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METABOLISM OF STANOZOLOL: IDENTIFICATION AND SYNTHESIS OF URINARY METABOLITES

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Summary—Urinary metabolites of stanozolol (17α-methyl-17β-hydroxy-5α-androst-2-eno(3,2-c)-pyrazole) following oral administration were isolated by chromatography on XAD-2 and by preparative high-performance liquid chromatography (HPLC) and identified by gas chromatography–mass spectrometry (GC/MS) with electron impact (EI)-ionisation.

Stanozolol is excreted as a conjugate but is metabolized to a large extent. All identified metabolites are hydroxylated, namely at C-3′ of the pyrazole ring and at C-4β, C-16α and C-16β of the steroid.

Less than 5% of the metabolites are found in the unconjugated urine fraction: 3′-hydroxy-stanozolol (**II**) and 3′-hydroxy-17-epistanozolol (**III**).

Conjugated excreted metabolites are 3′-hydroxystanozolol (**II**), stanozolol (**I**), 4β-hydroxy-stanozolol (**IV**), 16β-hydroxystanozolol (**V**), 16α-hydroxystanozolol (**VI**), two isomers of 3′,16-dihydroxystanozolol (**VII, VIII**), two isomers of 4β,16-dihydroxystanozolol (**IX, X**) and a 3′,?-dihydroxystanozolol (**XI**).

3′-Hydroxystanozolol, 4α-hydroxystanozolol, 4β-hydroxystanozolol, 16α-hydroxy-, 16α-hydroxy-17-epi- and 16β-hydroxystanozolol were synthesised to confirm the structural assignment of the main metabolites.

INTRODUCTION

Stanozolol (Fig. 1) was first synthesised by Clinton *et al.* in 1959 [1, 2]. The condensation of 2-hydroxy-methylene-17α-methyl-17β-hydroxy-5α-androstan-3-one with hydrazine hydrate leads to stanozolol, a steroid with a condensed pyrazole ring.

Several authors [3–9] describe the anabolic effects of stanozolol. Clinically it is used in cases of osteoporosis and a deficiency in protein synthesis.

Stanozolol has been marketed for more than twenty years. In addition to indicated medical administration stanozolol, like other anabolic steroids is often abused in sports to enhance performance. National as well as international sport federations forbid the use of anabolic steroids including stanozolol.

All published articles about the detection of stanozolol are concerned with stanozolol itself and do not mention any metabolite [10–12].

This paper describes the detection and identification of hydroxylated stanozolol metabolites (Fig. 1) after oral application of stanozolol.

EXPERIMENTAL

Metabolism studies

Stanozolol was administered to male volunteers orally in doses of 20–100 mg. The main investigations were done with a male person (75 kg, 34 yr), who took 100 mg of stanozolol orally. The result from these studies were confirmed by further excretion studies: male person (40 mg orally, 75 kg, 35 yr), male person (40 mg orally, 74 kg, 37 yr), male person (20 mg orally, 66 kg, 32 yr). The collected urine samples were stored at 4°C.

Isolation of steroids for GC/MS screening

To isolate the unconjugated excreted steroids 0.5 g of solid buffer (sodium bicarbonate/potassium carbonate 2:1 pH 9.6) are added to 5 ml of urine and the mixture is extracted with 5 ml of diethyl ether (distilled from calcium hydride). The ether layer is evaporated to dryness *in vacuo* and derivatised.

The conjugated steroids are isolated by XAD-2-adsorption as described by Donike *et al.*[13]. Starting with 5 ml of urine the following enzyme preparations are used in this study for hydrolysis:

(a) 50 μl of β-glucuronidase/arylsulfatase from *Helix pomatia* (SERVA, D-6900 Heidelberg) in 1 ml of 0.2 m sodium acetate buffer pH 5.2;
(b) 25 μl of β-glucuronidase from *Escherichia coli* (Boehringer, D-6800 Mannheim) in 1 ml of 0.2 m sodium phosphate buffer pH 7.0 (routinely used);
(c) 50 μl of arylsulfatase from *Helix pomatia* (Boehringer) in 1 ml of 0.2 M sodium acetate buffer pH 5.2.

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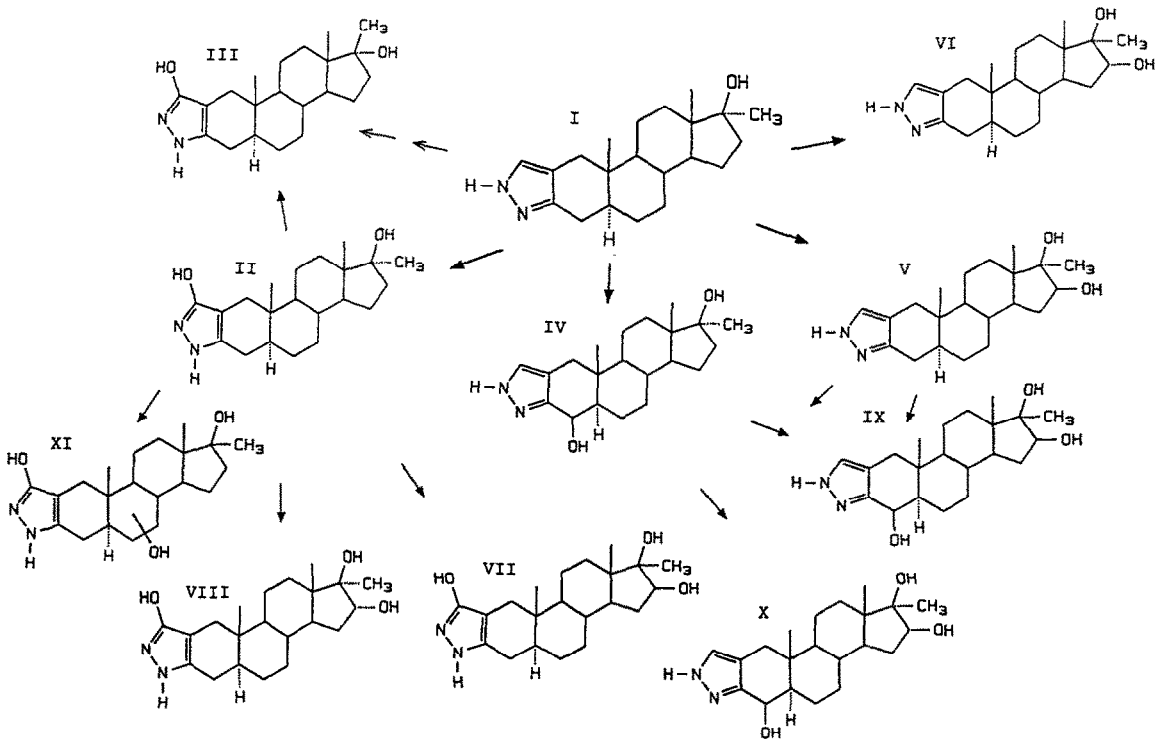

Fig. 1. Metabolism of stanozolol: **I** stanozolol; **II** 3′-hydroxystanozolol; **III** 3′-hydroxy-17-epistanozolol; **IV** 4β-hydroxystanozolol; **V** 16β-hydroxystanozolol; **VI** 16α-hydroxystanozolol; **VII** 3′,16-dihydroxystanozolol 1; **VIII** 3′,16-dihydroxystanozolol 2; **IX** 4,16-didihydroxystanozolol 1; **X** 4,16-dihydroxystanozolol 2; **XI** dihydroxystanozolol with one hydroxy-group in position 3′ of the pyrazole ring whereas the other position is still unknown.

Isolation of unconjugated metabolites

100 ml of urine are transferred into 6 separate XAD-2 columns (pasteur pipette, i.d. 5 mm, closed with a glass pearl, 3 cm bed height of XAD-2). Each XAD-2 column is washed with 10 ml of water and eluted with 2 ml of methanol. The methanol is evaporated to dryness *in vacuo* and the residues are dissolved in 1 ml of distilled water. To the aqueous layers about 100 mg of sodium bicarbonate/potassium carbonate (2:1) pH 9.6 are added and the metabolites are extracted with 5 ml of diethyl ether. The ether layers are combined, concentrated to dryness *in vacuo* and dissolved in 100 µl of methanol. The complete methanolic phase is injected on the HPLC-column. As external standards testosterone (Serva) and epitestosterone (Sigma) are used.

HPLC 1084A Hewlett & Packard; preparative column Nucleosil (R) RP-18, 7 µm, 25 × 1 cm (Macherey-Nagel, D-5160 Düren); solvent: (A) water, (B) acetonitrile/water 90:10 (v/v); flow: 6 ml/min, gradient: starting with 30% of B and linear gradient in 20 min to 80% of B.

The fractions are collected at 0.5 min intervals from 5 to 30 min and evaporated to dryness *in vacuo*.

Isolation of conjugated metabolites

The conjugated metabolites are separated by HPLC and hydrolysed. 100 ml of urine are adsorbed onto and eluted from XAD-2 as described above. The methanol is evaporated to dryness *in vacuo* and each residue is dissolved in 1 ml of distilled water. The unconjugated steroids and the lipid fraction are extracted twice with 8 ml of diethyl ether and discarded. The aqueous layers are combined and concentrated by evaporation *in vacuo*. The residue is dissolved in 100 µl of methanol and the methanolic layer is injected on the HPLC-column. Testosterone-β-D-glucuronide (Sigma), testosterone and epitestosterone are used as reference standards.

HPLC-condition: HPLC 1084 Hewlett & Packard solvent: (A) water/phosphoric acid 1000:0.1 (v/v) pH 2.0, (B) acetonitrile/water 90:10 (v/v), flow: 6 ml/min; gradient: starting with 20% of B and linear increase within 30 min to 60% of B, column: Nucleosil (R) RP-18, 7 µm, 25 × 1 cm.

The fractions are collected at 0.5 min intervals from 5 min to 30 min. They are diluted with 8 ml of water and adsorbed to XAD-2 columns as described. The eluted methanolic fractions are dried and hydrolysed with β-glucuronidase from *Escherichia coli* in 1 ml of 0.2 m phosphate buffer pH 7.0 for 2 h at 50°C. After hydrolysis 200 mg of solid buffer sodium bicarbonate/potassium carbonate (2:1) pH 9.6 are added and the steroids are extracted with 5 ml of diethyl ether. The ether layers are evaporated to dryness *in vacuo*.

Derivatisation for GC/MS-analysis

Trimethylsilylation. (a) The dry residue is dissolved in 50 µl of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide/trimethylsilylimidazole (100:2, v/v) and heated for 10 min at 60°C. (b) The same results are obtained by the use of 50 µl of *N*-methyl,*N*-trimethylsilyl-trifluoroacetamide/trimethyliodosilane (100:0.2, v/v) containing 0.2% of 1,4-dithioerythreitol and heating for 10 min at 60°C [14].

Selective derivatisation. The dry extract is dissolved in 50 µl of *N*-methyl-*N*-trimethylsilyl-heptafluorobutyramide/trimethylsilyl-imidazole (100:2, v/v) and heated for 15 min at 60°C. To the solution are added 5 µl of *N*-methyl-bis-heptafluorobutyramide and the mixture is heated for further 20 min at 60°C.

Methylation. Stanozolol and its metabolites are methylated using a reaction mixture of 200 µl of acetone, 20 µl of methyliodide and 100 mg of potassium carbonate and heating for 1 h at 60°C [15].

GC/MS-determination

Instrument: GC/MSD Hewlett & Packard (GC 5890/MS 5970), electron impact ionisation with 70 eV; column: fused silica capillary column crosslinked 5% phenyl 1% vinyl 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 or splitless injection; temperature program: initial temperature 200°C; program rate 40°C/min; final temperature 320°C; final time 5 min; injector temperature 300°C; interface temperature 300°C.

The presented mass spectra are not normalised.

Syntheses of reference compounds

Each synthesised substance is characterized by the mass spectrum of its per-TMS-derivative. The significant ions and their relative intensities are presented if the full mass spectrum is not shown.

3'-Hydroxystanozolol

3-Methoxycarbonylmethandrostanolone (XIII). To a solution of 10 g (32.8 mmol) of 17α-methyl-5α-androstan-17β-ol-3-one (**XII**, Serva) in 200 ml of dry dioxane, held under argon, are added 16.9 ml (200 mmol) of dimethylcarbonate (distilled from calcium hydride) and 1.5 g (65 mmol) of sodium in 18.5 ml of absolute methanol. The mixture is refluxed for 8 h, then cooled, poured into 500 ml of 10% aqueous acetic acid and extracted with 1 l of diethylether. The ether layer is washed twice with 300 ml of water and evaporated to dryness. The reaction product is separated on silica gel 60 (Merck, 35–70 mesh ASTM, bed 3 × 16 cm) using n-pentane/ethylacetate 75:25 (v/v). The isolated fraction is dried and dissolved in 50 ml of acetone. When the solution is concentrated and allowed to crystallise, 3.1 g (8.6 mmol) of (**XIII**), m.p. 128–129°C, are obtained (yield 26%). EI-spectrum of bis-TMS-**XIII**: 506 (2), 491 (100), 401 (8), 361 (15), 143 (68), 73 (71).

3'-Hydroxystanozolol (II). 2 g (5.52 mmol) of **XIII** are dissolved in 50 ml of methanol, 0.5 ml (10 mmol) of hydrazine hydrate (Fluka, CH-9470 Buchs) are added and the solution is refluxed for 2 h. After cooling and evaporation of the solvents the crude product is recrystallised from methanol to give 0.9 g (2.6 mmol) of **II**, m.p. 290–293°C, in 47.4% yield. EI-spectra see Figs 5 and 6.

4α-Hydroxystanozolol

4,5-Dehydrostanozolol (XVI). 5 g (16.5 mmol) of 17α-methyltestosterone (**XIV**) in 100 ml of absolute pyridine, 10 ml of (125 mmol) ethylformate, 780 mg (34 mmol) of sodium dissolved in 7 ml of abs. methanol, are stirred under argon for 24 h at ambient temperature. The solution is poured into 400 ml of 20% acetic acid and extracted with 1 l of diethyl ether. The ether layer is extracted five times with 200 ml of 2% sodium hydroxide solution. After addition of 50 ml of 99% acetic acid the resulting precipitate is filtered and washed with 1 l of water and dried. EI-spectrum of the 4,5-dehydro-oxymetholone (**XV**), Tris–TMS: 546 (100), 531 (7), 401 (16), 293 (9), 147 (11), 73 (62). The crude **XV** is dissolved in 50 ml of methanol, 1.2 ml (24.7 mmol) of hydrazine hydrate, refluxed for 2 h and evaporated to dryness. The dark red residue is recrystallised three times from acetone obtaining 2 g (6.1 mmol) of **XVI**, m.p. 165–170°C, in a yield of 37.2%. EI-spectrum of bis-TMS-**XVI**: 470 (100), 455 (32), 380 (70), 365 (22), 143 (78), 73 (100).

4α-Hydroxystanozolol (**XVII**). 800 mg (2.45 mmol) of **XVI** are dissolved in 250 ml of abs. diethyl ether under argon and 1.5 ml (11.9 mmol) of boron trifluoride-etherate (Merck) are added. A suspension of 300 mg of lithium aluminium hydride in 20 ml of diethyl ether is added dropwise within 20 min. After stirring for 1 h the solution is poured into 200 ml of a saturated aqueous sodium sulfate and the mixture is extracted with 250 ml of diethyl ether. The ether layer is dried over sodium sulfate and evaporated to dryness. The dry residue is dissolved in 75 ml of methanol, 5 ml of 6 N aqueous NaOH and 15 ml of 30% hydrogen peroxide and stirred for 1 h. The reaction mixture is diluted with 200 ml of water and extracted twice with 400 ml of diethyl ether. The ether layers are combined, dried over sodium sulfate and evaporated. The crude product is recrystallised from methanol yielding 140 mg (0.4 mmol) of pure **XVII**, m.p. 167–171°C, 16.6% of the theory. EI-spectrum of Tris–TMS-XVII see Fig. 12.

4β-Hydroxystanozolol

Stanozolol-4-one (XVIII). 100 mg (0.29 mmol) of **XVII** are dissolved in 2 ml of 96% acetic acid. 50 mg (0.5 mmol) of chromium trioxide dissolved in 3 ml of 96% acetic acid are added dropwise within 10 min. After stirring for 15 min at ambient temperature 50 ml of 10% potassium hydroxide solution are added. The resulting **XVIII** is extracted twice with

300 ml of diethyl ether. The ether layers are dried over sodium sulfate and evaporated. The residue is recrystallised from methanol (60 mg, m.p. 320–325°C, 60.5% of the theory). EI-spectrum of Tris–TMS-**XVIII**: 558 (19), 543 (100), 307 (33), 305 (23), 291 (38), 73 (41).

4β-Hydroxystanozolol (IV). 60 mg (0.175 mmol) of **XVIII** are dissolved in 15 ml of abs. dioxane held under argon. After addition of 1 g (3.9 mmol) of lithium tri-*t*-butoxy-aluminium hydride (Merck) and refluxing for 1 h the **XVIII** has reacted completely to a mixture of 54% of **IV** and 46% of **XVII** (the data are estimated by GC/FID of the per-TMS-derivatives). 200 ml of water are added and the reaction mixture is extracted with 400 ml of diethyl ether. The ether layers are dried over sodium sulfate and evaporated. **IV** is isolated by HPLC using a preparative column (RP-18, 7 μm, 25 × 1 cm). The isolated fractions containing **IV** are evaporated under reduced pressure and **IV** is recrystallised from methanol (18 mg, m.p. 270–272°C, yield 29.9%). EI-spectrum of Tris–TMS-**IV** see Fig. 11.

16α-Hydroxystanozolol and 16α-hydroxy-17-epistanozolol

20-Norstanozolol (XXI). 5 g (17.2 mmol) of 5α-dihydrotestosterone (**XIX**) are dissolved in 250 ml of absolute pyridine, 10 ml (125 mmol) of ethylformate and 805 mg (35 mmol) of sodium in 8.5 ml of absolute methanol are added and the mixture is stirred for 1 h at ambient temperature. 200 ml of 10% aqueous acetic acid are added and the reaction products are extracted twice with 600 ml of diethyl ether. The combined ether layers are evaporated to dryness (8.0 g of crude product). The GC/MS-analysis shows about 62% of 2-hydroxymethylene-5α-dihydrotestosterone (**XXa**), 29% of 2-hydroxymethylene-5α-dihydrotestosterone-17-formate (**XXb**) and 9% of side products. EI-spectrum of Tris–TMS-**XXa**: 534 (78), 519 (18), 281 (25), 267 (13), 129 (13), 73 (100), EI-spectrum of bis-TMS-**XXb**: 490 (80), 475 (19), 433 (10), 281 (22), 267 (11), 73 (100). The crude product is dissolved in 100 ml of ethanol containing 2.5 ml (51.5 mmol) of hydrazine hydrate and refluxed for 3 h. The reaction mixture is evaporated under reduced pressure and the residue is crystallised 3 times from 220 ml of methanol yielding 3.3 g (10.1 mmol, 58.5%) of **XXI** (m.p. 147–150°C). EI-spectrum of bis-TMS-**XXI**: 458 (94), 433 (19), 367 (7), 168 (74), 129 (22), 73 (100).

17-Oxo-5α-androst-2-eno(3,2-c)-pyrazole (XXII). To a solution of 3 g (9.55 mmol) of **XXI** in 45 ml of 96% acetic acid are added dropwise 2.4 g (24 mmol) of chromium trioxide in 58 ml of 96% acetic acid within 30 min. After stirring for 1 h at ambient temperature 300 ml of ice-cold aqueous 33% potassium hydroxide is added and the mixture is extracted 3 times with 1 l of diethyl ether. The ether layers are combined and evaporated to dryness. The residue is recrystallised twice from methanol to give 2.1 g (6.7 mmol) of **XXII** (m.p. 234–240°C, yield 70%). EI-spectrum of bis-TMS-**XXII**: 456 (65), 441 (100), 351 (13), 168 (16), 73 (42).

N-Acetyl-17-oxo-5α-androst-2-eno(3,2-c)-pyrazol-enol-acetate (XXIII). 2.0 g (6.4 mmol) of **XXII** are dissolved in 30 ml of isopropenyl-acetate, added with 3 ml of catalyst mixture (5 ml of isopropenylacetate and 0.1 ml of concentrated sulfuric acid) and refluxed. After 3, 6 and 9 h further 2 ml of catalyst solution are added and the mixture is refluxed at least for 12 h. The solution is diluted with 500 ml of n-pentane and filtered over acid-washed alumina N Akt.I (Woelm), bed 3 × 5 cm. The alumina is further washed with 100 ml of n-pentane, and the organic layers are evaporated to dryness yielding 2.2 g. The GC/MS-analysis of the crude product shows about 12.5% of *N*-mono-acetyl-product, 70% of **XXII**-bis-acetate (**XXIII**) and 18% of side-products. **XXIII** consists of a mixture of two isomers (*N*-1′-acetyl- and *N*-2′-acetyl-derivative). EI-spectra of **XXIII**: 396 (4, 4), 354 (100, 100), 339 (62, 58), 297 (37, 38), 43 (96, 83).

16α-Hydroxystanozolol (VI) and 16α-hydroxy-17-epistanozolol (XXVII). 1.1 g of raw **XXIII** are dissolved in 100 ml of 0.25 N perbenzoic acid in benzene [16]. After standing for 12 h at ambient temperature the solution is diluted with 200 ml of diethyl ether and extracted with 100 ml of water. The organic layer is washed with 100 ml of 10% potassium carbonate solution and twice with 100 ml of water. The organic layer is evaporated to dryness yielding 1.2 g of crude *N*-acetyl-16α,17α-epoxy-17β-acetoxy-5α-androst-2-eno(3,2-c)pyrazole (**XXV**). Two pairs of isomers were found by GC/MS: 412 (51, 47), 370 (33, 24), 255 (21, 14), 138 (23, 21), 96 (26, 22), 94 (23, 24) and 412 (46, 42), 370 (55, 47), 369 (31,23), 138 (56, 48), 96 (50, 42), 94 (21, 21). It is assumed that the first pair results from a thermal rearrangement of the epoxide.

The crude product is dissolved in 50 ml of methanol and 5 ml of 6 N sulfuric acid and kept at ambient temperature for 12 h. The solution is diluted with 500 ml of water and extracted three times with 1 l of diethyl ether. The ether layers are evaporated to dryness and the residue is separated over silicagel 60 (bed 50 × 1 cm) with isooctane/ethylacetate 50:50 (v/v) with increasing ethylacetate gradient. The fractions containing 17-oxo-16α-hydroxy-5α-androst-2-eno(3,2-c)-pyrazole (**XXVI**) are evaporated to dryness yielding 800 mg. EI-spectrum of bis-TMS-**XXVI**: 427 (11), 457 (8), 328 (100), 313 (8), 218 (8), 168 (17).

The crude **XXVI** is dissolved in 300 ml of abs. diethyl ether held under argon and added dropwise to a Grignard solution prepared from 2 g (87 mmol) of magnesium and 5.5 ml of methyliodide in 50 ml of diethyl ether. The mixture is stirred over night and poured onto 500 ml of ice-water. The solution is extracted three times with a mixture of 500 ml of ethylacetate and 200 ml of diethyl ether. The organic layers are combined, evaporated to dryness and

separated via silicagel column (bed 50 × 1 cm) with ethylacetate/n-pentane 70:30 (v/v). The reaction leads to a mixture of 16α-hydroxystanozolol (**VI**) and 16α-hydroxy-17-epistanozolol (**XXVII**) in a ratio of about 15:85. The fractions containing **XXVII** are recrystallised from methanol yielding 180 mg (523 μmol, 16.3% calculated from **XXII**) of 95% material (m.p. 282–286°C). EI-spectrum of Tris–TMS-**XXVII**: 560 (26), 470 (6), 381 (6), 231 (15), 218 (27), 117 (10). The fractions containing **VI** are separated by HPLC using a preparative reverse-phase column (25 × 1 cm). EI-spectrum of Tris–TMS-**VI** see Fig. 16.

16β-Hydroxystanozolol

To 1.1 g of the crude **XXIII** dissolved in 30 ml of 99.8% acetic acid are added 3 g (6.7 mmol) of lead tetraacetate and the solution is stirred at ambient temperature for 12 h. The solution is diluted with 200 ml of water and extracted with 500 ml of n-pentane. The organic layer is washed three times with 200 ml of water unitl the pH of the aqueous phase is at 6. The organic layer is evaporated to dryness yielding crude *N*-acetyl-17-oxo-16β-acetoxy-5α-androst-2-eno(3,2-c)-pyrazole (**XXIV**) and some side products. EI-spectrum of **XXIV**: 412 (8), 370 (16), 255 (14), 138 (28), 96 (41), 94 (32).

The crude reaction products are dissolved in 100 ml of abs. diethyl ether held under argon and dropped into a Grignard mixture prepared from 1.4 g (58.2 mmol) of magnesium and 8.3 g of methyl iodide in 30 ml of diethyl ether. After stirring for 10 h the solution is poured onto 400 ml of ice-water and extracted three times with 1 l of diethyl ether. The ether layers are combined, dried over sodium sulfate and evaporated to dryness. The main reaction product 16β-hydroxystanozolol (**V**) is recrystallised from methanol (256 mg, m.p. 302–310°C, 23.2% yield). EI-spectrum of Tris–TMS-**V** see Fig. 15.

The 16α-hydroxystanozolol (**VI**) is oxidised with chromium trioxide. To 10 mg of **VI** dissolved in 1 ml of 96% of acetic acid are added dropwise 50 mg of chromium trioxide in 2 ml of acetic acid. After stirring for 30 min at ambient temperature 50 ml of 6% potassium hydroxide solution are added and the mixture is extracted with 250 ml of diethyl ether. The ether is dried over sodium sulfate and evaporated to dryness yielding crude stanozolol-16-one (**XXVIII**). EI-spectrum of Tris–TMS-**XXVIII**: 558 (37), 543 (10), 244 (13), 147 (12), 73 (100).

Without purification **XXVIII** is dissolved in 50 ml of absolute diethyl ether held under argon. To this solution 50 mg of lithium aluminium hydride are added and after stirring for 1 h the mixture is diluted with 100 ml of water and extracted with 300 ml of diethyl ether. The ether is dried over sodium sulfate and evaporated to dryness. The residue is identified by GC/MS as 16β-hydroxystanozolol (**V**) and identical with V synthesized via lead tetraacetate.

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

The elimination of the 17-hydroxy group is easily performed in a 10% solution of trifluoroacetic acid in acetonitrile (Baker, HPLC-grade) by heating for 10 min at 60°C. The organic layer is evaporated to dryness *in vacuo*. In this way 3′-hydroxystanozolol and 3′-hydroxyepistanozolol are converted to 3′-hydroxy-13,14-dehydro-17,17-dimethyl-18-nor-5α-androst-2-eno(3,2-c)-pyrazole. EI-spectrum of the bis-TMS-derivative see Fig. 8.

RESULTS

HPLC-separation

The HPLC parameters of the separation of the unconjugated and conjugated urine fraction using a preparative column RP-18 are listed in Table 1. The fractions were cut in 0.5 min intervals. Complete separation of all the metabolites listed in Table 1 could be achieved except for 3′,16α-dihydroxystanozolol 2 and 16β-hydroxystanozolol.

3′-Hydroxystanozolol

3′-Hydroxystanozolol (**II**) was found in the unconjugated and in higher concentration in the conjugated fraction. It was synthesised in two steps (Fig. 2). The first step consists in the introduction of a methoxycarbonyl-group into position 2 of 17α-methyl-5α-androstan-17β-ol-3-one (**XII**) which then reacts with hydrazine hydrate to 3′-hydroxystanozolol.

Like pyrazol-3-one [17] the 3′-hydroxy-metabolite can exist theoretically in up to eight tautomeric forms, but only three are observed in practice (Fig. 3). With methyliodide and potassium carbonate in acetone three dimethyl derivatives could be prepared [18].

The extraction of 3′-hydroxystanozolol with organic solvents from an aqueous solution or from urine is pH-dependent with an optimum at 9.6 (Table 2), indicating that the pyrazol-3-one has acid character.

The concentration of the unconjugated 3′-hydroxystanozolol (**II**) in the urine samples increased during longer storage at 4°C, e.g. by a factor of about 5 within 105 days. The concentration of 3′-hydroxy-17-epistanozolol (**III**) remained constant (Table 3). In urine with a pH of 7 and in urine stored at −30°C the concentration of **II** was constant too.

Gas-chromatography and mass-spectrometry of stanozolol and 3′-hydroxystanozolol

Stanozolol (**I**) can be analysed by GC without derivatisation. The EI-spectrum (Fig. 4) with the molecular ion *m/z* 328 shows two fragment ions *m/z* 94 and *m/z* 96 with high abundances. The ion *m/z* 94 occurs from a retro Diels-Alder fragmentation (Fig. 4) as described by Budzikiewicz *et al.*[19]. The ion *m/z* 96 can be explained by a rearrangement of

Table 1. HPLC-elution of stanozolol metabolites from a preparative column: Nucleosil (R), RP-18, 7 μm, 25 × 1 cm

Unconjugated steroids		
Flow: 6 ml/min		
(A) water (B) acetonitrile/water (90/10)		
Gradient: 30% B in 20 min to 80% B		
Reference standards:		
Testosterone	13.90 min	
Epitestosterone	14.98 min	
Stanozolol	19.50 min	
Fraction		
10.0–11.0 min	3′-hydroxystanozolol	(II)
11.5–15.5 min	3′-hydroxyepistanozolol	(III)
Conjugated metabolites		
Flow: 6 ml/min		
(A) water/phosphoric acid pH 2.0		
(B) acetonitrile/water (90/10)		
Gradient: 20% B in 30 min to 60% B		
Reference standards:		
Testosterone-glucuronide	15.86 min	
Testosterone	25.84 min	
Epitestosterone	27.61 min	
Fraction	*Conjugate of*	
8.5–9.0 min	3′,16-dihydroxystanozolol 1	(VII)
9.5–10.0 min	4β,16-dihydroxystanozolol 1	(IX)
10.5–11.0 min	4β,16-dihydroxystanozolol 2	(X)
11.0–11.5 min	3′,16-dihydroxystanozolol 2	(VIII)
11.0–11.5 min	16β-hydroxystanozolol	(V)
12.0–12.5 min	16α-hydroxystanozolol	(VI)
12.5–13.0 min	3′,?-dihydroxystanozolol	(XI)
14.0–14.5 min	4β-hydroxystanozolol	(IV)
14.5–15.0 min	Stanozolol	(I)
16.5–17.0 min	3′-hydroxystanozolol	(II)

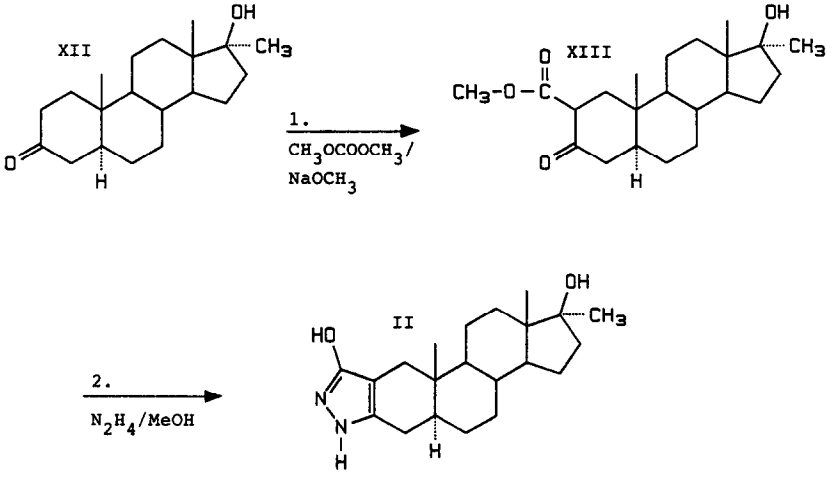

Fig. 2. Synthesis of 3′-hydroxystanozolol.

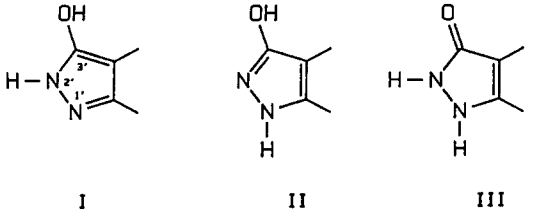

Fig. 3. Tautomers of 3′-hydroxystanozolol.

Table 2. Extraction profile of 3′-hydroxystanozolol

pH	Recovery
6.0	62%
8.0	70%
9.6	89%
11.0	12%
12.0	7%
13.5	<1%

To 4 ml of water with different pH 20 μg of 3′-hydroxystanozolol are added and extracted with 5 ml of diethyl ether.

Table 3. Increase of 3′-hydroxystanozolol (II) In the unconjugated fraction

From–to	pH of urine 6 days	pH of urine 105 days	II (ng/ml) 6 days	II (ng/ml) 105 days	III (ng/ml) 6 days	III (ng/ml) 105 days
			40 mg stanozolol orally			
0–6.5 h	6.0	5.9	2.2	3.4	1.9	1.9
6.5–15.5 h	5.0	6.0	6.6	29.5	6.7	8.1
15.5–24.0 h	6.0	5.7	8.5	38.6	7.8	13.0
24.0–31.0 h	6.0	5.5	4.9	26.4	11.0	13.7
31.0–39.0 h	5.5	5.7	5.1	25.5	11.5	8.6
39.0–50.0 h	6.0	5.7	4.5	10.1	11.4	8.4
6.5–15.5 h[a]	5.0	5.7	6.6	8.6	6.7	9.8
15.5–24.0 h[a]	6.0	5.8	8.5	9.4	7.8	7.9
			20 mg stanozolol orally			
3–7.5 h	5.5	5.5	3.9	13.3	3.2	2.9
7.5–15.5 h	5.5	5.5	2.8	7.0	4.0	3.4
15.5–23.5 h	7.5	7.5	1.1	1.0	2.6	2.6
23.5–29.5 h	6.0	6.2	2.5	3.9	6.5	6.0
29.5–39.5 h	5.5	5.5	4.5	17.5	9.9	10.8
39.5–45.5 h	7.5	7.5	1.4	1.6	4.2	3.9

Urine-storage at 4°C, [a]storage at −30°C.

the A-ring-fragment with two hydrogen atoms "captured". The origin of both hydrogens could not be evaluated.

The underivatised 3′-hydroxystanozolol cannot be analysed by gas-chromatography. An EI-spectrum was obtained by direct insertion technique (Fig. 5) and shows a base peak m/z 110. This ion results from a retro Diels-Alder fragmentation of ring A. The ion m/z 112 can be explained by a fragmentation of the A-ring with migration of two hydrogen atoms, the same fragmentation as described above for stanozolol.

To improve the gas-chromatographic behaviour of stanozolol and the stanozolol metabolites they are trimethyl silylated. In the EI-spectrum of 3′-hydroxystanozolol, Tris–TMS (Fig. 6) the most prominent ions can be attributed to the molecular ion m/z 560, an ionic fragment m/z 254 which may originate from a retro Diels-Alder process of the A-ring, similar to the ion m/z 94 in I, and a D-ring

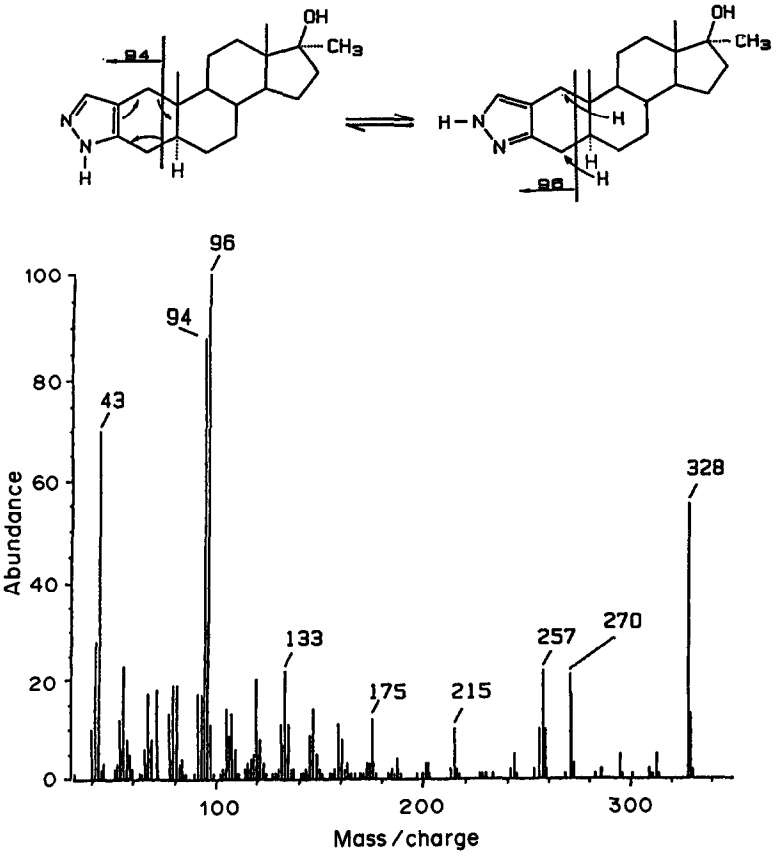

Fig. 4. EI-spectrum of stanozolol.

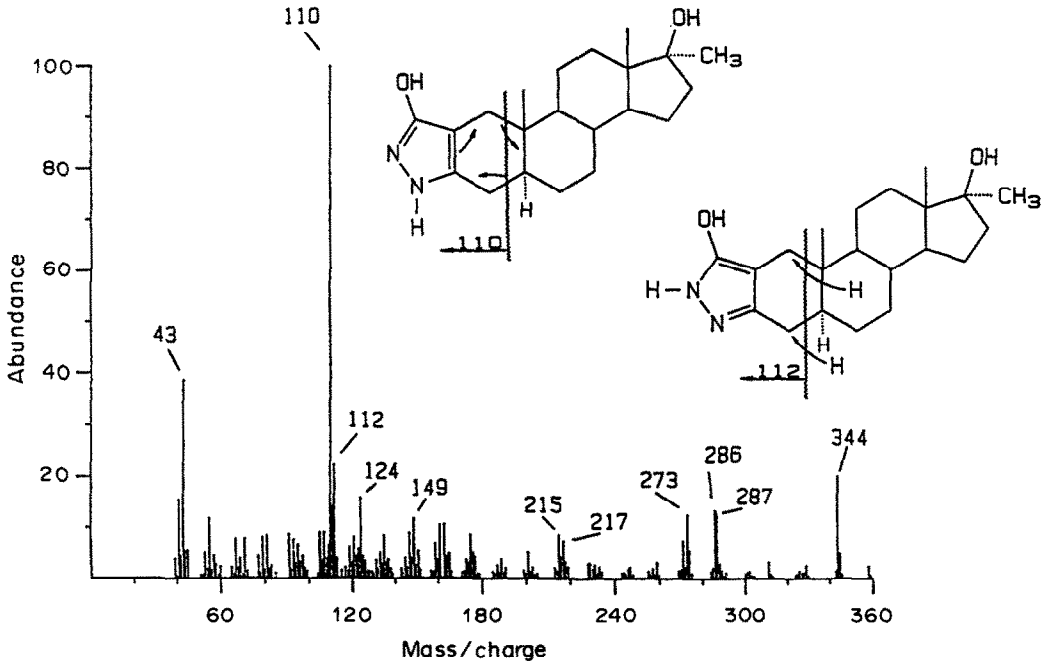

Fig. 5. EI-spectrum of 3′-hydroxystanozolol.

fragment m/z 143 characteristic for all trimethylsilylated 17-methyl-17-hydroxy-steroids. By high resolution mass spectrometry the molecular formula of the ion m/z 254 of 3′-hydroxystanozolol was shown to be C_{11}-H_{22}-N_2-O-Si_2.

In the mass spectrum of stanozolol, bis-TMS (Fig. 7) with the molecular ion m/z 472 an ion m/z 168 is recorded. The ion m/z 168 originates from a scission of the A-ring with migration of two hydrogen atoms. From this fragment-ion we assume that the TMS-group is located at position 2′ of the pyrazole-ring fixing the double bonds exocyclic in regard to the steroidal A-ring. When position 1′ is trimethylsilylated, a retro Diels-Alder fragmentation with m/z 166 should be favoured [18].

3′-Hydroxy-17-epistanozolol

3′-Hydroxy-17-epistanozolol (III) can only be detected in the unconjugated urine fraction. The identification of the metabolite isolated from the HPLC-

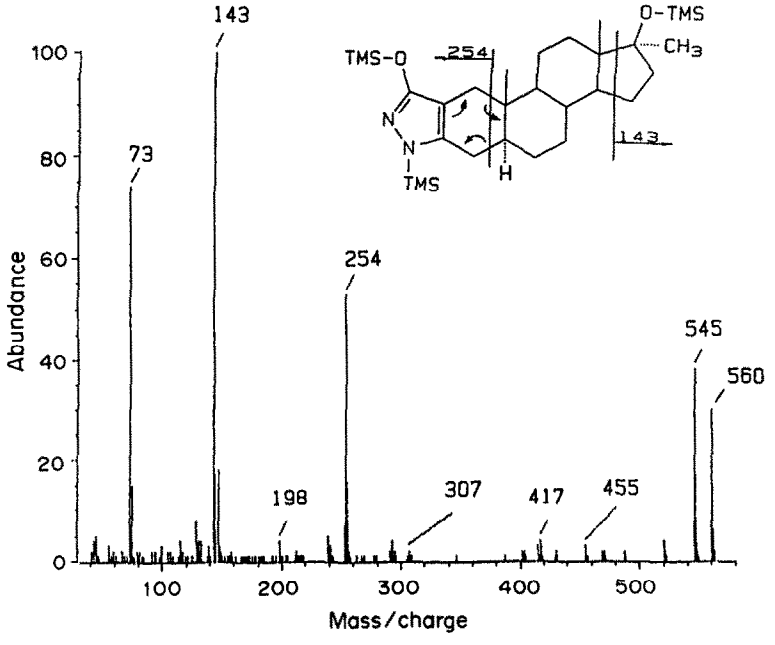

Fig. 6. EI-spectrum of 3′-hydroxystanozolol, Tris–TMS.

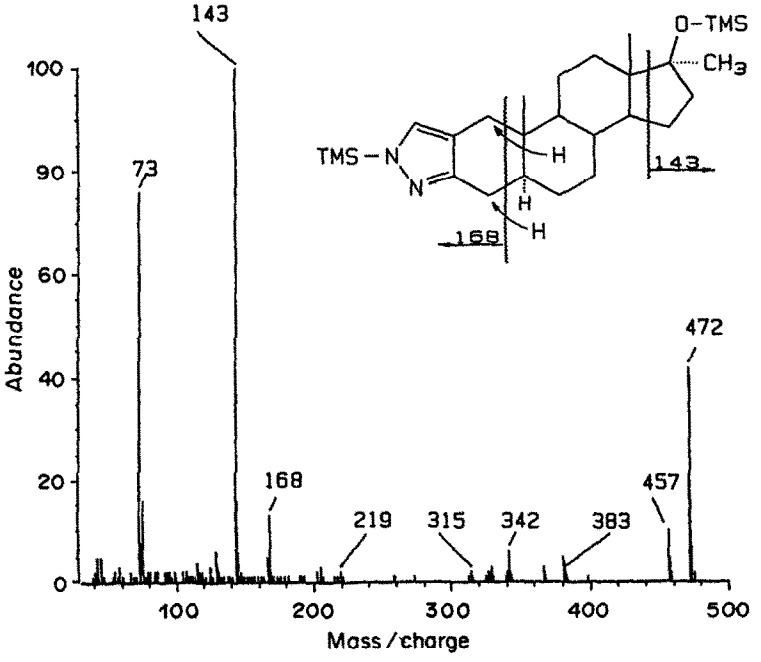

Fig. 7. EI-spectrum of stanozolol, bis-TMS.

fraction 11.5–12.5 min of the unconjugated fraction (Table 1) was performed by elimination of the 17-hydroxy-group with trifluoroacetic acid in acetonitrile. The same reaction product is obtained by dehydration with 1 N aqueous hydrochloric acid.

The reaction of 17-hydroxy-17-methyl-steroids with 1 N aqueous hydrochloric acid was published by Segaloff *et al.*[20] who found a rearrangement of 17-hydroxy-17-methyl-androstanes to 17,17-dimethyl-18-norandrost-13(14)-enes. The treatment of the isolated metabolite and the synthetic 3′-hydroxy-stanozolol with trifluoroacetic acid/acetonitrile yields the same product: 3′-Hydroxy-13,14-dehydro-17,17-dimethyl-18-nor-5α-androst-2-eno(3,2-c)-pyrazole, which was analysed by GC/MS as its bis-TMS-derivative (Fig. 8).

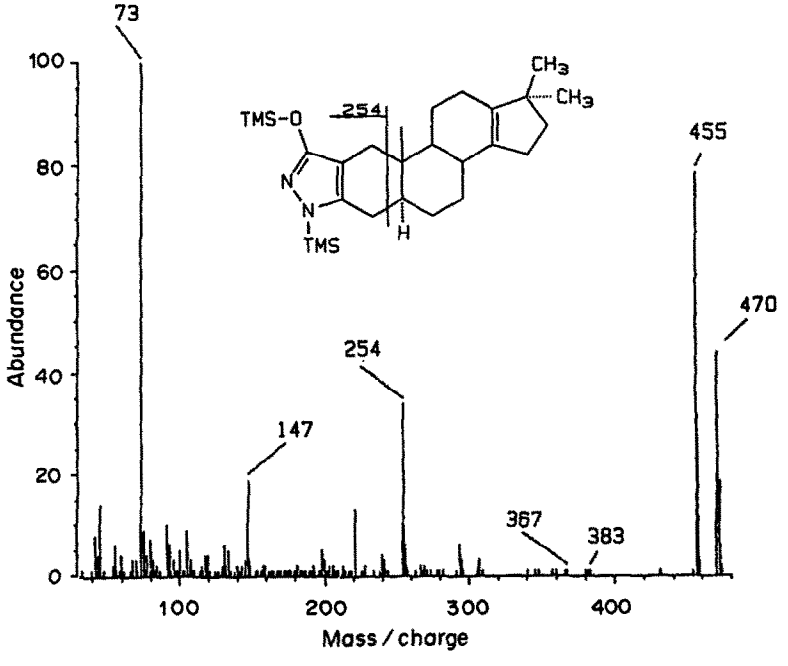

Fig. 8. EI-spectrum of 3′-hydroxy-13,14-dehydro-17,17-dimethyl-18-nor-5α-androst-2-eno(2,3-c)-pyrazole, bis-TMS.

Table 4. Kovats-indices of stanozolol and mono-hydroxystanozolol per-TMS-derivatives

Substance	Index
17-epistanozolol, bis-TMS[a]	3112
3′-hydroxy-17-epistanozolol, Tris–TMS[a]	3173
Stanozolol, bis-TMS	3232
16β-hydroxy-17-epistanozolol, Tris–TMS[a]	3281
3′-hydroxystanozolol, Tris–TMS	3307
4β-hydroxystanozolol, Tris–TMS	3325
16α-hydroxy-17-epistanozolol, Tris–TMS	3343
4α-hydroxystanozolol, Tris–TMS	3344
16α-hydroxystanozolol, Tris–TMS	3432
16β-hydroxystanozolol, Tris–TMS	3444
17-epi-metandienone, bis-TMS[a]	2666
17-epi-metandienone, TMS[a]	2687
Metandienone, bis-TMS	2768
Metandienone, TMS	2807

Column: fused silica capillary crosslinked 5% phenyl methyl silicone (SE 54) (Macherey-Nagel) 25 m × 0.25 mm, 0.22 μm film thickness, flow: 2 ml/min, split: 1:15, head pressure: 27 psi, TPG initial value: 280°C, program rate: 4°C/min, final temperature: 320°C.

[a]The publication of the synthesis of the marked substances is in preparation.

These results confirm that the only difference between this metabolite and 3′-hydroxystanozolol is the configuration at position 17.

In GC with SE 54 stationary phase Tris–TMS-III is eluted before Tris–TMS-II (Table 4). This result is in agreement with the elution pattern of other trimethylsilylated 17-hydroxy-epimers, e.g. epimetandienone, bis-TMS is eluted earlier than metandienone, bis-TMS. On the other hand the elution pattern in HPLC on a RP 18 column is inverse (Table 1): 3′-Hydroxy-17-epistanozolol is eluted later than 3′-hydroxystanozolol, i.e. in the same order as testosterone and epitestosterone.

The EI-spectrum of the 3′-hydroxy-17-epistanozolol, Tris–TMS (Fig. 9) shows no significant differences to the mass spectrum of the 3′-hydroxystanozolol, Tris–TMS.

4β-Hydroxystanozolol

4β-Hydroxystanozolol (**IV**) and 4α-hydroxystanozolol (**XVII**) were synthesised (Fig. 10) to confirm the structure of the metabolite isolated from the HPLC-fraction 14.0–14.5 min of the conjugated urine fraction (Table 1). The structural assignment based on the mass spectrum indicating a hydroxyl function in the A-ring fragment and on the fact that the hydroxy-group did not react with methyliodide excluding the positions in the pyrazole ring.

The synthesis of 4β-hydroxy-stanozolol was performed in five steps. Starting with methyltestosterone (**XIV**) the first two steps correspond to the synthesis of stanozolol as described by Clinton *et al.*[2]. The resulting 4,5-dehydrostanozolol (**XVI**) was hydroxylated to 4α-hydroxystanozolol (**XVII**) via the anti-Markownikoff hydroboration published by Wolfe *et al.*[21] who prepared cholestan-4α-ol from cholest-4-ene. **XVII** was oxidised to stanozolol-4-one (**XVIII**) which then was reduced to a mixture of 46% of 4α-hydroxystanozolol (**XVII**) and 54% of 4β-hydroxystanozolol (**IV**). The use of lithium aluminium hydride instead of lithium tri-*t*-butoxy-aluminium hydride under the same reaction conditions did not favour the production of one isomer (46% of the 4β-hydroxy- and 54% of the 4α-hydroxy-isomer).

The EI-spectrum of the persilylated synthesised 4β-hydroxystanozolol (Fig. 11) is identical with the mass-spectrum of the per-TMS-derivative of the isolated metabolite. A comparison of the main fragment ions *m/z* 254, 365, 380, 430, 455, 470, 471, 545 and

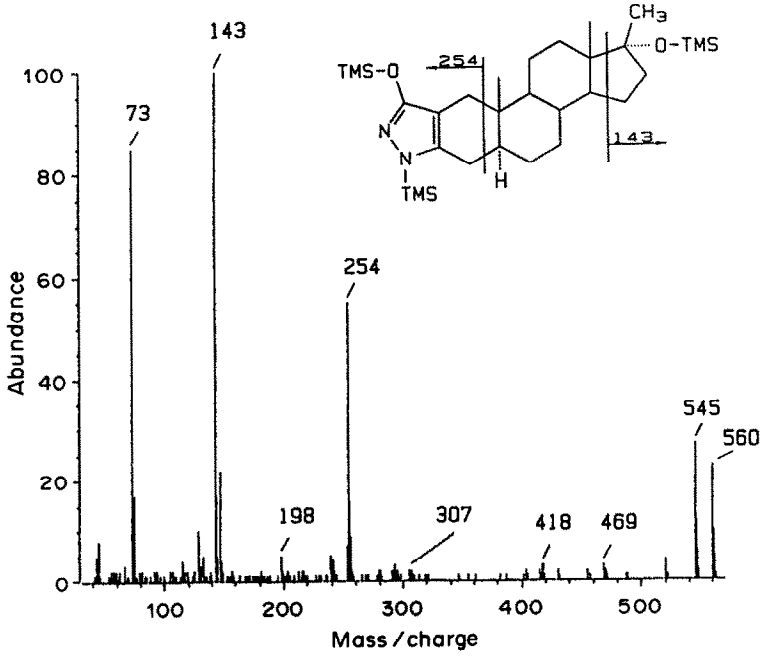

Fig. 9. EI-spectrum of 3′-hydroxy-17-epistanozolol, Tris–TMS.

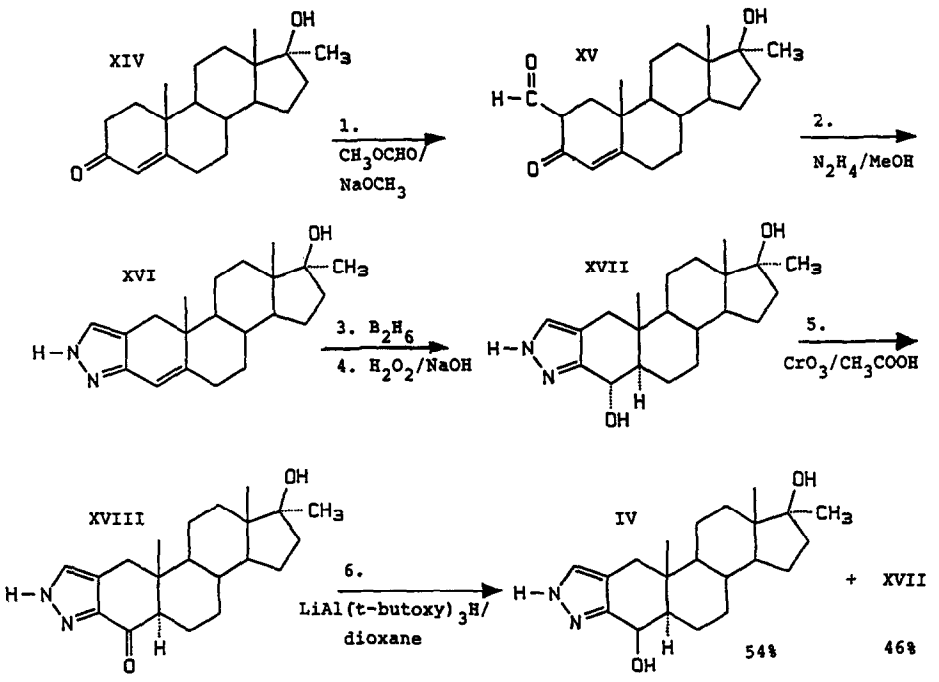

Fig. 10. Synthesis of 4α-hydroxy- and 4β-hydroxystanozolol.

the molecular ion m/z 560 by selected ion monitoring is presented in Table 5.

4α-Hydroxystanozolol, Tris–TMS has a stronger retention than the 4β-hydroxystanozolol, Tris–TMS (Table 4) in gas chromatography and its EI-spectrum (Fig. 12) differs in the abundances of the main ionic fragments (Table 5).

16β-Hydroxystanozolol

16β-Hydroxystanozolol (V) was synthesised in 6 steps (Fig. 13) starting from 5α-dihydrotestosterone (**XIX**). The condensation to a pyrazole ring corresponds to the synthesis of stanozolol described by Clinton *et al.*[2] followed by oxidation of the 17-hydroxy-group. The introduction of a 16β-hydroxy-

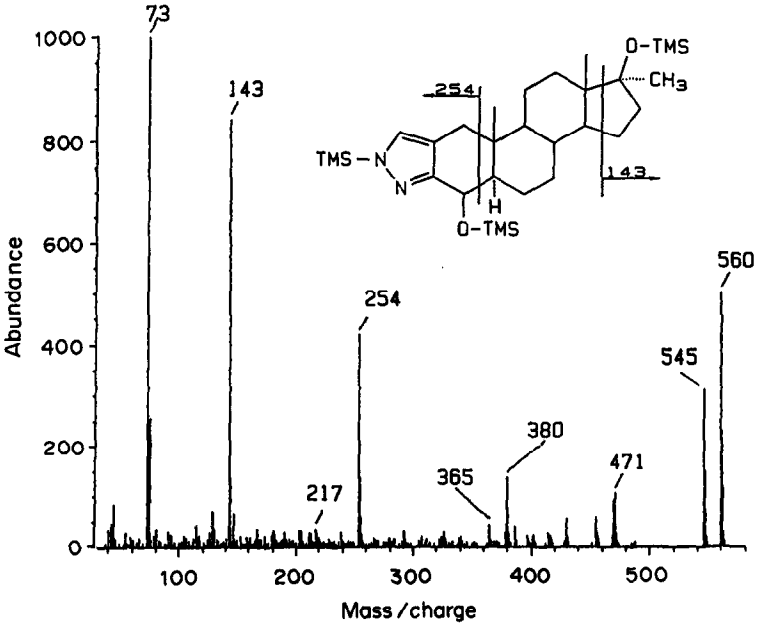

Fig. 11. EI-spectrum of 4β-hydroxystanozolol, Tris–TMS.

Table 5. Relative intensity of the 9 most intense fragment ions (without *m/z* 143) of the Tris–TMS-derivatives of isolated metabolite from HPLC-fraction 14.0–14.5 min (Table 1) of the conjugated fraction, synthesized 4β-hydroxy- and 4α-hydroxystanozolol

m/z	560	545	471	470	455	430	380	368	254
Metabolite	48.8	30.6	11.5	11.1	7.5	6.8	16.9	5.7	100
4β-HO-	49.8	31.0	11.6	11.9	7.5	6.7	17.1	5.7	100
4α-HO-	88.2	87.5	30.7	19.3	13.8	15.4	24.1	7.1	100

group into a 17-keto-steroid was investigated by Johnson *et al.*[22]. The attack of lead tetraacetate on a 17-keto-enol-acetate yields a 16β-acetoxy-17-one. This derivative was treated with methyl magnesium iodide to obtain 16β-hydroxystanozolol (**V**).

The isolated metabolite and the synthesised 16β-hydroxystanozolol form both an acetonide when they are heated in acetone with perchloric acid as catalyst (0.2% v/v). The EI-spectrum of the N-TMS-derivative of the acetonide with a molecular ion *m/z* 456 is presented in Fig. 14.

The 16β-hydroxystanozolol, Tris–TMS has the highest retention-index (Table 4). The EI-spectrum (Fig. 15) displays a molecular ion of *m/z* 560. The ionic fragments *m/z* 218 and *m/z* 231 indicate that position 16 of the D-ring is hydroxylated. Compared to the D-ring fragmentation of the parent compound this corresponds to an increment of 88 amu which is typical for the introduction of one hydroxy-group and its trimethylsilylation.

16α-Hydroxystanozolol

In the synthesis of 16α-hydroxystanozolol (**VI**, Fig. 13) the first four steps follow the synthesis of 16β-hydroxystanozolol. Step 5 and 6 correspond to the published synthesis of 1,3,5(10)-estatriene-3,16α-diol-17-one and 5α-androstane-3β,16α-diol-17-one by Leeds *et al.*[23]. The obtained 17-oxo-16α-hydroxy-5α-androst-2-eno(3,2-c)-pyrazole (**XXVI**) was treated with methyl magnesium iodide yielding a mixture of two 17-methyl-isomers. Isomer 1 (85%) was confirmed as 16α-hydroxy-17-epistanozolol (**XXVII**) and isomer 2 (15%) as 16α-hydroxystanozolol (**VI**).

The structural assignment is based on the following:

(1) With acetone/perchloric acid isomer 1—a *cis*-diol—forms an acetonide whereas isomer 2 does not react due to its *trans* arrangement of the hydroxy groups.
(2) With chromium trioxide/acetic acid isomer 1 cannot be oxidised to the 16-oxo-derivative. Like with other steroids of this structure the reaction leads to a cleavage of the carbon–carbon bond between position 16 and 17. Isomer 2 however is oxidised to a 16-oxo-derivative (**XXVIII**) indicating trans-configuration.
(3) The 16-keto-derivative can be reduced with lithium hydride yielding a 16β-hydroxy-derivative as described by Leeds *et al.*[23]. This reduction product was identical with the synthesised 16β-hydroxystanozolol.

The EI-spectrum of the TMS-derivative of the isolated metabolite from the HPLC-fraction

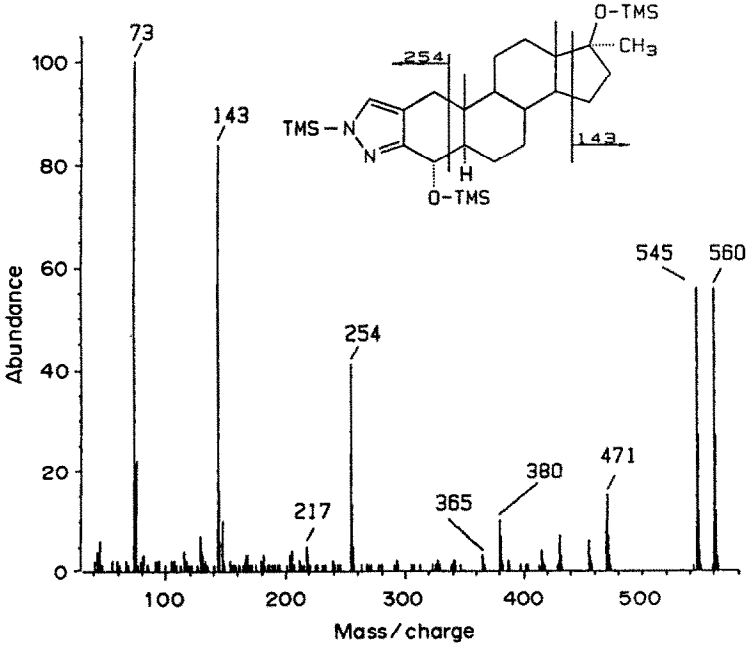

Fig. 12. EI-spectrum of 4α-hydroxystanozolol, Tris–TMS.

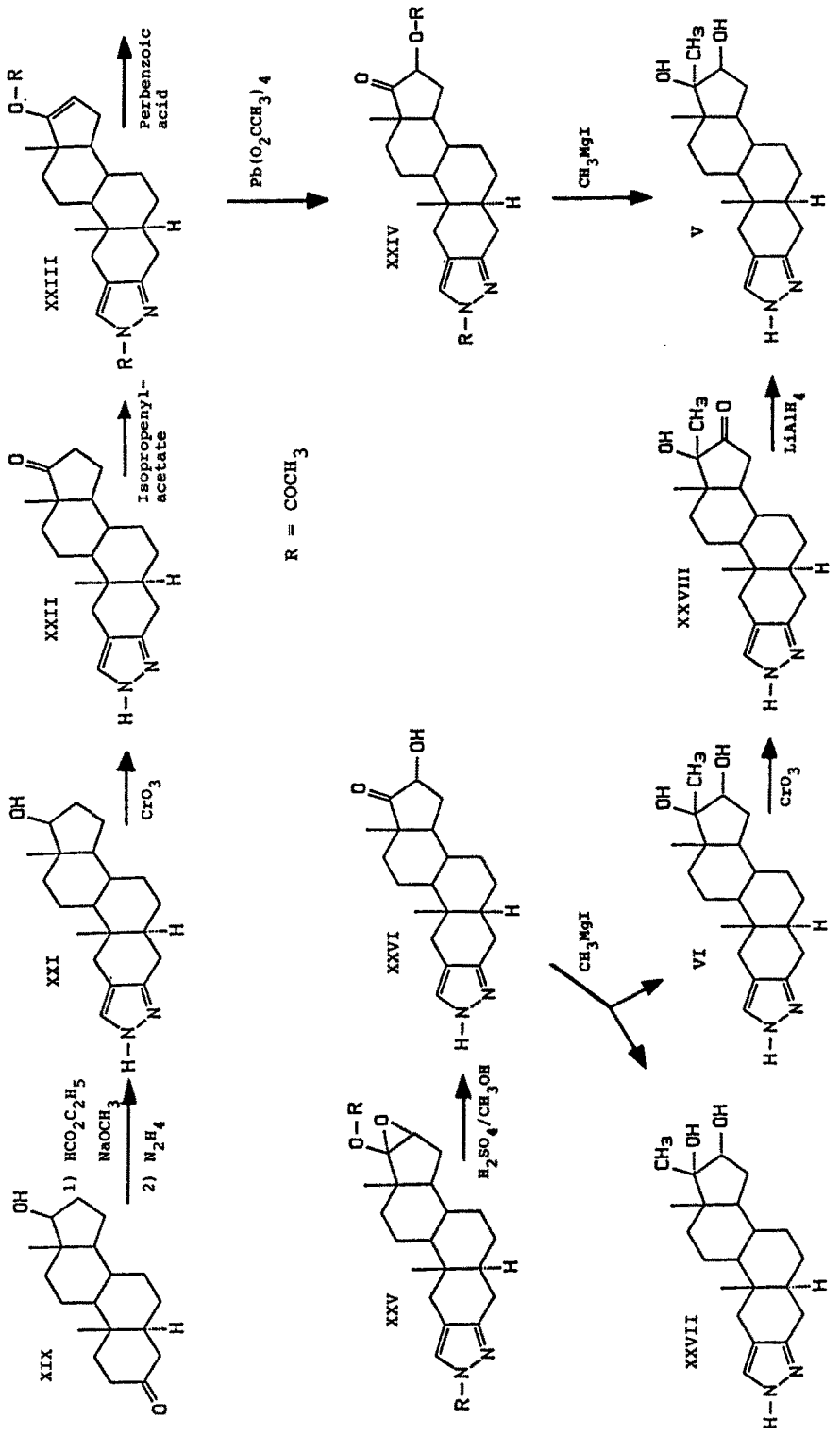

Fig. 13. Synthesis of 16α-hydroxy-, 16α-hydroxy-17-epi- and 16β-hydroxystanozolol.

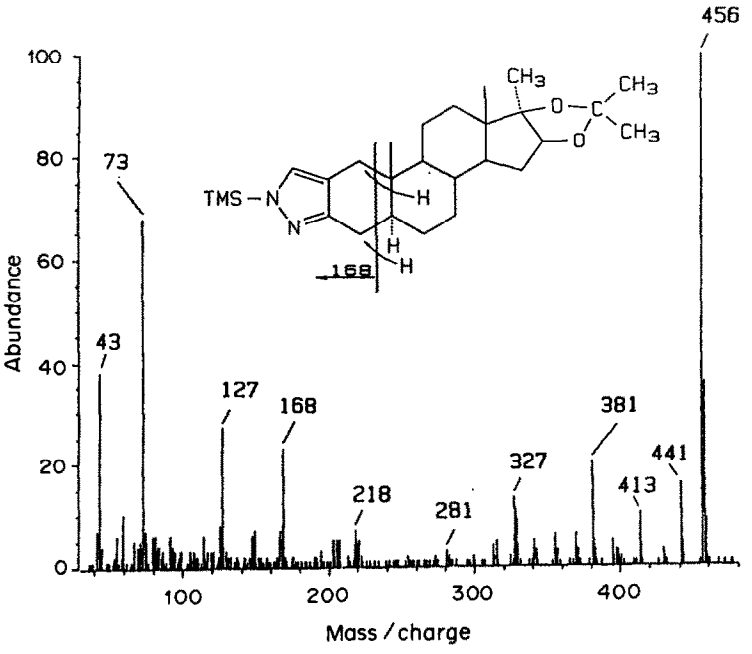

Fig. 14. EI-spectrum of 16β-hydroxystanozolol-acetonide, mono-TMS.

12–12.5 min (Table 1) of the conjugated urine fraction was identical with the mass spectrum of the per-silylated synthesised 16α-hydroxystanozolol (Fig. 16).

16α-Hydroxystanozolol, mainly detected after hydrolysis with β-glucuronidase/arylsulfatase from *Helix pomatia*, was also detected after HPLC-separation in the glucuronide fraction, but with less intensity.

The Kovats-indices of the Tris–TMS-derivatives of the 16-hydroxystanozolol-isomers (Table 4) show on a SE 54 column as on a OV 1 column the following elution order: 16β-Hydroxy-17-epistanozolol < 16α-hydroxy-17-epistanozolol < 16α-hydroxystanozolol < 16β-hydroxystanozolol.

Bis-hydroxy-metabolites

Besides mono-hydroxy-metabolites five bis-hydroxy-metabolites are detected in the conjugated urine fraction after enzymatic hydrolysis with β-glucuronidase from *E. coli*. The molecular ions *m/z* 648 and the fragment *m/z* 254 (Figs 17–21) have all five

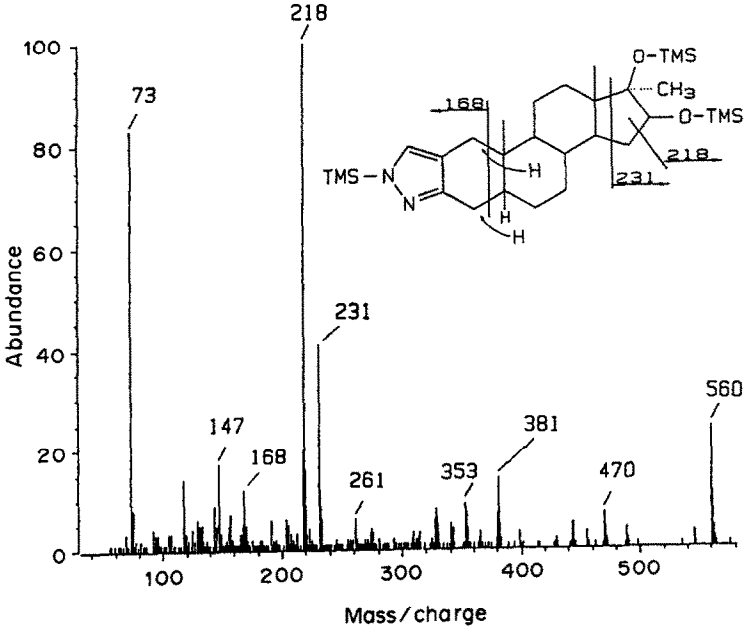

Fig. 15. EI-spectrum of 16β-hydroxystanozolol, Tris–TMS.

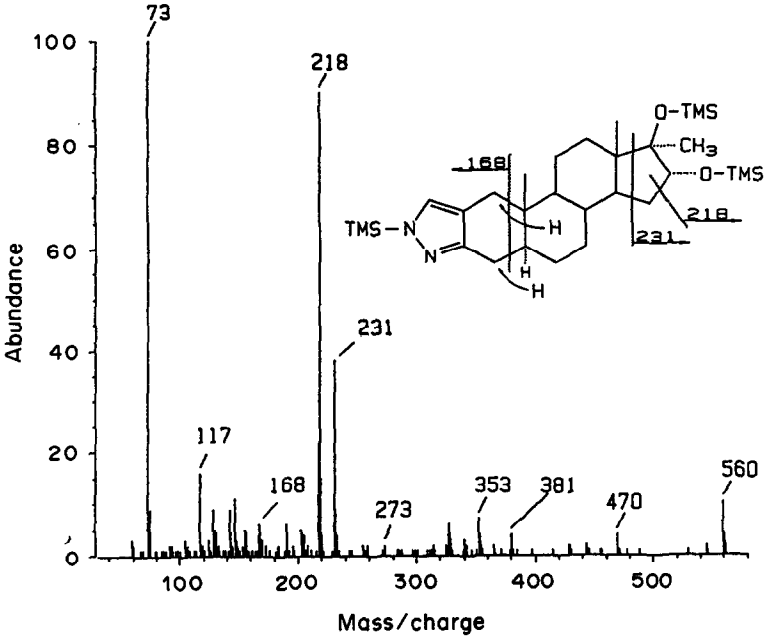

Fig. 16. EI-spectrum of 16α-hydroxystanozolol, Tris–TMS.

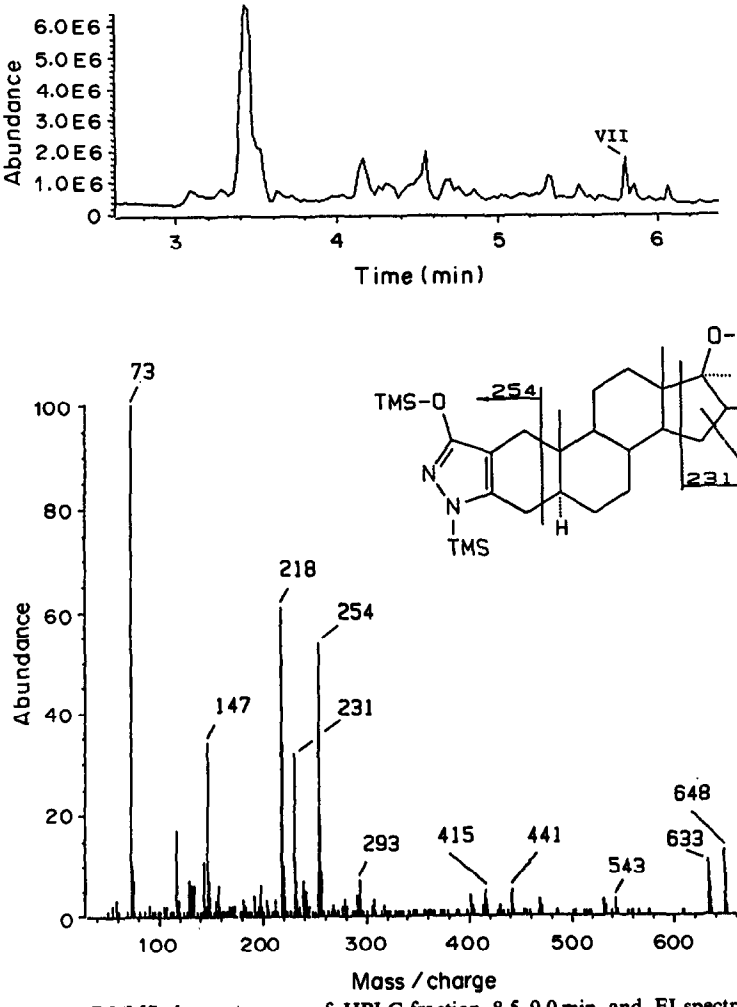

Fig. 17. GC/MS-chromatogram of HPLC-fraction 8.5–9.0 min and EI-spectrum of 3′,16-dihydroxystanozolol 1, tetra-TMS.

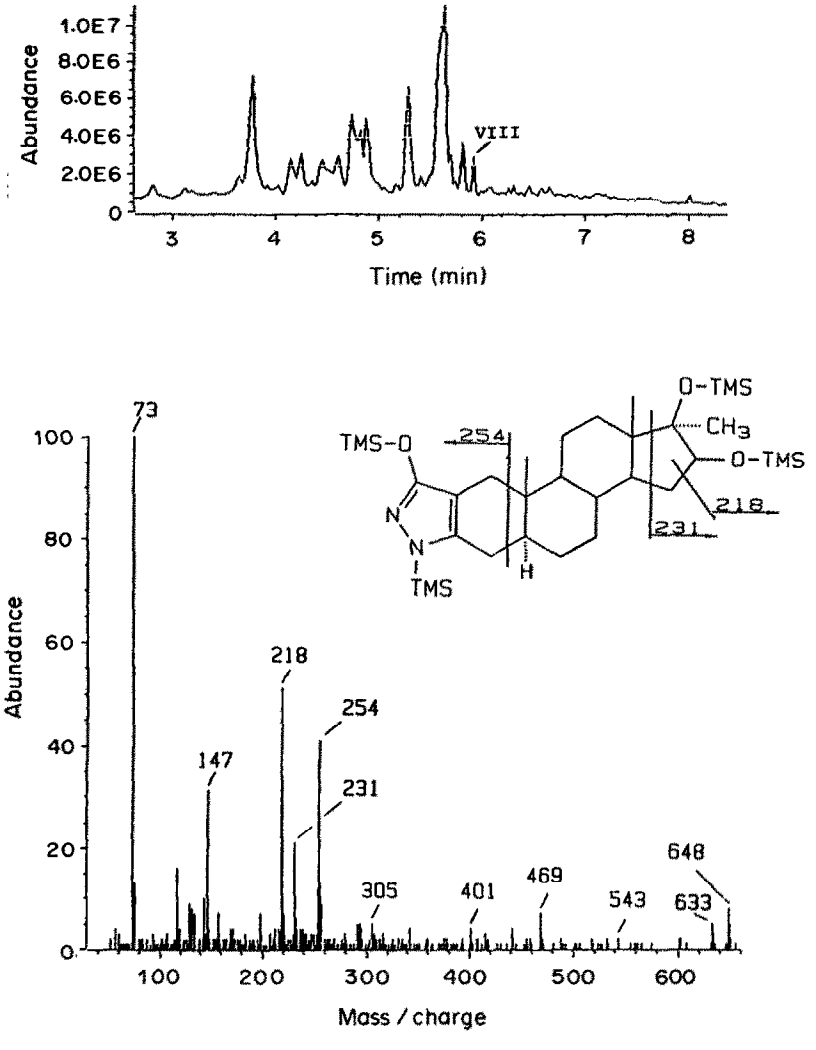

Fig. 18. GC/MS-chromatogram of HPLC-fraction 11.0–11.5 min and EI-spectrum of 3′,16-dihydroxystanozolol 2, tetra-TMS.

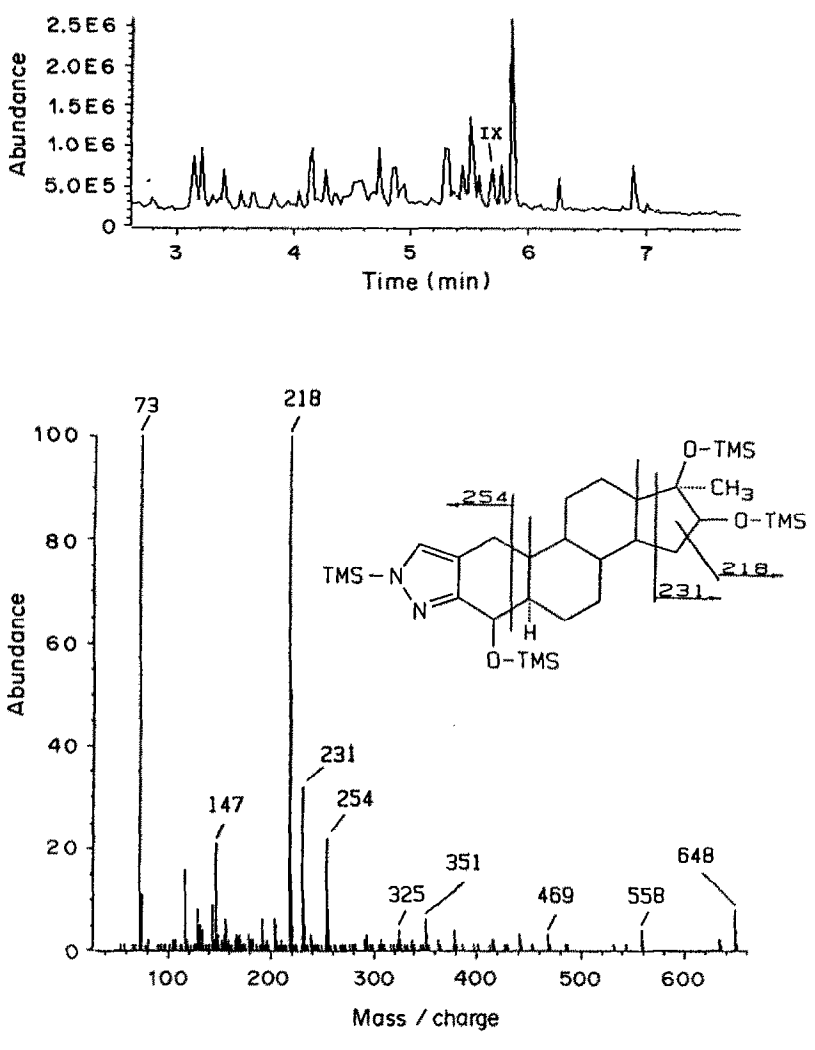

Fig. 19. GC/MS-chromatogram of HPLC-fraction 9.5–10.0 min and EI-spectrum of 4β,16-dihydroxystanozolol 1, tetra-TMS.

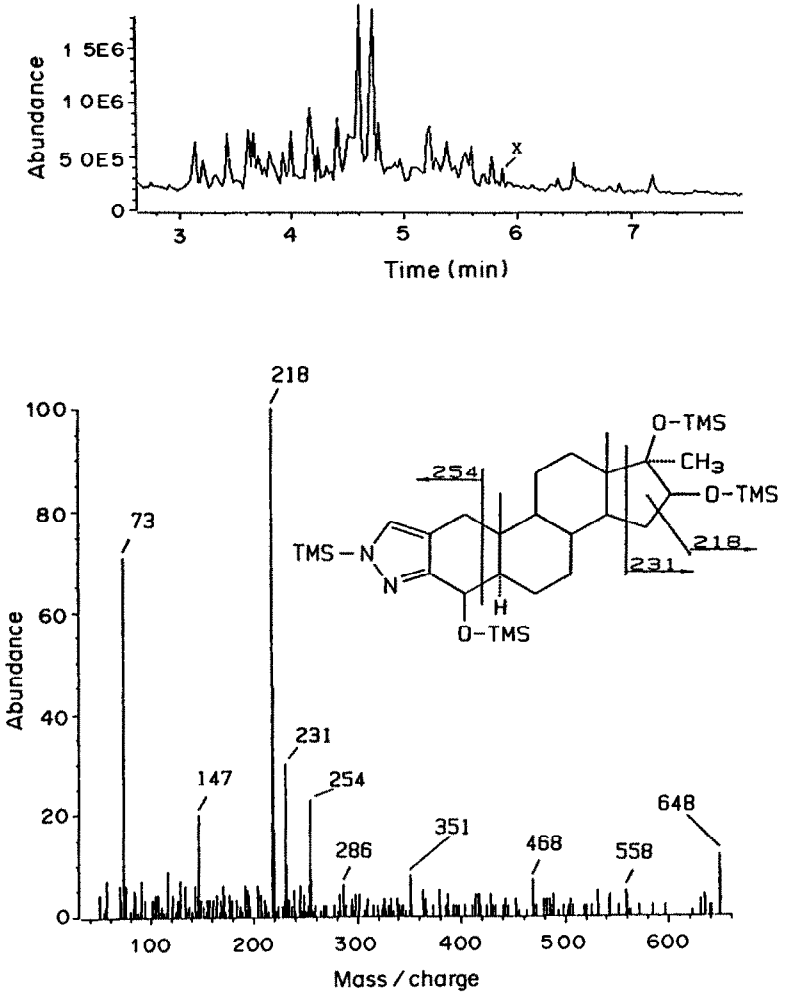

Fig. 20. GC/MS-chromatogram of HPLC-fraction 10.5–11.0 min and EI-spectrum of 4β,16-dihydroxystanozolol 2, tetra-TMS.

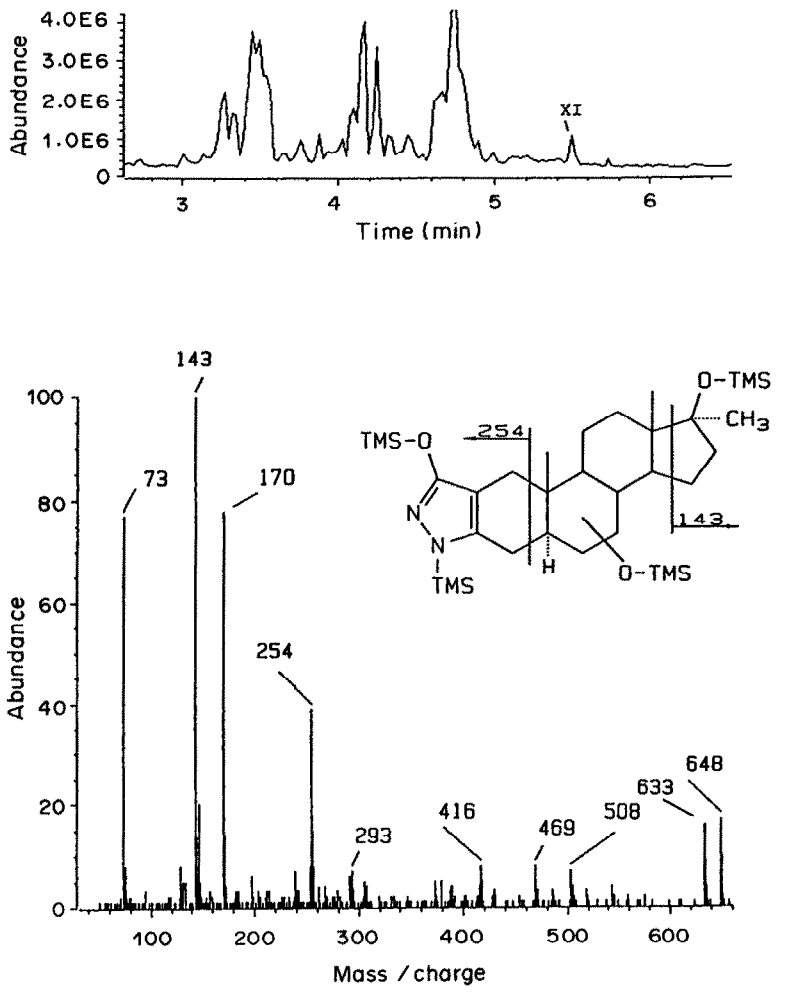

Fig. 21. GC/MS-chromatogram of HPLC-fraction 12.5–13.0 min and EI-spectrum of 3',12(?)-dihydroxystanozolol, tetra-TMS.

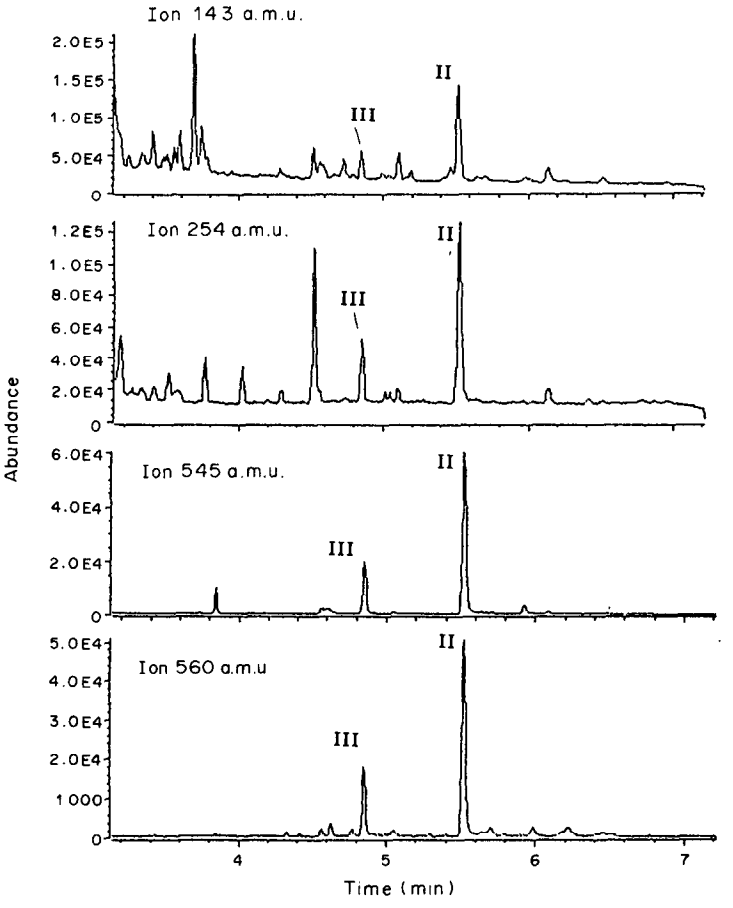

Fig. 22. Selected ion chromatogram of the unconjugated urine fraction 15.5–24 h after oral application of 40 mg of stanozolol (after 5 months storage at 4°C) with *m/z* 560, 545, 245 and 143, column and temperature program: see method, splitless-injection (**II**) 3′-hydroxystanozolol, Tris–TMS, (**III**) 3′-hydroxy-17-epistanozolol, Tris–TMS.

pertrimethylsilylated metabolites. Four of these five metabolites show the ionic fragments *m/z* 218 and *m/z* 231 indicating hydroxylation in position 16α or 16β.

The second hydroxy-group should be located either in position 3′ of the pyrazole-ring or in position 4, which is evident by the ionic fragment *m/z* 254. To determine the position of the second hydroxy-group the metabolites were methylated with methyl-iodide/acetone. Two methyl-groups are introduced into the molecule if the hydroxy-group is at C-3′. However, only one methyl-group is introduced if the hydroxy-group is at C-4. So by the comparison of pertrimethylsilylation and methylation of the 3′-hydroxy-group with subsequent trimethylsilylation the following structural assignments can be made: Figs 17 and 18, 3′,16-dihydroxystanozolol 1 and 2; Figs 19 and 20, 4,16-dihydroxystanozolol 1 and 2.

The fifth metabolite isolated from HPLC-fraction 12.5–13.0 min (Table 1) shows the ionic fragments *m/z* 143, 170, 254, 633 and 648 in the mass spectrum of the per-TMS-derivative (Fig. 21). This bis-hydroxy-metabolite has one hydroxy-group in position 3′ because two methyl-groups are introduced by methylation. The ionic fragment *m/z* 170 is still unexplained and it appears also after methylation of the pyrazole ringe and subsequent silylation. The location of the second hydroxy-group remains unknown, but position 1, 4, 15, 16, 20 can be excluded due to the appearance of *m/z* 254 and *m/z* 143.

The ionic fragment *m/z* 170 is also observed in a persilylated metabolite of metandienone and of chlorodehydromethyltestosterone [24]. The latter corresponds most probably to a metabolite assumed to be 6β,12-bis-hydroxychlorodehydromethyltestosterone [25].

GC/MS-screening

After oral application of stanozolol 3′-hydroxy-stanozolol (**II**) and 3′-hydroxy-17-epistanozolol (**III**) are detected in the unconjugated urine fraction 14.5–24 h after application (Fig. 22). The selected ion chromatogram shows the traces of the Tris–TMS-derivatives with the characteristic ions *m/z* 560 (molecular ion), 545, 254 and 143.

After hydrolysis of the same urine sample with β-glucuronidase from *E.coli* the aglycones of four metabolites are detected:

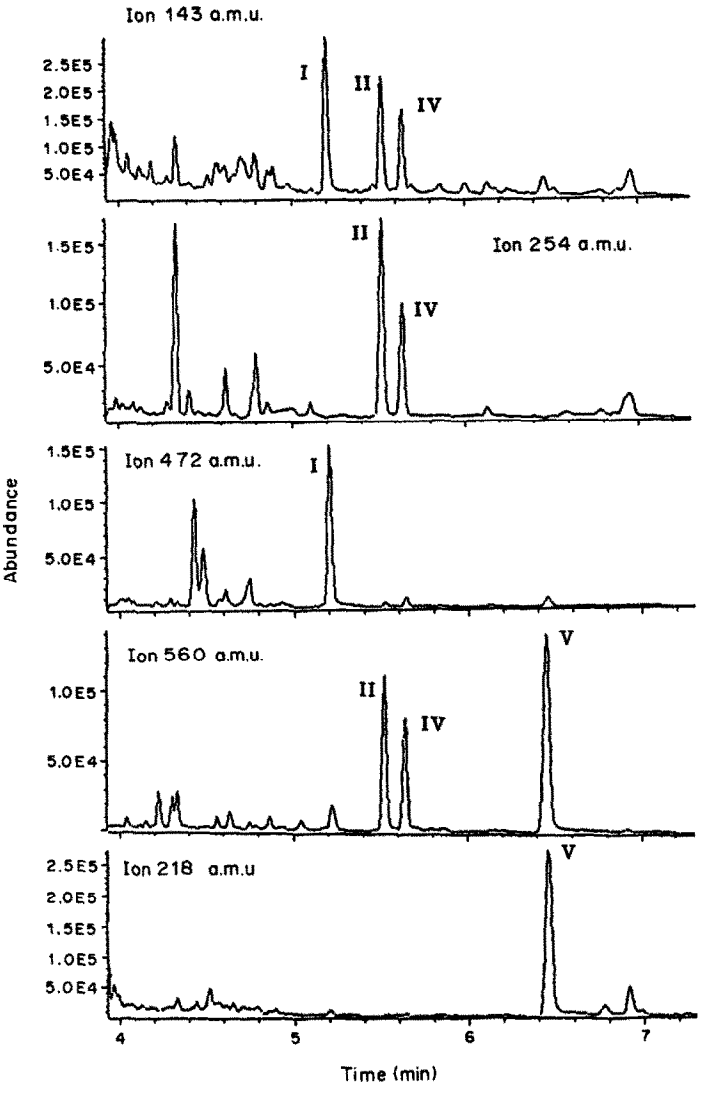

Fig. 23. Selected ion chromatogram of the conjugated urine fraction 15.5–24 h after oral application of 40 mg of stanozolol with *m/z* 560, 472, 254, 218 and 143, hydrolysis with β-glucuronidase from *E. coli*, column and temperature program: see method, (I) stanozolol, bis-TMS, (II) 3′-hydroxystanozolol, Tris-TMS, (IV) 4β-hydroxystanozolol, Tris-TMS, (V) 16β-hydroxystanozolol, Tris-TMS.

Stanozolol (I) with *m/z* 472 (molecular ion) and 143; 3′-hydroxystanozolol (II) with *m/z* 560, 254 and 143; 4β-hydroxystanozolol (IV) with *m/z* 560, 254 and 143 and; 16β-hydroxystanozolol (V) with *m/z* 560, and 218 (Fig. 23).

Kinetic studies

Two kinetic studies with quantification of urine concentrations of stanozolol and the mono-hydroxy-metabolites were performed after oral administration of 40 mg of stanozolol. In the first study only the unconjugated excreted metabolites were analysed after 10 day storage of the urine at 4°C. The detection limit of 1 ng of metabolite per ml of urine was reached only by preparing N-HFB, bis-*O*-TMS-derivatives of 3′-hydroxystanozolol and 3′-hydroxy-17-epistanozolol.

The second kinetic study with quantification of the conjugated excreted metabolites only was performed one year later when the conjugated metabolites 4β-hydroxy-, 16β-hydroxy- and 16α-hydroxystanozolol were synthesised enabling the registration of a calibration curve from spiked urine. The latter was necessary because stanozolol and the hydroxy-metabolites (mainly 16β-hydroxystanozolol) did not show linear calibration curves apparently due to adsorption effects in the gas chromatographic system.

The results are listed in Table 6 for the unconjugated excreted metabolites and in Table 7 for the conjugated excreted stanozolol and mono-hydroxy-metabolites. The better detection limit of the unconjugated metabolites may be due to a lower biological background and to the use of N-HFB, *O*-TMS-derivatives. By application of 40 mg of stanozolol

Table 6. Excretion of 3′-hydroxystanozolol (II) and of 3′-hydroxy-17-epistanozolol (III) after oral administration of 40 mg of stanozolol (unconjugated fraction)

Time after administration	II (ng/min)	III (ng/min)
0–6.5 h	3.2	2.6
6.5–15.5 h	5.9	6.6
15.5–24.0 h	8.3	7.6
24.0–31.0 h	2.7	6.2
31.0–39.0 h	3.9	8.8
39.0–50.0 h	2.5	6.3
50.0–54.5 h	4.6	5.3
54.5–63.0 h	2.6	5.2
63.0–69.5 h	4.4	6.0
89.0–92.0 h	3.0	4.2
122.0–126.0 h	1.5	1.6
137.0–141.0 h	0.6	0.7
Total excretion	23 μg	33 μg

Quantification by height of SIM-chromatograms of the *N*-heptafluorobutyryl,*O*-TMS derivatives (*m/z* 669) using 6β-hydroxymetandienone as internal standard (SIM at *m/z* 209 for the bis-TMS-derivative).

about 0.15% of the applied stanozolol are recovered in the unconjugated fraction as 3′-hydroxy-metabolites whereas in the conjugated fraction about 0.8% are excreted as stanozolol and 4.7% as mono-hydroxy-metabolites.

The main metabolite is 16β-hydroxystanozolol with a total amount of about 874 μg. The 3′-hydroxy-17-epistanozolol has its highest excretion rate between 31 and 39 h. The unconjugated excreted 3′-hydroxystanozolol seems to arise from hydrolysis of its conjugate. All four mono-hydroxy-metabolites and stanozolol have an excretion maximum between 8 and 11 h.

Hydrolysis of conjugates and nature of the conjugates

The conjugated metabolites were hydrolysed with different enzyme preparations. The main metabolites can be hydrolysed with β-glucuronidase from *E. coli* or *Helix pomatia* juice. Only 16α-hydroxystanozolol is hydrolysed to a larger extent with β-glucuronidase/arylsulfatase from *Helix pomatia* and to a less amount with the β-glucuronidase.

DISCUSSION

In the literature no information about stanozolol metabolites was found. Speculations such as a minor absorption in the gastrointestinal tract cannot explain the well documented anabolic effect of stanozolol [6–9]. Other speculations as complete, even irreversible binding to the plasma protein fraction or a distribution favouring the lipid compartments of the body are not in agreement with the metabolism of other anabolic steroids.

That the metabolites of stanozolol could not be detected by the conventional analytical GC/MS-technique of steroid analysis is to be explained by the chemical properties of the pyrazole nucleus condensed to the A-ring, the polarity of some of the metabolites and the instability of the TMS-derivatives performed prior to gas-chromatography.

Stanozolol is metabolised in man to a large extent (Fig. 1). The parent compound is also excreted as a conjugate. No stanozolol could be detected by the

Table 7. Excretion of conjugates after oral application of 40 mg of stanozolol (I): I and mono-hydroxy-metabolites

Collection period (h)	ST (I) (ng/min)	3′-HOST (ng/min)	4β-HOST (ng/min)	16β-HOST (ng/min)	16α-HOST (ng/min)
0–1.0	36	3	9	17	0
1.0–2.0	115	54	164	169	15
2.0–4.0	220	104	334	424	27
4.0–6.0	230	158	387	483	73
6.0–8.0	168	136	230	414	74
8.0–11.0	319	222	493	935	158
11.0–13.1	189	155	284	542	115
13.1–21.2	99	103	94	302	66
21.2–25.5	64	103	110	225	38
25.5–30.3	39	73	80	142	28
30.3–36.0	58	62	110	135	27
36.0–45.0	46	55	47	123	24
45.0–50.7	29	59	40	106	25
50.7–56.0	—	56	32	113	20
56.0–61.5	—	57	36	103	15
61.5–69.0	—	32	13	48	10
69.0–77.5	—	23	10	35	6
77.5–85.0	—	27	9	40	—
85.0–93.0	—	13	3	25	—
93.0–101.8	—	17	2	13	—
101.8–107.7	—	—	—	—	—
107.7–117.2	—	—	—	—	—
Total	314 μg	367 μg	468 μg	874 μg	164 μg

3′-HOST = II, 4β-HOST = IV, 16β-HOST = V, 16α-HOST = VI. Quantification by height of selected ion traces of the per-TMS-derivatives: *m/z* 472 for ST, *m/z* 545 for 3′-HOST, *m/z* 560 for 4β-HOST and *m/z* 218 for 16β-HOST and 16α-HOST and *m/z* 435 for the internal standard 17α-methy-5α-androstane-3β,17β-diol.

analytical methods used in this study in the unconjugated fraction.

After oral administration of 40 mg of stanozolol about 97% of mono-hydroxy-metabolites and stanozolol were excreted as conjugates. Only 3% of metabolites are detected in the unconjugated urine fraction. This estimation does not consider the amount of excreted bis-hydroxy-metabolites, which seem to be in lower concentrations but may be underestimated by the analytical method used. Only 5.4% of the administered stanozolol was excreted as parent compound and as mono-hydroxy-metabolites.

In the unconjugated fraction 3′-hydroxystanozolol and 3′-hydroxy-17-epistanozolol were identified. The occurrence of 3′-hydroxystanozolol is in agreement with the metabolism of pyrazole where a 3-hydroxy-metabolite was identified by Clay *et al.*[26].

Excreted 3′-hydroxystanozolol was quantified after oral application of 40 mg of stanozolol. 23 μg of 3′-hydroxystanozolol were excreted unconjugated within six days, but a much higher amount of 367 μg conjugated. The concentration of the unconjugated 3′-hydroxystanozolol was higher when the samples were stored over a longer period of time at 4°C and when the pH-value of the urine was lower than six. From this result we assume that 3′-hydroxystanozolol is excreted as an unstable conjugate which will be slowly hydrolysed under the above mentioned conditions.

It was not confirmed at what position of the pyrazolone ring 3′-hydroxystanozolol is conjugated and whether it is conjugated with glucuronic acid. As it is hydrolysed with β-glucuronidase a conjugation with glucuronic acid seems probable. A conjugation at the 3′-hydroxy-group may explain the instability of the conjugate. This assumption is supported by results obtained from mono-methylated isomers of 3′-hydroxystanozolol [24]: The *O*-methyl-derivative is unstable whereas the two *N*-methyl-derivatives are stable.

3′-Hydroxy-17-epistanozolol is the only metabolite of stanozolol with a 17α-hydroxy-17β-methyl-configuration. No further mono-hydroxy metabolite with a 17-epi-configuration was found. The configuration of the bis-hydroxy-metabolites could not be established since no reference substances are yet available. The proposed structure for the 16-hydroxy-isomers **VII/VIII** and **IX/X** were confirmed only for the A-ring.

Nearly all conjugated metabolites can be hydrolysed with β-glucuronidase. From this result we suggest a conjugation of stanozolol, 4β-hydroxystanozolol and 16β-hydroxystanozolol with glucuronic acid. A conjugation at the pyrazole ring is possible as Clay *et al.* identified a *N*-glucuronide as a major metabolite of pyrazole [26]. Only 16α-hydroxystanozolol is mainly hydrolysed with β-glucuronidase/arylsulfatase from *Helix pomatia*. An excretion of 16α-hydroxy- and 16β-hydroxystanozolol as bis-conjugated metabolites is also possible.

The hydroxylation of stanozolol in position 4β was not expected. But the 4β-hydroxylation can be understood when it is compared to the enzymatic 6β-hydroxylation of metandienone [27]. The A-ring of metandienone and the pyrazole ring of stanozolol are conjugated systems, the pyrazole even has aromatic character. A further comparison shows that the 4β-position in stanozolol and the 6β-position in metandienone are both axial and both in α-position to the conjugated system ("allylic oxidation").

The 16-hydroxylation of stanozolol is preferred in the β-position (Table 7). The ratio of 16β-hydroxystanozolol to 16α-hydroxystanozolol was estimated with 5.3 when 40 mg of stanozolol were applied orally. This result of the 16-hydroxylation is in agreement with the observations of Watabe *et al.*[28], who found that the 16-hydroxylation of methyltestosterone in rabbits leads preferentially to the 16β-position. Whether the 16β-hydroxylation of 17-methylsteroids in man is the main 16-hydroxylation too is under investigation for other 17α-methyl-17β-hydroxy steroids. Up to now 16-hydroxy-metabolites have been found in human urine in metabolism studies with metandienone, chlorodehydromethyltestosterone, oxandrolone and furazabol [24].

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