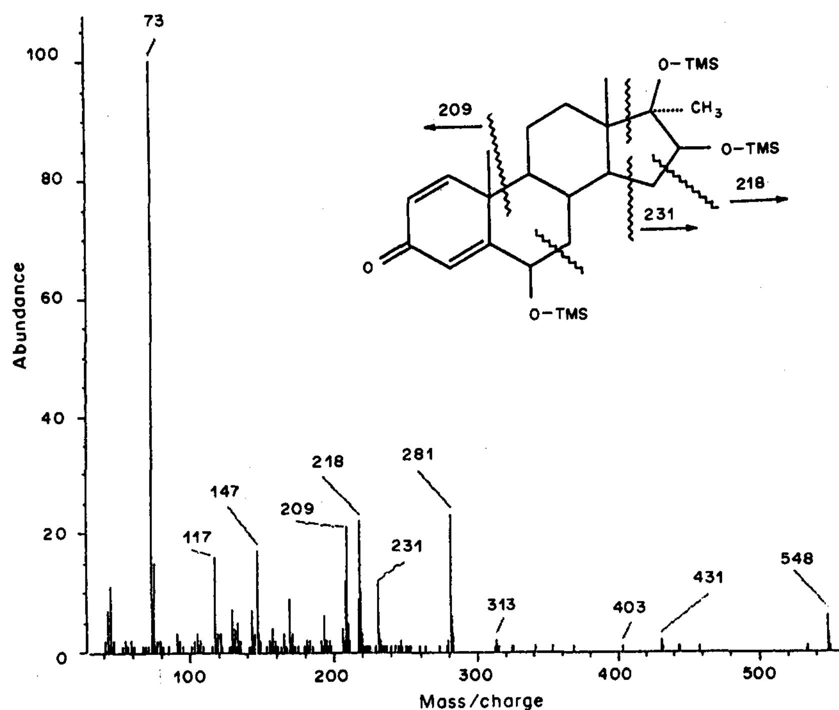

Fig. 9. EI-spectrum of 6β,16β-dihydroxy-metandienone,Tris-TMS (X).

fraction from 410 to 800 ml containing the 5α- and 5β-isomers are concentrated and dried. The residue (450 mg) contains 77% of the 5β-isomer, 17% of the 5α-isomer and 6% of side products.

(3) 17α-Methyl-5β-androst-9,11-en-17β-ol-3,12-dione. Without further purification the residue (450 mg) is dissolved in 20 ml of acetone containing 500 mg of chromium trioxide and stirred for 4 days at ambient temperature. The

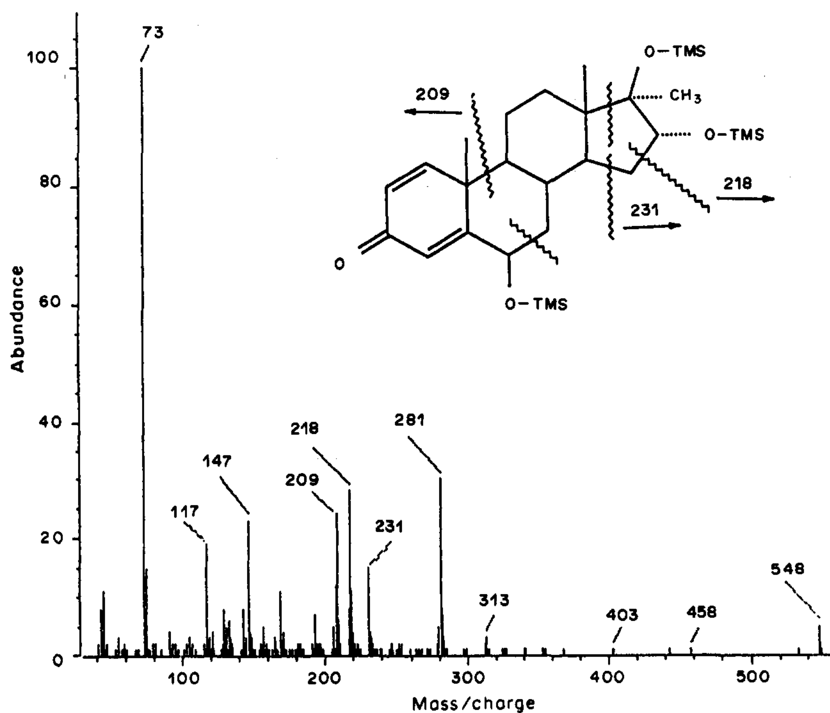

Fig. 10. EI-spectrum of 6β,16α-dihydroxy-metandienone,Tris-TMS (XI).

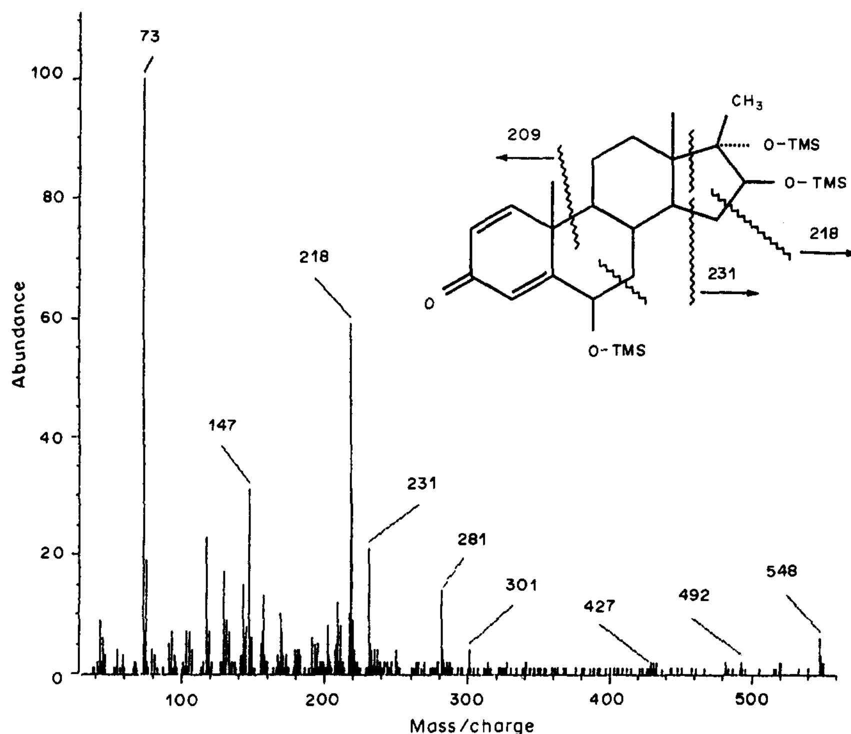

Fig. 11. EI-spectrum of 6β,16β-dihydroxy-17-epimetandienone,Tris-TMS (XII).

mixture is diluted with 100 ml of bidistilled water and extracted with 200 ml of diethyl ether. The ether layer is dried and the reaction products are purified over silicagel 60 (bed 50 × 1 cm) with *n*-pentane/ethyl acetate 40:60 (v/v). The fractions from 500 to 1100 ml containing the oxidation product are combined and dried yielding 44 mg of crude product with 65% of 17α-methyl-5β-androst-9,11-en-17β-ol-3,12-dione. EI-spectrum of the Tris-TMS-derivative:

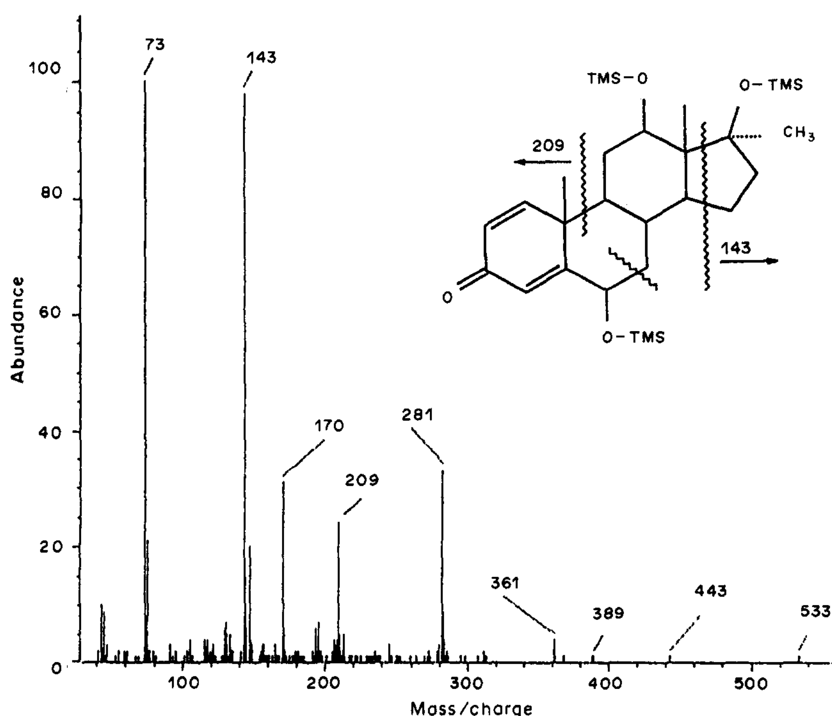

Fig. 12. EI-spectrum of 6β,12-dihydroxy-metandienone,Tris-TMS (IX).

532 (21), 402 (6), 390 (9), 300 (10), 285 (11), 260 (77), 246 (38), 245 (29), 144 (43), 143 (12), 73 (100).

(4) 17α-Methyl-5β-androstan-17β-ol-3,12-dione. The crude product (44 mg) of 3. is dissolved in 10 ml of methanol containing 20 mg of palladium on charcoal (10%) as catalyst and reduced with hydrogen within 1 h. The reaction mixture is diluted with 100 ml of bidistilled water and extracted with 200 ml of diethyl ether. The ether layer is dried. The GC/MS-chromatogram shows two per-TMS-isomers of the 17α-methyl-5β-androstan-17β-ol-3,12-dione in a ratio of 3:1. The isomers are suggested to be enolization isomers: 2,3-ene and 3,4-ene. EI spectrum of the Tris-TMS-derivative of the main isomer: 534 (11), 444 (5), 429 (5), 391 (30), 389 (30), 302 (16), 301 (23), 193 (45), 144 (25), 143 (15), 73 (100).

(5) 17α-Methyl-5β-androstane-3,12,17β-triol. The crude product of 4. is dissolved in 50 ml of absolute diethyl ether. To this solution 100 mg of lithium aluminium hydride are added. After 1 h stirring at ambient temperature 100 ml of distilled water are added and the mixture is extracted with 200 ml of diethyl ether. The ether is washed with 100 ml of water and evaporated to dryness. The reaction products are not further purified. The GC/MS analysis of the per-TMS-derivatives shows two isomers in a ratio of 2:1.

The first isomer is suggested to be the 3α-isomer concluded from the GC-elution of silylated 3α- and 3β-hydroxy-isomers of 17α-methyl-5β-androstan-3,17β-diols [13].

Both Tris-TMS-derivatives show a strong fragment at m/z 170.

EI-spectrum of the Tris-TMS-drivative of 17α-methyl-5β-androstane-3α,12,17β-triol: see Fig. 14.

EI-spectrum of the Tris-TMS-derivative of 17α-methyl-5β-androstane-3β,12,17β-triol: 538 (0.2), 523 (1.4), 448 (2), 433 (2.4), 358 (2), 269 (3), 268 (4), 170 (77), 143 (100).

Hydrogenation of androst-1-enes

The metabolites III and IV isolated by HPLC are dissolved in 10 ml of methanol and reduced within 10 min with hydrogen using 5 mg of platinum dioxide as catalyst. After filtration the solution is evaporated to dryness *in vacuo*.

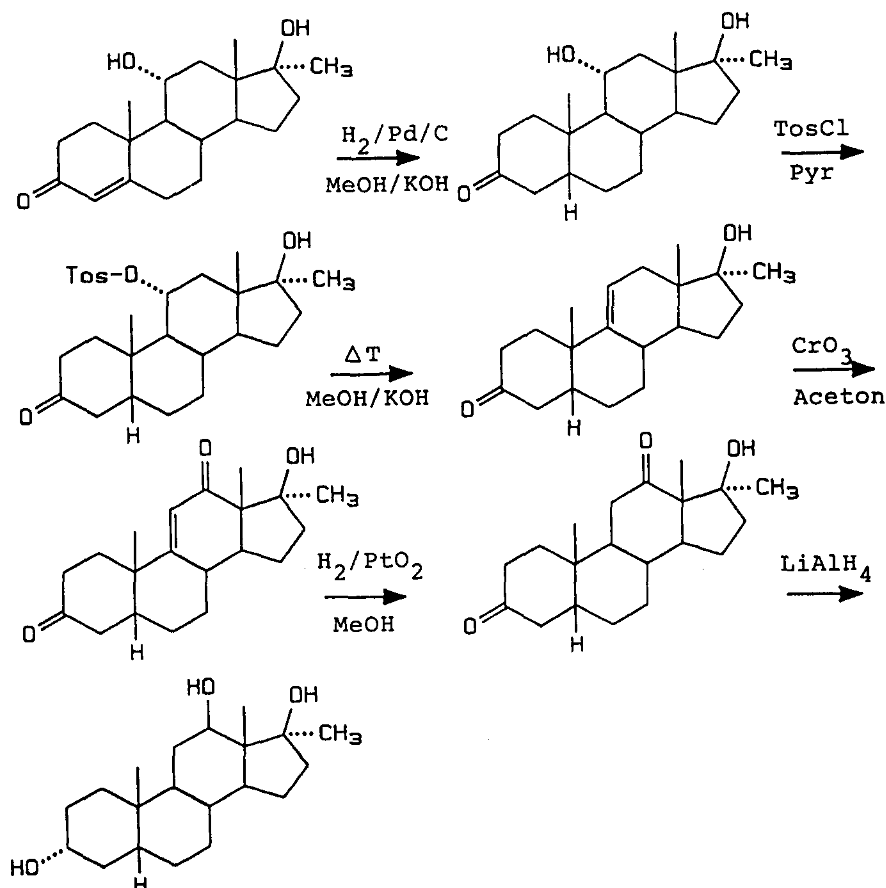

Fig. 13. Synthesis of 17α-methyl-5β-androstane-3α,12,17β-triol.

Rearrangement of 17-methyl-17-hydroxy-androstanes to 17,17-dimethyl-18-norandrost-13-enes

The dry sample is dissolved in 0.5 ml of acetonitrile (Baker, HPLC-grade)/trifluoroacetic acid 90:10 (v/v) and heated for 10 min at 60°C. The organic layer is removed by evaporation *in vacuo*.

RESULTS

HPLC separation

The results of the HPLC-separation on a preparative column RP-18 are listed in Table 1. Four metabolites are eluted at a higher retention time than metandienone itself. Table 2 shows the elution of the unconjugated metabolites including bis-hydroxy-metabolites which have been found in the unconjugated fraction after administration of 40 mg of metandienone.

Hydrolysis of conjugates

The conjugated metabolites are hydrolyzed with different enzyme preparations. The use of β-glucuronidase from *E. coli* or arylsulfatase/β-glucuronidase from *H. pomatia*, respectively, yields the same amount of hydrolyzed metabolites indicating that these metabolites are excreted as glucuronides.

Kovats indices

Retention indices of metandienone and all metandienone metabolites as their TMS-derivatives are estimated on two fused silica capillary columns OV-1 and SE-54 (Table 3). The suggested structures of the four 6β,16-bis-hydroxy-metabolites of I are based on comparison of the GC-behaviour with synthesized 16-hydroxy-stanozolol isomers [14]. The Kovats indices of other per-silylated androgens are also shown in Table 3.

Table 1. HPLC-elution of hydrolyzed conjugated metandienone-metabolites

Preparative column: Nucleosil (R), RP-18, 7 μm, 25 × 1 cm. (A) water, (B) acetonitrile/water (90/10), flow: 6 ml/min gradient: 30% B in 25 min to 80% B.

Reference substance	Retention time
Cortisol	7.79 min
Cortisone	8.21 min
Fluoxymesterone	10.95 min
19-Nortestosterone	14.13 min
Metandienone (I)	14.71 min
Testosterone	15.60 min
Epitestosterone	16.88 min
4-Chlorodehydromethyltestosterone	18.47 min
4-Chloro-testosterone	19.06 min

Fraction collected	
15.5–16.0 min	Testosterone
16.5–17.0 min	Epitestosterone
17.0–17.5 min	Dehydroepiandrosterone
17.5–18.0 min	Dehydroandrosterone and 17α-Methyl-5β-androst-1-ene-3α,17β-diol (III)
18.0–18.5 min	17α-Methyl-5β-androstane-3α,17β-diol (V)
18.5–19.0 min	17α-Methyl-5β-androst-1-en-17β-ol-3-one (II)
19.0–19.5 min	Etiocholanolone
20.0–20.5 min	Androsterone
22.5–23.0 min	17β-Methyl-5β-androst-1-ene-3α,17α-diol (IV)

Table 2. HPLC-elution of unconjugated metandienone-metabolites

Preparative column: Nucleosil (R), RP-18, 7 μm, 25 × 1 cm. (A) water, (B) acetonitrile, flow: 6 ml/min, gradient: 20% B in 20 min to 70% B and 10 min to 100% B.

Reference substance	Retention time
6β-Hydroxy-metandienone (VII)	10.28 min
Cortisol	10.57 min
Cortisone	11.05 min
Fluoxymesterone	13.39 min
19-Nortestosterone	16.40 min
Metandienone (I)	16.89 min
Testosterone	17.68 min
17-Epitestosterone	18.52 min
4-Chlorodehydromethyltestosterone	19.28 min
4-Chloro-testosterone	20.18 min
17,17-Dimethyl-18-norandrosta-1,4,13-trien-3-one	30.61 min

Fraction collected	Substance
6.0–6.5 min	6β,16-Dihydroxy-metandienone 3 (16α) (XI) 6β,12-Dihydroxy-metandienone (IX)
6.5–7.0 min	6β,16-Dihydroxy-metandienone 1 (16β-, 17-epi) (XII)
7.5–8.0 min	6β,16-Dihydroxy-metandienone 4 (16β) (X)
10.0–10.5 min	6β-Hydroxy-metandienone (VII)
11.0–11.5 min	6β-Hydroxy-17-epimetandienone (VIII)
16.0–16.7 min	17α-Methyl-androsta-1,4,6-trien-17β-ol-3-one
16.5–17.0 min	Metandienone (I)
18.0–18.5 min	17β-Methyl-androsta-1,4,6-trien-17α-ol-3-one
18.5–19.0 min	17-Epimetandienone (VI)
30.0–31.0 min	17,17-Dimethyl-18-norandrosta-1,4,13-trien-3-one

Conjugated excreted metabolites

17α-Methyl-5β-androst-1-en-17β-ol-3-one (II). This substance as the first intermediate in the metabolism of metandienone to the 17α-methyl-5β-androst-1-ene-3α,17β-diol was only detected after administration of 40 mg of metandienone in the conjugated fraction.

The EI-spectrum of the bis-TMS-derivative (Fig. 2) displays a molecular ion m/z 446, an ionic fragment m/z 431 resulting from a loss of methyl, m/z 143 (a D-ring fragment) and an ion m/z 194 which can be explained by scissions between carbons 9 and 10 and carbons 6 and 7.

To confirm the structure of this metabolite 17α-methyl-5β-androst-1-ene-17β-ol-3-one is synthesized. A selective hydrogenation of metandienone in methanol/potassium hydroxide with palladium on charcoal as catalyst was used (Fig. 3). 17α-Methyltestosterone reacts under these conditions to a mixture of 90% of 17α-methyl-5β-androstan-1-en-17β-ol-3-one and 10% of the 5α-isomer [13]. Metandienone is at last converted to the same substances. As the

Table 3. Kovats indices of metandienone, its metabolites and androgens

Substance	Capillary column: Temperature program:	SE 54 180/5/300	OV 1 180/4/300
17,17-Dimethyl-18-nor-5β-androsta-1,13-dien-3α-ol,TMS[a]		2278	2241
17,17-Dimethyl-18-norandrosta-1,4,13-trien-3-one,TMS[a]		2394	NE
17,17-Dimethyl-18-norandrosta-1,4,13-trien-3-one[a]		2428	NE
17β-Methyl-5β-androst-1-ene-3α,17α-diol,bis-TMS[a]		2477	2459
17β-Methyl-5β-androst-1-en-17α-ol-3-one,bis-TMS[a]		2488	2468
17α-Methyl-5β-androst-1-en-17β-ol-3-one,bis-TMS[a]		2570	2554
17α-Methyl-5β-androst-1-ene-3α,17β-diol,bis-TMS[a]		2633	2604
17α-Methyl-5β-androstane-3α,17β-diol,bis-TMS[a]		2635	2613
17-Epimetandienone,bis-TMS[a]		2666	2632
17β-Methyl-androsta-1,4,6-trien-17α-ol-3-one,TMS[b]		2678	NE
17-Epimetandienone,TMS[a]		2687	2621
6β-Hydroxy-17-epimetandienone,bis-TMS[a]		2767	2734
6β-Hydroxy-17-epimetandienone,Tris-TMS[a]		2779	2755
Metandienone,bis-TMS		2768	2726
Metandienone,TMS		2807	2733
17α-Methyl-androsta-1,4,6-trien-17β-ol-3-one,TMS[b]		2808	NE
6β,16-Dihydroxy-metandienone 1 (16β-,17-epi),Tris-TMS[b]		2867	NE
6β-Hydroxy-metandienone,bis-TMS		2886	2840
6β-Hydroxy-metandienone,Tris-TMS		2910	2884
6β,16-Dihydroxy-metandienone 2 (16α-,17-epi),Tris-TMS[c]		2930	NE
6β,12-Dihydroxy-metandienone,Tris-TMS[b]		2993	NE
6β,16-Dihydroxy-metandienone 3 (16α),Tris-TMS[b]		3011	NE
6β,16-Dihydroxy-metandienone 4 (16β),Tris-TMS[b]		3067	NE
5α-Androstan-3α-ol-17-one,bis-TMS (androsterone)		2536	2520
5β-Androstan-3α-ol-17-one,bis-TMS (etiocholanolone)		2543	2530
Androst-5-en-3β-ol-17-one,bis-TMS (DHEA)		2614	2587
5α-Androstane-3,17-dione,bis-TMS		2638	2604
Androst-4-en-17α-ol-3-one,bis-TMS (epitestosterone)		2641	2611
Androst-4-ene-3,17-dione,bis-TMS		2673	2635
Androst-4-en-17β-ol-3-one,bis-TMS (testosterone)		2688	2653
5α-Androstane-3α,11β-diol-17-one,Tris-TMS		2681	2671
5β-Androstane-3α,11β-diol-17-one,Tris-TMS		2689	2681

[a]Synthesized substance.
[b]Obtained from an excretion study with metandienone.
[c]Obtained from an excretion study with epimetandienone [17].
NE = not estimated on OV-1 column.
GC-conditions and column parameters see Methods.

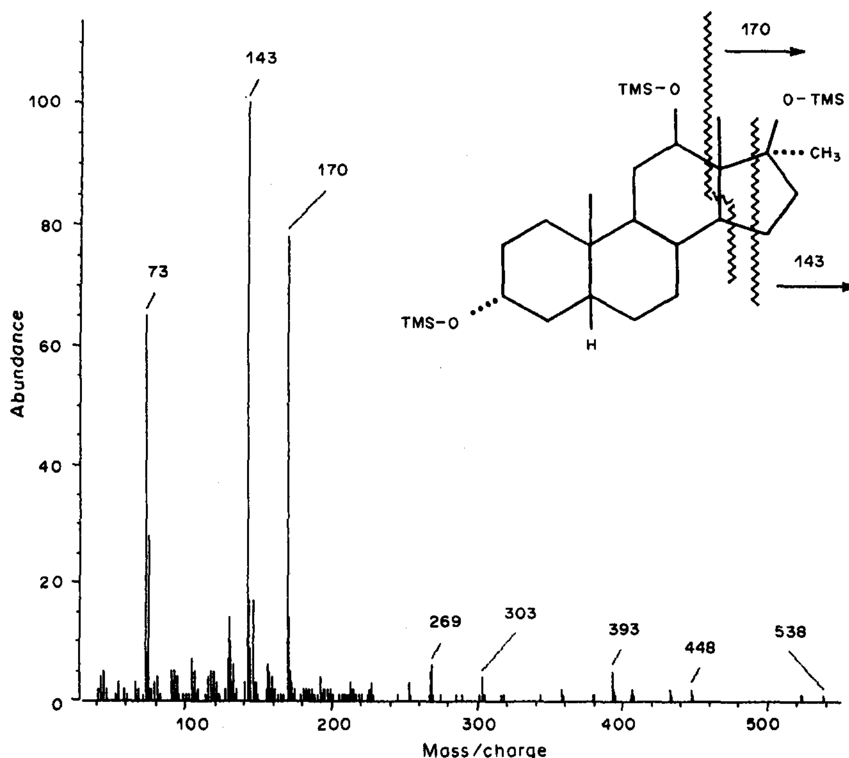

Fig. 14. EI-spectrum of 17α-methyl-5β-androstane-3α,12,17β-triol,Tris-TMS.

double bonds in the A-ring react with comparable velocity first 17α-methyltestosterone and II are formed in nearly the same amounts and both are subsequently reduced. When the hydrogenation is stopped after the use of 33% of the theoretical amount of hydrogen necessary to reduce both double-bonds 17α-methyl-5β-androst-1-en-17β-ol-3-one (II) is obtained in a yield of about 20%.

17α-Methyl-5β-androst-1-ene-3α,17β-diol (III). The EI-spectrum of the silylated metabolite III (Fig. 4) from HPLC-fraction 17.5–

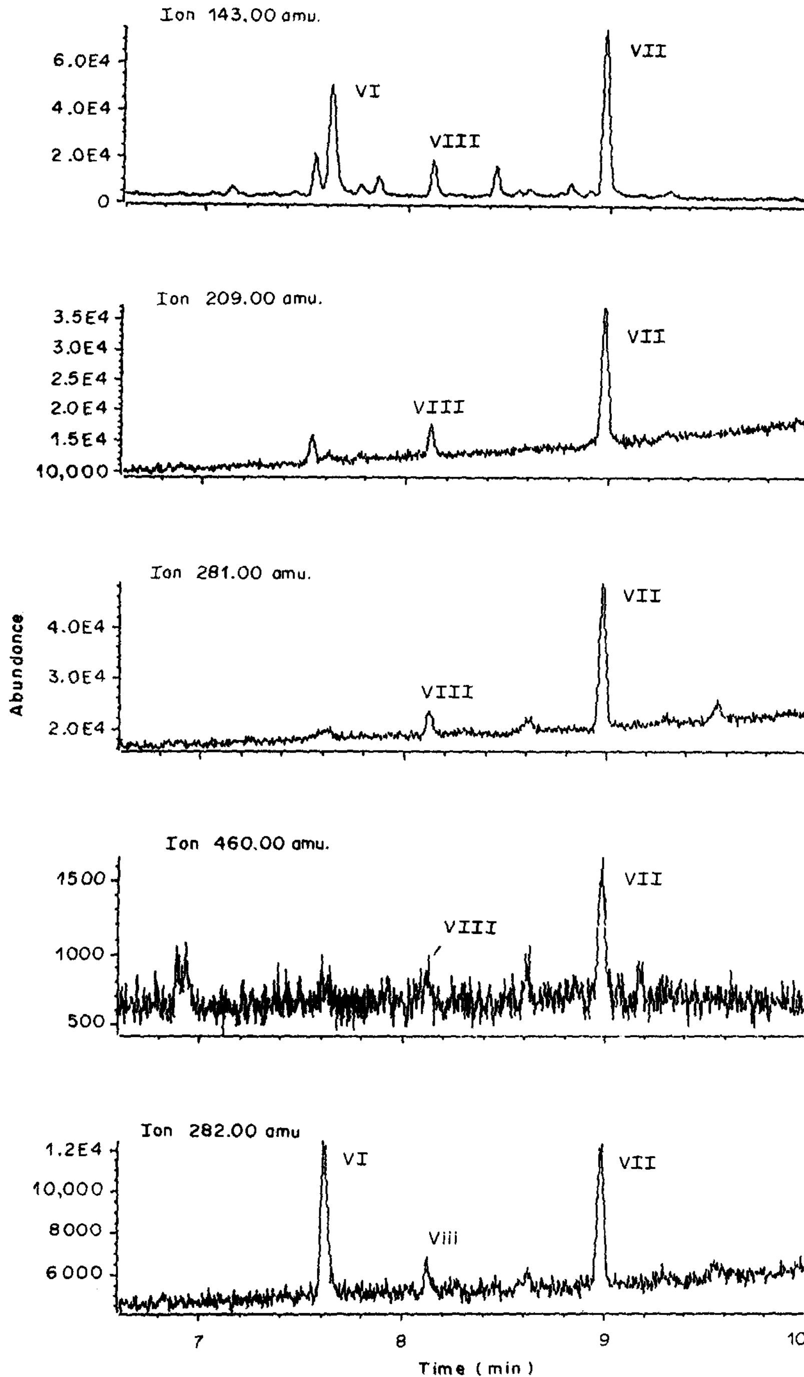

Fig. 15. SIM-chromatogram of the unconjugated urine fraction of a metandienone positive urine sample, GC 5890/MS 5970, column: 15 m fused silica capillary column 5% phenyl methyl silicone, i.d. 0.22 mm, film thickness 0.33 μm, carrier gas: helium 1.5 ml/min, split: 1:10, head pressure: 83 kPa, temperature program: initial temperature: 180°C, temperature rate: 10°C/min, final temperature: 320°C. Selected ions: *m/z* 143, 209, 281, 282, 460. VI 17-Epimetandienone,TMS. VII 6β-Hydroxy-metandienone,bis-TMS. VIII 6β-Hydroxy-17-epimetandienone,bis-TMS.

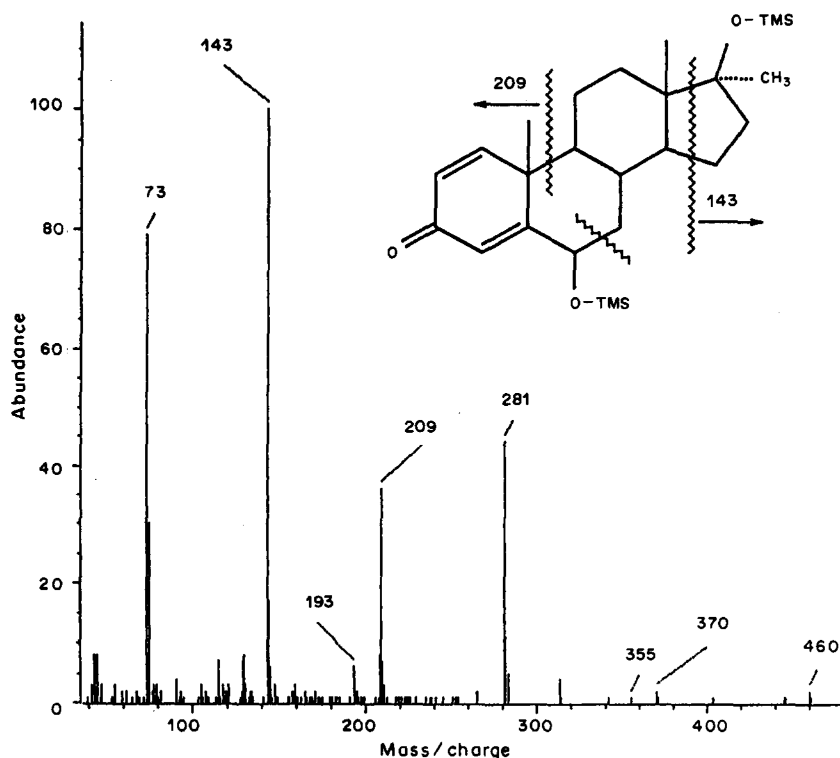

Fig. 16. EI-spectrum of 6β-hydroxy-metandienone,bis-TMS (VII).

18.0 min shows a molecular ion m/z 448 and a base peak m/z 143. The reduction of this metabolite with hydrogen in methanol using platinum dioxide as catalyst yields only one product which is identical with 17α-methyl-5β-androstane-3α,17β-diol (V). As the hydrogenation of androst-4-enes under the same conditions leads to 2 isomers with 5α- and

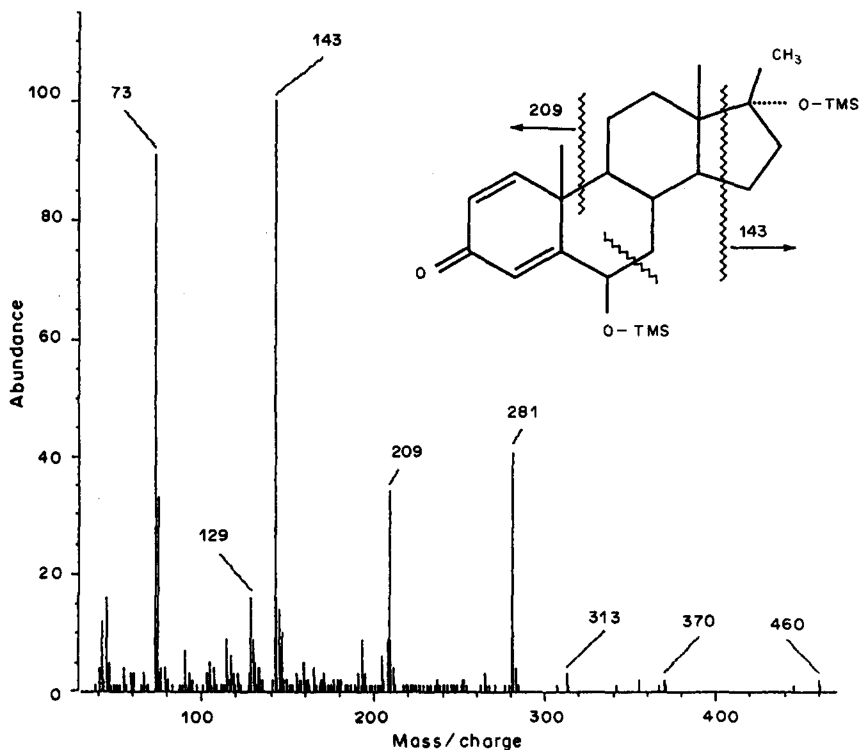

Fig. 17. EI-spectrum of 6β-hydroxy-17-epimetandienone,bis-TMS (VIII).

5β-configuration [13] it is concluded that the double bond of the metabolite III is located between carbon 1 and 2.

To confirm the structure of this metabolite the 17α-methyl-5β-androst-1-en-17β-ol-3-one was reduced with lithium aluminium hydride to the 3α- and the 3β-hydroxy-isomer in a ratio of about 85:15. The subsequent reduction of the 3α-hydroxy-isomer with hydrogen using platinum dioxide as catalyst yields 17α-methyl-5β-androstane-3α,17β-diol (V).

17β-Methyl-5β-androst-1-ene-3α,17α-diol (IV). This epimeric metabolite is detected in the conjugated fraction as bis-TMS-derivative with a much shorter retention time (Kovats index 2477) than 17α-methyl-5β-androst-1-ene-3α,17β-diol,bis-TMS (Kovats index 2633). Compared to endogenous excreted androgens it is eluted from a SE-54 or OV-1 capillary column before androsterone,bis-TMS (Table 3, Kovats index 2536). This elution pattern is characteristic for trimethylsilylated 17-methyl-17-hydroxy-isomers: the 17β-methyl-17α-TMS ether is eluted much earlier than the 17α-methyl-17β-TMS ether (Table 3). The EI-spectrum of the bis-TMS-derivative of this metabolite (Fig. 5) is identical with the EI-spectrum of 17α-methyl-5β-androst-1-ene-3α,17β-diol,bis-TMS (Fig. 4).

The hydrogenation of this metabolite (IV) obtained from the HPLC-fraction 22.5–23.0 min with platinum dioxide as catalyst in methanol leads to 17β-methyl-5β-androstane-3α,17α-diol (XIII) whereas the same reaction of the reference substance III yields 17α-methyl-5β-androstane-3α,17β-diol (V), the 17-epimer (Fig. 6).

The treatment of V and XIII with trifluoroacetic acid/acetonitrile leads under rearrangement of the D-ring [15] to the same product: 18-nor-17,17-dimethyl-5β-androst-13-en-3α-ol (XIV). The EI-spectrum of XIV as TMS-derivative is presented in Fig. 7.

The 17β-methyl-5β-androst-1-ene-3α,17α-diol (IV) was synthesized via two different reaction pathways in μg amount without further purification for GC/MS-analysis (Fig. 6). In both reactions the 17-epimer is obtained via a 17-sulfate which spontaneously epimerized in water. The inversion of a 17α-methyl-17β-sulfate to a 17β-methyl-17α-hydroxy-configuration when treated with water was published in 1989 by Edlund *et al.* for metandienone [16]. Starting with III the 3α-hydroxy-group is protected by an acetyl-group which after inversion is hydrolyzed. Starting with II the 17-epimer can be obtained after inversion and reduction with lithium aluminium hydride. The last reaction yields a mixture of 3α-hydroxy- and 3β-hydroxy-isomers in a ratio of 10:1.

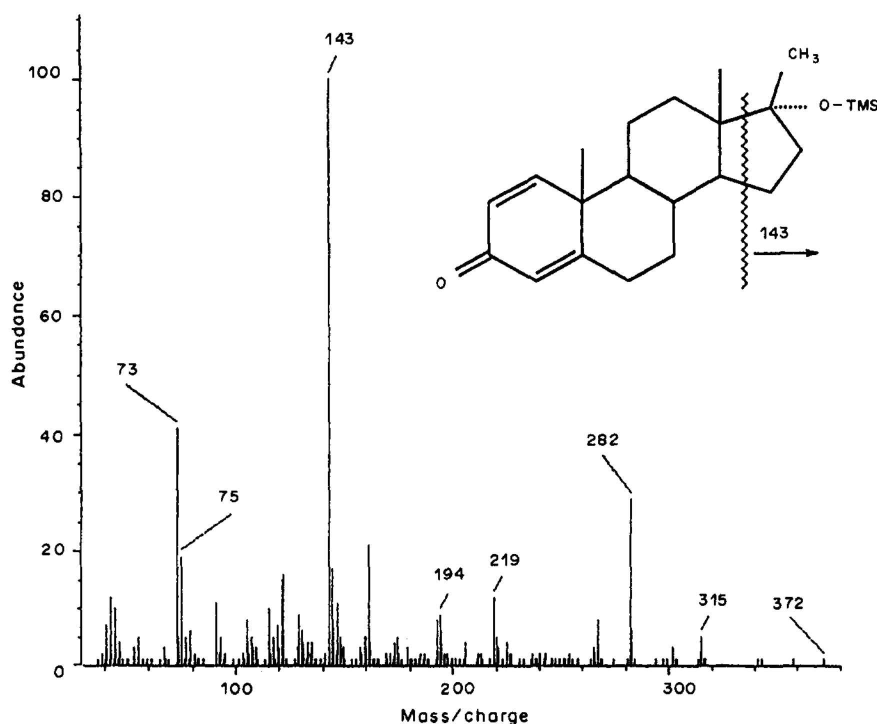

Fig. 18. EI-spectrum of 17-epimetandienone, TMS (VI).

17α-Methyl-5β-androstane-3α,17β-diol (V). The isolated metabolite from the HPLC-fraction 18.0–18.5 min is identified as 17α-methyl-5β-androstane-3α,17β-diol (V). The EI-spectrum and the retention time of the bis-TMS-derivative (Fig. 8) was identical with the EI-spectrum and the retention time of the synthesized substance.

Unconjugated excreted metabolites

In the unconjugated fraction 6β-hydroxymetandienone, 6β-hydroxy-17-epimetandienone and 17-eipmetandienone are detected and quantified by GC/MS with selected ion monitoring (SIM).

Further four bis-hydroxy-metabolites were detected in the unconjugated fraction after administration of 40 mg of I. The EI-spectra of all four Tris-TMS-metabolites display an ion m/z 209 indicating that they all have a 6-hydroxy-group. The fragments m/z 218 and 231 registrated in three of the four Tris-TMS-metabolites are characteristic for a 16-hydroxy-group (Figs 9–11).

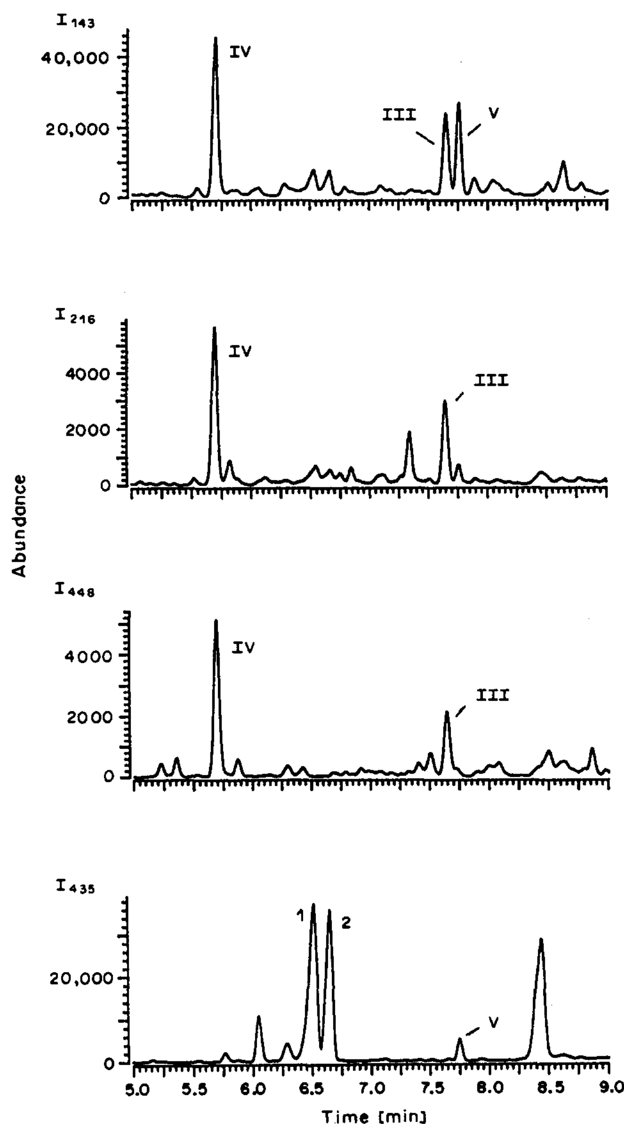

Fig. 19. SIM-chromatogram of the conjugated urine fraction of a metandienone positive urine sample, GC/MS 5996B: column: 17 m fused silica capillary column crosslinked methyl silicone, film thickness: 0.11 μm, i.d. 0.2 mm, carrier gas: hydrogen 2 ml/min, split 1:10, head pressure: 70 kPa, temperature program: initial temp. 180°C, temp. rate: 4°C/min, final temp. 250°C. Selected ions m/z 143, 216, 435, 448. III 17α-methyl-5β-androst-1-ene-3α,17β-diol,bis-TMS. IV 17β-methyl-5β-androst-1-ene-3α,17α-diol,bis-TMS. V 17α-methyl-5β-androstane-3α,17β-diol,bis-TMS. 1. Androsterone,bis-TMS. 2. Etiocholanolone, bis-TMS.

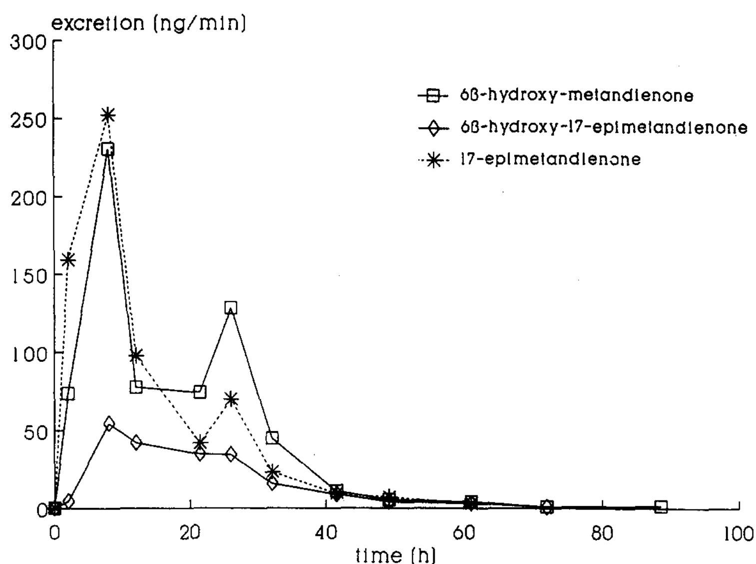

Fig. 20. Excretion curve of 6β-hydroxy-metandienone (VII), 6β-hydroxy-17-epimetandienone (VIII) and 17-epimetandienone (VI) after oral application of 12 mg of metandienone.

The fourth possible 6,16-bis-hydroxy-metandienone isomer was obtained after administration of 17-epimetandienone [17].

No 16-mono-hydroxy-metandienone was found either in the conjugated or in the unconjugated fraction.

The EI-spectrum of the fourth bis-hydroxy-metabolite (Fig. 12) shows the ions m/z 143 and 170: These fragments are assigned to 17-methyl-androstane-12,17-diols. To elucidate this assumption 17α-methyl-5β-androstane-3α,12,17β-triol was synthesized in μg amount as reference compound (Fig. 13).

The introduction of a 12-keto-group into 11α-hydroxy-17α-methyl-testosterone and removal of the 11-hydroxy-group was performed according to the published synthesis of 3β-acetoxy-11α-deutero-5α-androstan-12-one by Shapiro *et al.* [18].

The last reaction step with lithium aluminium hydride yielded in over 80% the 3α-hydroxy-compound and 20% of the 3β-isomer. The configuration of the 3-hydroxy-group is suggested in comparison to the reduction of 17α-methyl-5β-androstan-17β-ol-3-one with lithium aluminium hydride which yielded 85%

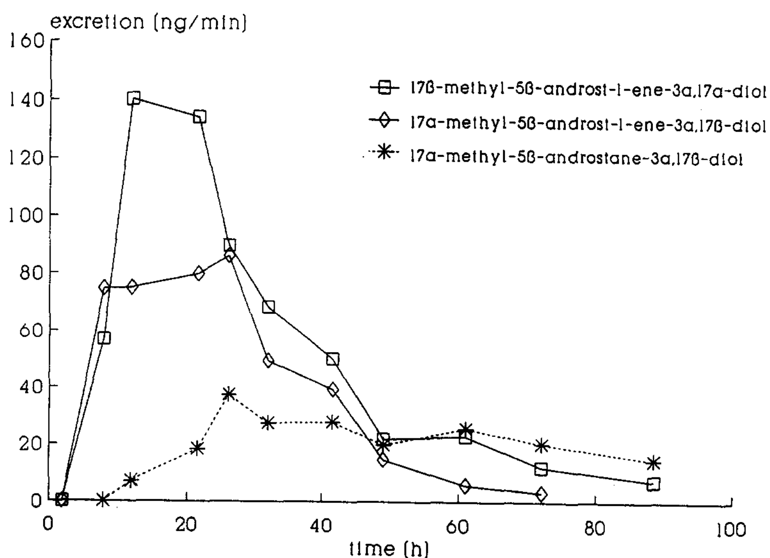

Fig. 21. Excretion curve of 17α-methyl-5β-androst-1-ene-3α,17β-diol (III), 17β-methyl-5β-androst-1-ene-3α,17α-diol (IV) and 17α-methyl-5β-androstane-3α,17β-diol (V) after oral application of 12 mg of metandienone.

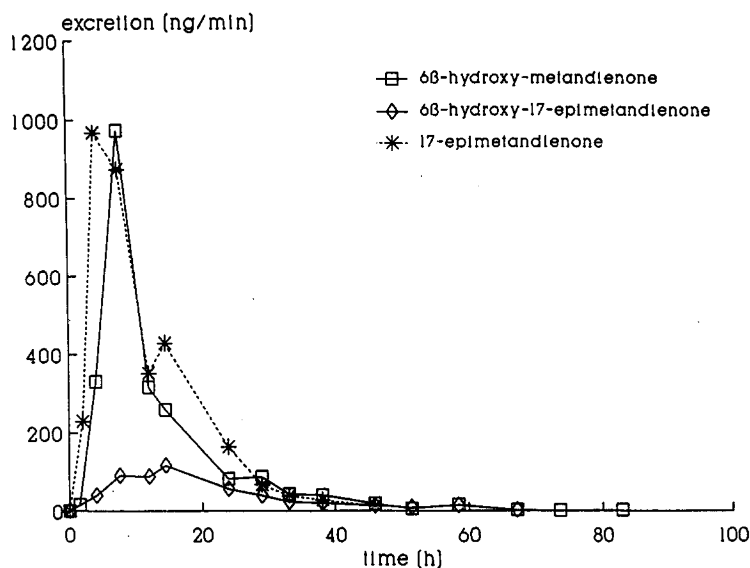

Fig. 22. Excretion curve of 6β-hydroxy-metandienone (VII), 6β-hydroxy-17-epimetandienone (VIII) and 17-epimetandienone (VI) after oral application of 24 mg of metandienone.

of the 3α-isomer and 15% of the 5β-isomer [13]. The 12-keto-group is reduced with over 95% to one 12-hydroxy-isomer (expected to be 12β-hydroxy-isomer). The EI-spectrum of the Tris-TMS-derivative is shown in Fig. 14 and dominated by the ions m/z 143 and 170.

The fragment m/z 170 is still difficult to explain. The following results [13] can be used for further discussions:

(1) Introduction of a deuterium in position 12 of 17α-methyl-5β-androstane-3,12,-17β-triol (by reduction of the 12-ketone with $LiAlO_4$) did not change the fragment m/z 170.

(2) In 20,20,20-trideutero-6β,12-dihydroxy-metandienone (obtained from an excretion study with 20,20,20-trideutero-I) the fragment m/z 170 is shifted to m/z 173 [17].

(3) In 16,16-dideuterated IX (obtained from an excretion study with 16,16-dideutero-I) the fragment m/z 170 is shifted to 171 (30%) and 172 (70%).

Obviously the fragment m/z 170 contains a TMS-oxy-group and 6 carbons. Three of them

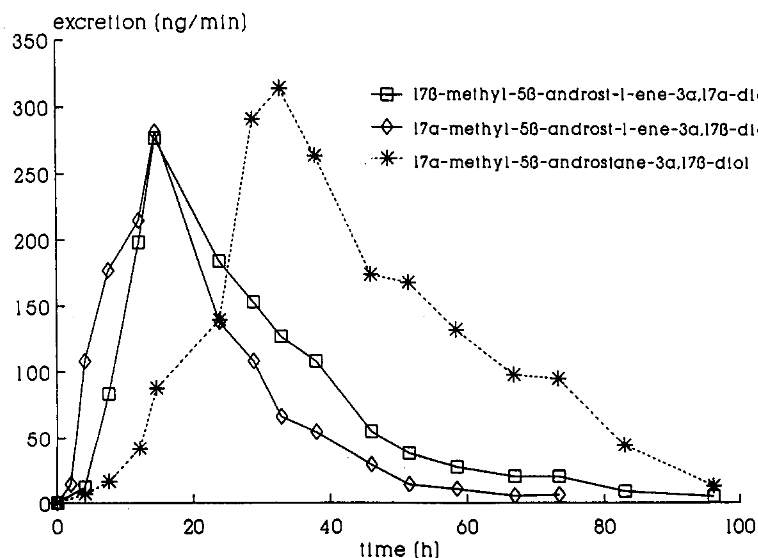

Fig. 23. Excretion curve of 17α-methyl-5β-androst-1-ene-3α,17β-diol (III), 17β-methyl-5β-androst-1-ene-3α,17α-diol (IV) and 17α-methyl-5β-androstane-3α,17β-diol (V) after oral application of 24 mg of metandienone.

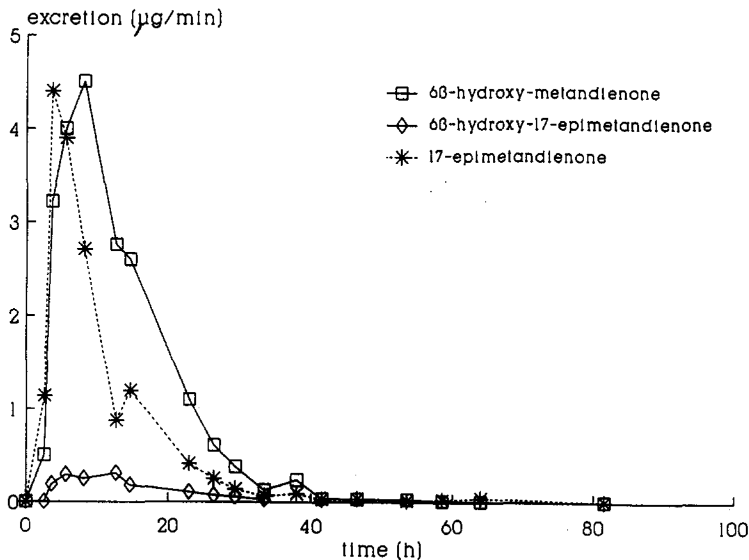

Fig. 24. Excretion curve of 6β-hydroxy-metandienone (VII), 6β-hydroxy-17-epimetandienone (VIII) and 17-epimetandienone (VI) after oral application of 40 mg of metandienone.

are the carbons 16, 17 and 20 and carbon 12 is not included.

GC/MS-screening

Figure 15 shows the GC/MS-screening of a positive urine for metandienone metabolites in the unconjugated fraction. The metabolites are detected as TMS-derivatives with SIM of the most characteristic ions: 6β-Hydroxy-metandienone,bis-TMS (VII, Fig. 16) the 6β-hydroxy-17-epimetandienone,bis-TMS (VIII, Fig. 17) with a molecular ion m/z 460 and the fragment ions 143, 209 and 281 and 17-epimetandienone, TMS (VI, Fig. 18) with m/z 143 and 282. The results of the corresponding conjugated fraction are displayed in Fig. 19: 17α-methyl-5β-androst-1-ene-3α,17β-diol (III) and 17β-methyl-5β-androst-1-ene-3α,17α-diol (IV) are registered as their bis-TMS-derivatives with abundant fragment ions m/z 143, 216 and 448 and 17α-methyl-5β-androstane-3α,17β-diol (V) with m/z 143 and 435.

The screening of the unconjugated fraction is performed on a capillary column SE-54 whereas the conjugated fraction is measured on a capillary column OV-1. An OV-1 column is used to

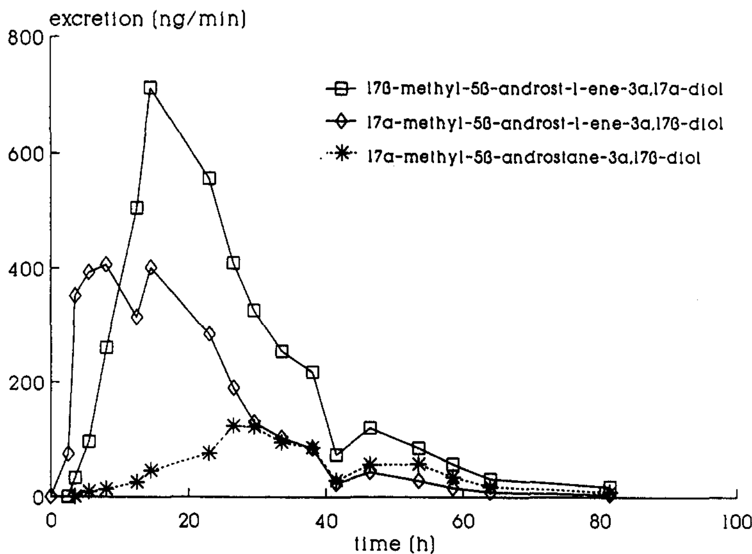

Fig. 25. Excretion curve of 17α-methyl-5β-androst-1-ene-3α,17β-diol (III), 17β-methyl-5β-androst-1-ene-3α,17α-diol (IV) and 17α-methyl-5β-androstane-3α,17β-diol (V) after oral application of 40 mg of metandienone.

separate the TMS ethers of 17α-methyl-5β-androst-1-ene-3α,17β-diol (III) and 17α-methyl-5β-androstane-3α,17β-diol (V) which are coeluted on a SE-54 column (Table 3).

Excretion studies

(A). The excretion of the unconjugated and conjugated metabolites after oral administration of 12, 24 and 40 mg of metandienone are presented in Figs 20–25. In all three excretion studies the unconjugated metabolites show the same profile with a maximum excretion rate between 3 and 6 h. The conjugated metabolites are excreted later with excretion maxima between 5.3–26 h for III, 8–14.5 h for IV and 21.5–33 h for V. The total amounts of the excreted metabolites are listed in Table 4.

(B). After oral application of 10 mg of III about 0.85 mg of III and 0.32 mg of V are excreted into urine within 53 h (Table 5).

Both are conjugated and could be hydrolyzed with β-glucuronidase from *E. coli.* No 17-epimer of III was detected in this experiment.

DISCUSSION

Whereas the 6β-hydroxylation of metandienone as well as the 17-epimerization of metandienone and 6β-hydroxy-metandienone lead to unconjugated excreted metabolites the metabolic hydrogenation of metandienone leads to metabolites excreted as conjugates. The reduction of the double bonds and the keto-group in the A-ring followed by conjugation with glucuronic acid is compared to unconjugated excreted metabolites not a minor metabolic pathway. About 4–6% of the applied drug are excreted as reduced metabolites independent of the amount of metandienone administered.

In contrast to this observation the amount of unconjugated excreted metabolites such as 6β-hydroxy-metandienone and 17-epimetandienone seems to be dependent on the amount of metandienone applied. About 4% of the administered drug are detected as unconjugated excreted metabolites after oral application of 12 mg of metandienone whereas the administration of 24 and 40 mg increases the excreted amount to 5.4 and 14.3%. In the latter case also unconjugated excreted bis-hydroxy-metabolites are obtained.

Dürbeck *et al.* observed such bis-hydroxy-metabolites in the unconjugated urine fraction of an excretion study with single doses of 25 mg of chlorodehydromethyltestosterone [19] but not in a study with single doses of 10 mg of metandienone [7]. They found a compound assumed to be 6β,12-dihydroxy-chlorodehydromethyltestosterone and a 6β,16-dihydroxy-metabolite. They searched for but could not detect the 16β-mono-hydroxy-metabolite previously described by Schubert *et al.* [20] after administration of high doses of chlorodehydromethyltestosterone.

Five bis-hydroxy-metabolites of stanozolol have been detected by Schänzer *et al.* [14] in the conjugated fraction. This anabolic steroid with a condensed pyrazole nucleus should be considered as exceptional because also the parent

Table 4. Total and relative amount of urinary conjugated and unconjugated metabolites after oral application of 12, 24 and 40 mg of metandienone. Collection period 72 h

	Conjugated fraction					
Oral administration of	17α-Methyl-5β-androstane-3α,17β-diol (V) (μg)	(%)	17α-Methyl-5β-androst-1-ene-3α,17β-diol (III) (μg)	(%)	17β-Methyl-5β-androst-1-ene-3α,17α-diol (IV) (μg)	(%)
12 mg	94	0.78	163	1.35	243	2.02
24 mg	704	2.93	327	1.37	404	1.70
40 mg	210	0.53	570	1.42	940	2.34
	Unconjugated fraction					
Oral administration of	17-Epi-metandienone (VI) (μg)	(%)	6β-Hydroxymetandienone (VII) (μg)	(%)	6β-Hydroxy-17-epimetandienone (VIII) (μg)	(%)
12 mg	195	1.63	214	1.78	74	0.66
24 mg	662	2.76	489	2.04	138	0.58
40 mg	2080	5.20	3400	8.50	260	0.65

Quantification by peak heights of selected ion profiles: conjugated fraction with m/z 448 for bis-TMS-III and bis-TMS-IV and with m/z 435 for bis-TMS-V and the internal standard (200 ng/ml) bis-TMS-17α-methyl-5α-androstane-3β,17β-diol; unconjugated fraction with m/z 143 for TMS-VI, bis-TMS-VII, and bis-TMS-VIII and 17α-methyl-5α-androstane-3β,17β-diol as internal standard with 1 μg/ml. The response factors for V, III, VI and VII were estimated by using reference substances whereas for IV and VIII the response factors of their 17-epimers were used. Sample preparation was performed as described by Donike *et al.* [12]. The unconjugated and conjugated fractions were separated. Derivatization of the unconjugated and conjugated fraction see Method (a).

Table 5. Urinary excretion of 17α-methyl-5β-androst-1-ene-3α,17β-diol (III) and 17α-methyl-5β-androstane-3α,17β-diol (V) after oral administration of 10 mg of 17α-methyl-5β-androst-1-ene-3α,17β-diol (III)

Period after administration	17α-Methyl-5β-androst-1-ene-3α,17β-diol (III) (ng/min)	17α-Methyl-5β-androstane-3α,17β-diol (V) (ng/min)
0–3.5	956.0	51.4
3.5–6.0	858.0	55.6
6.0–9.0	702.0	50.8
9.0–12.5	575.0	83.3
12.5–14.0	411.0	103.0
14.0–17.0	355.0	126.4
17.0–21.5	246.0	157.0
21.5–27.0	153.0	203.0
27.0–32.0	28.9	59.8
32.0–35.5	44.2	108.2
35.5–45.0	22.0	68.5
45.0–49.0	36.2	137.7
49.0–53.0	14.4	73.7
Total amount	850 μg	320 μg

Quantification by peak heights of selected ion profiles of the bis-TMS-derivatives: m/z 448 for III, m/z 435 for V and 17α-methyl-5α-androstane-3β,17β-diol as internal standard with 200 ng/ml. The response factors of III and V were estimated by the use of synthesized reference substances. Samples were prepared as described by Donike et al. [12]. Derivatization see Method (a).

compound as well as four mono-hydroxy-metabolites including 16α- and 16β-hydroxy-stanozolol are excreted as conjugates.

The structural assignment of the bis-hydroxy-metabolites of metandienone is based on the EI-spectra and on the methylene units of their TMS-derivatives. All four have the 6-hydroxy-group indicated by the fragments m/z 209 and 281, one shows the ion m/z 170 which is interpreted as a fragment of 6β,12-bis-hydroxy-metandienone, Tris-TMS. Three have a 16-hydroxy-group as indicated by the ions m/z 218 and 231. Four isomers of 16,17-dihydroxy-androstanes can exist and the fourth was found in an excretion study with 17-epimetandienone [17].

The final assignment was performed by comparison of the Kovats indices of the four 6β,16,17-trihydroxy-androsta-1,4-dien-3-ones, Tris-TMS (Fig. 26A) with them of the four synthesized 17-methyl-16,17-dihydroxy-5α-androstan-3-ols (Fig. 26B) [13] and of the four synthesized 17-methyl-16,17-dihydroxy-androst-2-eno(3,2-c)-pyrazoles,Tris-TMS (Fig. 26C) [14]. The elution pattern of the per-trimethylsilylated isomers is similar: 16β,17α-dihydroxy- < 16α,17α-dihydroxy- < 16α,17β-dihydroxy- < 16β,17β-dihydroxy-.

Apparently 6β-hydroxy-metandienone is hydroxylated in positions 16α and 16β but only the 16β-hydroxy-isomer of 6β-hydroxy-17-epimetandienone was detected in this study.

The identification of one bis-hydroxy-metabolite as a 12-hydroxylated metabolite of 6β-hydroxy-metandienone is based on its EI-spectrum of the persilylated compound with a relative intense fragment ion m/z 170 besides the D-ring fragment m/z 143. The fragment m/z 170 is elucidated by the synthesized reference substance 17α-methyl-5β-androstane-3α,12,17β-triol which also shows an abundant ion m/z 170. This fragment m/z 170 is also observed in a bis-hydroxylated metabolite of 4-chloro-dehydromethyltestosterone [13] and also in one bis-hydroxylated metabolite of stanozolol [14].

The mechanism of the 17-epimerization in man is up to now not elucidated but recently Edlund et al. [16] reported that in horse metandienone is excreted as 17-sulfate into the urine where it is spontaneously hydrolyzed to 17-epimetandienone and several dehydration products, e.g. 17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one already described as an artifact [7].

Furthermore 17-epimetandienone was not detected in blood [13] and the authors suggest that the epimerization of 17-methyl-steroids in man follows a similar mechanism: first a 17-conjugation and then urinary inversion. The nature of the conjugate in man is not yet established but it is assumed that it can be further metabolized. In agreement with this suggestion is the detection of a 17-epimer within the bis-hydroxy-metabolites (6β,16β-dihydroxy-17-epimetandienone) and within the reduced metabolites (17β-methyl-5β-androst-1-ene-3α,17α-diol).

The reduced metandienone metabolites all have a 5β-configuration and no 5α-androstane metabolite is detected. This is different to the metabolism of methyltestosterone [9–11] where beside of the main metabolite 17α-methyl-5β-androstane-3α,17β-diol also the 5α-isomer is obtained.

The first step in the reduction of the metandienone is the hydrogenation of the double bond between carbon 4 and 5 by a 5β-reductase to II. The following reduction of the 3-keto-group by a 3α-dehydrogenase is very quick so that II is excreted in low amount and is detected only within the first 6 h after application of a high dose (40 mg) of metandienone. The conjugation with glucuronic acid is only possible as an enol-conjugate.

In relatively high and comparable amount the 17α-methyl-5β-androst-1-ene-3α,17β-diol (III) and its 17-epimer (IV) are found both excreted as glucuronide. Two pathways (Fig. 1) are to be taken in account for the genesis of the 17-epimer: First the 17-conjugation of 17α-methyl-5β-androst-1-ene-3α,17β-diol (III) and second

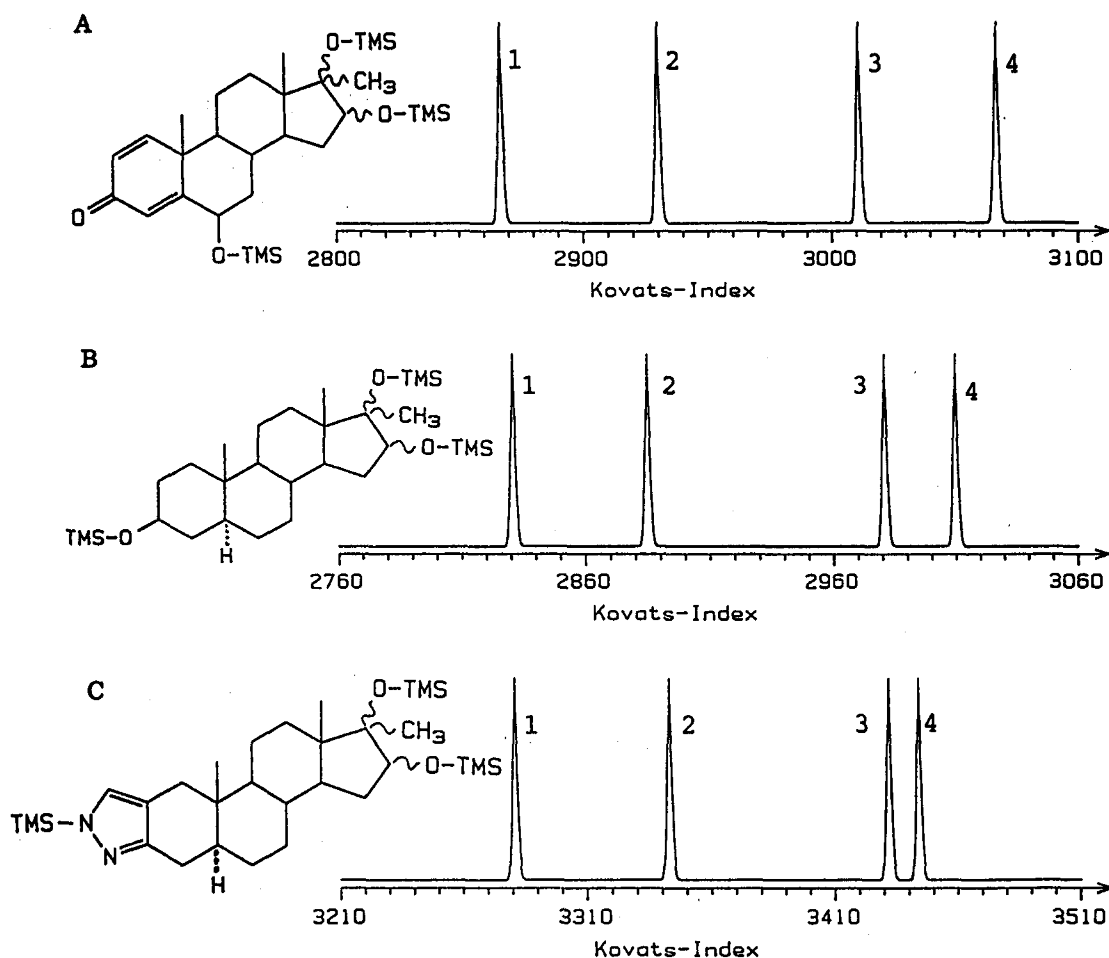

Fig. 26. Reconstruction of methylene units (SE 54) of 17-methyl-16,17-dihydroxy-,bis-TMS-isomers of (A) 6β-hydroxy-metandienone-metabolites isolated from urine, (B) synthesized 17-methyl-5α-androstane-3β,16,17-triol, (C) synthesized 16-hydroxy-stanozolol.

Methylene units of	A	B	C
1 16β,17α-dihydroxy	2867	2831	3281
2 16α,17α-dihydroxy	2930	2885	3343
3 16α,17β-dihydroxy	3011	2981	3432
4 16β,17β-dihydroxy	3067	3010	3444

the reduction of a metandienone-17-conjugate. In both cases the epimer (IV) is obtained after excretion and urinary inversion.

The oral administration of 10 mg of III to a male volunteer results in the excretion of the administered substance and the reduced metabolite V (Table 5). No 17-epimer of the administered substance is detected. This result shows very clear that the second metabolic pathway via reduction of the metandienone-17-conjugate occurs.

The last step in the reduction of metandienone is the hydrogenation of the double bond between carbon 1 and 2. This reduction occurs with 17α-methyl-5β-androst-1-ene-3α,17β-diol whereas the double bond of its 17-conjugate is not hydrogenated in a detectable amount.

The time-course of the appearance of the reduced metabolites with excretion maxima between 5.3 and 26 h for 17α-methyl-5β-androst-1-ene-3α,17β-diol, between 8 and 14.5 h for 17α-methyl-5β-androst-1-ene-3α,17β-diol and between 21.5 and 33 h for 17α-methyl-5β-androstane-3α,-17β-diol shows that the enzymatic reduction of metandienone and its 17-conjugate are much slower than the 6β-hydroxylation and 17-conjugation or that it takes much more time for metandienone and its 17-conjugate to enter and to leave those compartments where the reduction takes place.

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REFERENCES

1. Vischer E., Meystre Ch. and Wettstein A.: Herstellung weiterer 1-Dehydro-steroide auf mikrobiologischem Wege. *Helv. Chim. Acta* **38** (1955) 1502–1505.
2. Meystre Ch., Frey H., Voser W. and Wettstein A.: Gewinnung von 1,4-Bisdehydro-3-oxo-steroiden. *Helv. Chim. Acta* **39** (1956) 734–742.
3. Rongone E. and Segaloff A.: *In vivo* metabolism of Δ^1,17α-methyltestosterone in man. *Steroids* **1** (1963) 179–184.
4. Macdonald B. S., Sykes P. J., Adhikary P. M. and Harkness R. A.: The identification of 17α-hydroxy-17-methyl-1,4-androstadien-3-one as a metabolite of the anabolic steroid drug 17α-hydroxy-17-methyl-1,4-androstadien-3-one in man. *Steroids* **18** (1971) 753–766.
5. Macdonald B. S., Sykes P. J., Adhikary P. M. and Harkness R. A.: The identification of 17α-hydroxy-17β-methyl-androsta-1,4-dien-3-one as a metabolite of 17β-hydroxy-17α-methylandrosta-1,4-dien-3-one in man. *Biochem. J.* **122** (1971) 26P.
6. Dürbeck H. W., Büker I., Scheulen B. and Telin B.: Gas chromatographic and capillary column gas chromatographic-mass spectrometric determination of synthetic anabolic steroids. *J. Chromat.* **167** (1978) 117–124.
7. Dürbeck H. W. and Büker I.: Studies on anabolic steroids. The mass spectra of 17α-methyl-17β-hydroxy-1,4-androstadien-3-one (Dianabol) and its metabolites. *Biomed. Mass Spectrosc.* **10** (1980) 437–445.
8. Steele J. W. and Schlesinger U. P.: Anabolic steroids. Part 1—The identification of some methandrostanolone metabolites in the rat. *Can. J. Pharm. Sci.* **14**(2) (1979) 50–52.
9. Quincey R. V. and Gray C. H.: The metabolism of (1,2-(3)H)17α-methyltestosterone in human objects. *J. Endocr.* **37** (1967) 37–55.
10. Segaloff A., Gabbard B., Carriere B. and Rongone E.: The metabolism of 4-(14)C-17α-methyltestosterone. *Steroids* **1** (1965) 149–158.
11. Rongone E. and Segaloff A.: Isolation of urinary metabolites of 17α-methyltestosterone. *J. Biol. Chem.* **237** (1962) 1066–1067.
12. Donike M., Zimmerman J., Bärwald K.-R., Schänzer W., Christ V., Klostermann K. and Opfermann G.: Routinebe-stimmung von Anabolika im Harn. *D. Z. Sportmed.* **1** (1984) 14–23.
13. Schänzer W.: Unpublished results (1988–90).
14. Schänzer W., Opfermann G. and Donike M.: Metabolism of stanozolol: identification and synthesis of urinary metabolites. *J. Steroid Biochem.* **36** (1990) 153–174.
15. Segaloff A. and Gabbard R. B.: Anti-androgenic activity of 17,17-dimethyl-18-norandrost-13-enes, *Steroids* **4** (1964) 433–443.
16. Edlund P. O., Bowers L. and Henion J.: Determination of methandrostanolone and its metabolites in equine plasma and urine by coupled-column liquid chromatography with ultraviolet detection and confirmation by tandem mass spectrometry. *J. Chromat.* **487** (1989) 341–356.
17. Cavallo J. R. and Frischhorn C. G. B.: Metandienone metabolism. Manuscript in preparation.
18. Shapiro R. H., Williams D. W., Budzikiewicz H. and Djerassi C.: Mass spectrometry in structural and stereochemical problems. LIII. Fragmentation and hydrogen transfer reactions of a typical 3-keto-steroid, 5α-androstane-3-one. *J. Am. Chem. Soc.* **86** (1964) 2837–2845.
19. Dürbeck H. W., Büker I., Scheulen B. and Telin B.: GC and capillary column GC/MS determination of synthetic anabolic steroids. II. 4-Chloro-metan-dienone (Oral Turinabol) and its metabolites. *J. Chromat. Sci.* **21** (1983) 405–410.
20. Schubert K. and Wehrberger K.: Stoffwechsel von Steroidpharmaka. II. Isolierung und Charakterisierung der Metaboliten von 4-Chlor-17α-methyl-17β-hydroxy-1,4-androstadien-3-on. *Endokrinologie* **55** (1970) 257–269.