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Metabolism of anabolic steroids in man: synthesis and use of reference substances for identification of anabolic steroid metabolites

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Abstract

The use of anabolic steroids was banned by the International Olympic Committee for the first time at the Olympic Games in Montreal in 1976 Since that time the misuse of anabolic steroids by athletes has been controlled by analysis of urine extracts by gas chromatography–mass spectrometry (GC–MS) The excreted steroids or their metabolites, or both, are isolated from urine by XAD-2 adsorption, enzymatic hydrolysis of conjugated excreted metabolites with β-glucuronidase from *Escherichia coli*, liquid–liquid extraction with diethyl ether, and converted into trimethylsilyl (TMS) derivatives The confirmation of an anabolic steroid misuse is based on comparison of the electron impact ionization (EI) mass spectrum and GC retention time of the isolated steroid and/or its metabolite with the EI mass spectrum and GC retention time of authentic reference substances For this purpose excretion studies with the most common anabolic steroids were performed and the main excreted metabolites were synthesized for bolasterone, boldenone, 4-chlorodehydromethyltestosterone, clostebol, drostanolone, fluoxymesterone, formebolone, mestanolone, mesterolone, metandienone, methandriol, metenolone, methyltestosterone, nandrolone, norethandrolone, oxandrolone and stanozolol The metabolism of anabolic steroids, the synthesis of their main metabolites, their GC retention and EI mass spectra as TMS derivatives are discussed

Keywords Gas chromatography, Gas chromatography–mass spectrometry, Anabolic steroids, Metabolism

The misuse of testosterone and anabolic steroids by athletes to improve their performance has led to a ban on anabolic steroids by the International Olympic Committee (IOC) and national and international sports federations Anabolic steroids were banned for the first time at the Olympic Games 1976 in Montreal and testosterone at the Olympic Games 1984 in Los Angeles To control the misuse of an anabolic steroid the urines of athletes are collected directly after competition or out of competition and have to be analysed by IOC accredited laboratories The anabolic steroids and their metabolites are isolated from urine, derivatized with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) and analysed by gas chromatography (GC) and mass spectrometry (MS) using electron impact (EI) ionization at 70 eV Because most anabolic steroids are completely metabolized and no parent steroid is excreted, the metabolism of the anabolic steroids must be known Papers on the metabolism of anabolic steroids in man were available for boldenone [1], 4-chlorodehydromethyltestosterone [2,3], metandienone [4,5], methyltestosterone [6], nandrolone [7] and norethandrolone [8] The identification of these metabolites was based on the isolation and purification of urinary excreted metabolites after application of large amounts of

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the anabolic steroid and characterization by melting points and infrared (IR), ^{1}H nuclear magnetic resonance (NMR) and mass spectrometry At least the proposed structure of the metabolite was elucidated by its synthesis

GC with fused-silica capillary columns combined with MS with EI ionization allows very fast and relatively simple sample preparation to isolate small amounts of anabolic steroids and their metabolites from urine [9–11] The isolated metabolites are characterized by their GC retention times and EI mass spectra A metabolite is identified when the comparision with an authentic reference substance shows the same GC retention time and EI mass spectrum

As most of the anabolic steroid metabolites were not commercially available in earlier times, so-called "reference substances" were obtained from urine in excretion studies with anabolic steroids A criticism of this method was the low accuracy of a urinary "reference substance" for which the structure is not exactly known and the possible impurity of the urinary extract

To circumvent this disadvantage we started in 1987 the synthesis of the main metabolites of those anabolic steroids which were mainly misused in sports The synthesis of metabolites of boldenone [12], metandienone [13], stanozolol [14] and the synthesis of 17-epimeric steroids [15] have been published Further publications are in preparation

This paper gives an overview of the main metabolic pathways, the synthesis of the main metabolites of anabolic steroids and their trimethylsilyl (TMS) derivatization for GC–MS and presents the GC retention indices and EI mass spectra of the anabolic steroids excreted unchanged and their main metabolites, which are routinely used for screening and confirmation

The pharmacokinetics of the excreted steroids and metabolites will not be discussed

EXPERIMENTAL

Steroids and chemicals

K-Selektride (1 M potassium tri-*sec*-butylborohydride in tetrahydrofuran), lithium aluminium hydride, chromium trioxide, platinum dioxide and 10% palladium on charcoal were purchased from Aldrich (Steinheim) Bolasterone was purchased from Upjohn, 4-chlorodehydromethyltestosterone was a gift from Jenapharm (Jena), fluoxymesterone was a gift from Ciba-Geigy, furazabol was a gift from Daiichi (Tokyo), oxandrolone was a gift from Searle, oxymesterone was a gift from Carlo Erba, mesterolone, metenolone and metenolone acetate were gifts from Schering (Berlin), formebolone was a gift from A H Beckett (London), drostanolone propionate was a gift from Grunenthal (Aachen), boldenone, clostebol acetate, mestanolone, metandienone, methandriol, methandriol dipropionate, methyltestosterone, nandrolone and stanozolol were purchased from Sigma (Deisendorf), norethandrolone, 6β-hydroxytestosterone and 6β-hydroxyandrostenedione were purchased from Steraloids via Paesel (Frankfurt)

N-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was synthesized in the laboratory All other reagents and solvents were of analytical-reagent grade

Metabolism studies

Metabolism studies were performed by oral application of the anabolic steroids **I–XX** to male volunteers and by self-administration Usually a single dose of 20 mg of steroid was applied and the following urine samples were collected and stored at 4°C

Isolation of anabolic steroids and their metabolites

The steroids and/or their metabolites were isolated as described by Donike et al [9] with some modifications The actual sample preparation is described

Isolation of unconjugated excreted steroids

To 5 ml of urine are added 0 5 g of sodium hydrogencarbonate–potassium carbonate (2 1, w/w) solid buffer and 50 ng of 4α-hydroxystanozolol as internal standard and the unconjugated steroids are extracted with 5 ml of diethyl ether (distilled over calcium hydride) The ether

layer is transferred after centrifugation and evaporated to dryness under vacuum

See also the isolation of conjugated steroids

Isolation of conjugated excreted steroids and metabolites

To 2 ml of urine is added 1 μg of methyltestosterone (internal standard) and the steroids are adsorbed on Amberlite XAD-2 polystyrene resin The XAD-2 columns (Pasteur pipette, closed with a glass pearl, bed height 2 cm) are washed with 2 ml of doubly distilled water and eluted with 2 ml of methanol The methanolic eluate is evaporated to dryness and the residue is dissolved in 1 ml of 0 2 M sodium phosphate buffer (pH 7)

At this stage a separation of conjugated and unconjugated excreted steroids is possible The unconjugated steroids can be extracted with 5 ml of diethyl ether The remaining ether must be removed by evaporation of the buffer solution for a short time To the buffer solution 50 μl of β-glucoronidase from *E coli* (K12, Boehringer, Mannheim) are added and after 1 h at 50°C the buffer solution is made alkaline (pH 9–10) with 250 μl of 5% potassium carbonate solution and the steroids are extracted with 5 ml of diethyl ether on a vibro mixer for 30 s After centrifugation the ether layer is transferred and evaporated to dryness under vacuum

Derivatization for GC–MS analysis

Unconjugated fraction The dry residue is derivatized with 50 μl of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide–imidazole (MSTFA–Imi) (100 2, v/w) and heated for 30 min at 80°C [9]

Conjugated fraction The dry residue is derivatized with 100 μl of MSTFA–ammonium iodide–dithioerythritol (1000 2 4, v/w/w) and heated for 15 min at 60°C This reaction mixture is equivalent to a mixture of MSTFA–trimethyliodosilane (TMIS) (1000 2, v/v) [16]

GC–MS parameters

Unconjugated fraction A Hewlett-Packard HP 5890 gas chromatograph and an HP 5970 mass spectrometer were used. The carrier gas was helium at 1 ml/min at 180°C, splitting ratio 1 10 Column C was used (see GC–FID parameters) The column temperature was programmed from 200°C at 40°C/min to 320°C (held for 3 min) The injector temperature was 300°C and the interface temperature 320°C

Conjugated fraction The instruments and carrier gas were the same as above Column A was used (see GC–FID parameters) The column temperature was programmed from 180°C at 4°C/min to 240°C and at 15°C/min to 320°C The injector temperature was 300°C and the interface temperature 320°C

GC–FID parameters

An HP 5880 gas chromatograph was used The carrier gas was helium at 1 5 ml/min at 180°C, splitting ratio 1 : 10, pressure 27 psi The column temperature was programmed from 180°C at 5°C/min to 320°C Column A was Hewlett–Packard Ultra-1 fused silica, cross-linked methylsilicone (OV-1), 17 m × 0 2 mm i d , film thickness 0 11 μm Column B was Hewlett–Packard HP-1 fused silica, cross-linked methylsilicone (OV-1), 17 m × 0 25 mm i d , film thickness 0 33 μm Column C was Chrompack WCOT fused silica CP-SIL 8CB, cross-linked 5% phenyl–methylsilicone (SE-54), 17 m × 0 25 mm i d , film thickness 0 25 μm Column D was Macherey–Nagel Permabond, fused silica, cross-linked 5% phenyl–methylsilicone (SE-54), 17 m × 0 25 mm i d , film thickness 0 25 μm Column E was Hewlett–Packard HP-5, fused silica, 5% phenyl–methylsilicone (SE-54), 17 m × 0 2 mm i d , film thickness 0 33 μm

Synthesis of reference substances (general description)

Oxidation of secondary hydroxy groups with chromium trioxide The substance which was to be oxidized was dissolved in 96% acetic acid and with stirring a solution of 10% chromium trioxide in 96% acetic acid was added within 10 min In general an equimolar addition of chromium trioxide was sufficient, yielding quantitative oxidation After 30 min of reaction the reaction mixture was neutralized and then made alkaline (pH 12–14) with a cooled aqueous solution of 20% potassium hydroxide The reaction products were extracted

with a tenfold volume of diethyl ether The ether layer was washed with water, dried over sodium sulphate and evaporated to dryness under vacuum

Reduction of the 4,5-double bond Reduction of the 4,5-double bond in 3-keto-4-ene steroids was performed with hydrogen using methanol or methanol–6 M potassium or sodium hydroxide (10 1, v/v) as solvent and platinum dioxide or 10% palladium on charcoal as catalyst

Reduction of 3-keto groups with lithium aluminium hydride Reduction with lithium aluminium hydride was performed in absolute dry diethyl ether or in tetrahydrofuran (both freshly distilled over calcium hydride) depending on the solubility of the substance which was to be reduced The reaction mixture was held under argon and while stirring solid lithium aluminium hydride was added (1 equimolar excess) After a further 15 min the reaction mixture was poured into the same volume of water and the reaction products were extracted with a tenfold volume of diethyl ether The ether layer was washed with water, dried over sodium sulphate and then evaporated to dryness under vacuum

Reduction of 3-keto groups with K-Selectride The reaction and sample preparation were performed in the same way as the reaction with lithium aluminium hydride. The K-Selektride was added in an equimolar amount to the stirred solution held under argon within 10 min

Reduction of 3-keto groups with hydrogen The reduction of 3-keto groups with hydrogen using platinum dioxide or 10% palladium on charcoal was performed in the same way as the reduction of the 4,5-double bond, but the reaction was prolonged to 3 days

6β-Hydroxylation. Stereospecific 6β-hydroxylation of androst-4-en-3-ones and androsta-1,4-diene-3-ones was performed with the following procedure The substance was dissolved in ethyl acetate–MSTFA–ammonium iodide (100 10 0 5, v/v/w) and refluxed for 15 min The obtained androsta-3,5-dien-3-enol and androsta-1,3,5-trien-3-enol TMS-ethers were extracted with *n*-pentane after addition of potassium carbonate and water to the reaction mixture The *n*-pentane phase was evaporated to dryness and the residue dissolved in ethanol or isopropanol. The ethanolic solution was exposed to sunlight while stirring for about 4–8 h The androsta-3,5-dien-3-enol and androsta-1,3,5-trien-3-enol TMS-ethers were autooxidized under these conditions at C-6, yielding a 6β-hydroxyandrost-4-en-3-one or 6β-hydroxyandrosta-1,4-dien-3-one structure During further sample preparation remaining TMS groups, e g , 17β-*O*-TMS, were hydrolysed A detailed description of this synthesis is in preparation.

RESULTS AND DISCUSSION

Synthesis of reference substances

A general description of the often used reaction schemes for the synthesis of anabolic steroid metabolites will be described The synthesis of boldenone [12], conjugated metandienone [13], stanozolol [14] metabolites and 17-epimerization of 17α-methyl-17β-hydroxy steroids [15] have already been published The synthesis of other metabolites will be published elsewhere.

All the synthesized metabolites were obtained as pure crystals with a yield between 60 and 2000 mg

Oxidation of secondary hydroxy groups with chromium trioxide

A selective oxidation of only the 3-hydroxy group or 17β-hydroxy group when both hydroxy groups were present was not possible As all synthesized metabolites have a 17-keto- or 17-hydroxy-17-methyl group, this unspecific oxidation was chosen followed by a group-selective reduction of the 3-keto group only

Reduction of the 4,5-double bond

The reduction performed in methanol yielded in general a mixture of 5α- and 5β-isomers in comparable amounts whereas the reaction in methanol–aqueous potassium hydroxide or sodium hydroxide favours the production of the 5β-isomer To obtain a 5α-isomer methanol was chosen as solvent, yielding 5α- and 5β-isomers, which were then separated by column chromatography

on silica gel followed by repeated crystallization The commonly used Birch reaction which yields mainly the 5α-isomer was not applied

Reduction of 3-keto groups with lithium aluminium hydride

Reduction with lithium aluminium hydride is not group selective If more than one keto group is present, e g , in 5α-estrane-3,17-dione, both keto groups will react immediately with the reagent The reduction is stereospecific to 3-keto groups which will be reduced mainly to 3β-hydroxy (Table 1) when the A-ring has a 5α or 4-ene configuration but the 3-keto group will be reduced in over 80% yield to 3α-hydroxy when the A-ring has a 5β configuration (Table 1)

TABLE 1

Reduction yields of 3-keto groups with $LiAlH_4$ and K-Selektride

Substance	$LiAlH_4$		K-Selektride	
	3α-OH (%) [b]	3β-OH (%) [b]	3α-OH (%) [b]	3β-OH (%) [b]
5α-Configuration				
5α-Androstan-17β-ol-3-one	12	88	88	12
5α-Androstane-3,17-dione	11	89	93	7
5α-Estrane-3,17-dione [a]	13	87	93	7
1-Methylene-5α-androstane-3,17-dione [a]	7	93	95	5
1α-Methyl-5α-androstan-17β-ol-3-one	49	51	> 99	< 1
1α-Methyl-5α-androstane-3,17-dione [a]	49	51	> 99	< 1
2α-Methyl-5α-androstan-17β-ol-3-one	13	87	> 99	< 1
2α-Methyl-5α-androstane-3,17-dione [a]	8	92	99	1
17α-Methyl-5α-androstan-17β-ol-3-one	11	89	88	12
17β-Methyl-5α-androstan-17α-ol-3-one [a]	11	89	93	7
17α-Ethyl-5α-estran-17β-ol-3-one [a]	14	86	93	7
7α,17α-Dimethyl-5α-androstan-17β-ol-3-one [a]	10	90	87	13
5β-Configuration				
5β-Androstan-17β-ol-3-one	89	11	4	96
5β-Estrane-3,17-dione [a]	90	10	6	94
17α-Methyl-5β-androstan-17β-ol-3-one	87	13	9	91
17β-Methyl-5β-androstan-17α-ol-3-one [a]	88	12	6	94
17α-Ethyl-5β-estran-17β-ol-3-one [a]	90	10	5	95
7α,17α-Dimethyl-5β-androstan-17β-ol-3-one [a]	80	20	4	96
5α-Androst-1-ene-configuration				
1-Methyl-5α-androst-1-en-17β-ol-3-one	9	91	10	90
1-Methyl-5α-androst-1-ene-3,17-dione [a]	10	90	10	90
Androst-4-en-3-one-configuration				
Androst-4-en-17β-ol-3-one	13	87	30	70
Androst-4-en-17α-ol-3-one	14	86	38	62
Estr-4-en-17β-ol-3-one	19	81	42	58
17α-Methylandrost-4-en-17β-ol-3-one	10	90	28	72
17β-Methylandrost-4-en-17α-ol-3-one [a]	14	86	36	64
Androst-4-ene-6β,17β-diol-3-one	14	86	15	85
Androst-4-en-6β-ol-3,17-dione	19	81	16	84
9α-Fluoro-17α-methylandrost-4-ene-11β,17β-diol-3-one	28	72	90	10
9α-Fluoro-17α-methylandrost-4-ene-6β,11β,17β-triol-3-one [a]	27	73	72	28
4-Chloroandrost-4-en-17β-ol-3-one	11	89	14	86
4-Chloroandrost-4-ene-3,17-dione [a]	13	87	19	81

[a] Synthesized reference substance [b] Percentage values were calculated from GC–FID analysis after reduction (see Experimental) of 3 mg of each reference compound

TABLE 2

Screening for anabolic steroids (Status Cologne Laboratory, 1st March 1992)

Anabolic steroid	Main excreted substance parent and/or metabolite [a]	Origin of substance used for confirmation [b]	Excretion in urine [c]
Bolasterone	7α,17α-Dimethyl-5β-androstane-3α,17β-diol (I)	Synthesized	Conjugated
Boldenone	Boldenone (II)	Parent	Conjugated
	5β-Androst-1-en-17β-ol-3-one (III)	Synthesized	Conjugated
4-Chlorodehydro-methyltestosterone	6β-Hydroxy-4-chloro-dehydromethyl-testosterone (IV)	Synthesized	"Free"
Clostebol	4-Chloro-androst-4-en-3α-ol-17-one (V)	Synthesized	Conjugated
Drostanolone	2α-Methyl-5α-androstan-3α-ol-17-one (VI)	Synthesized	Conjugated
Fluoxymesterone	9α-Fluoro-18-nor-17,17-dimethyl-androsta-4,13-dien-11β-ol-3-one (VII)	Synthesized	"Free"
	9α-Fluoro-17α-methyl-androst-4-ene-3α,6β, 11β,17β-tetrol (VIII)	Synthesized	"Free"
Formebolone	2-Hydroxymethyl-17α-methyl-androsta-1,4-diene-11α,17β-diol-3-one (IX)	Synthesized	"Free"
Furazabol	16z-Hydroxyfurazabol	Urine ex study	Conjugated
Mestanolone	17α-Methyl-5α-androstane-3α,17β-diol (X)	Synthesized	Conjugated
Mesterolone	1α-Methyl-5α-androstan-3α-ol-17-one (XI)	Synthesized	Conjugated
Metandienone	17-Epimetandienone (XII)	Synthesized	"Free"
	6β-Hydroxymetandienone (XIII)	Synthesized	"Free"
	17α-Methyl-5β-androstane-3α,17β-diol (XIV)	Synthesized	Conjugated
	17β-Methyl-5β-androst-1-ene-3α,17α-diol (XV)	Synthesized	Conjugated
Methandriol	17α-Methyl-5β-androstane-3α,17β-diol (XIV)	Synthesized	Conjugated
Metenolone	Metenolone (XVI)	Parent	Conjugated
	1-Methylene-5α-androstan-3α-ol-17-one (XVII)	Synthesized	Conjugated
Methyltestosterone	17α-Methyl-5α-androstane-3α,17β-diol (X)	Synthesized	Conjugated
	17α-Methyl-5β-androstane-3α,17β-diol (XIV)	Synthesized	Conjugated
Nandrolone	5α-Estran-3α-ol-17-one (XVIII)	Synthesized	Conjugated
	5β-Estran-3α-ol-17-one (XIX)	Synthesized	Conjugated
Norethandrolone	17α-Ethyl-5β-estrane-3α,17β-diol (XX)	Synthesized	Conjugated
Oxandrolone	Oxandrolone (XXI)	Parent	"Free"
	17-Epioxandrolone (XXII)	Synthesized	"Free"
Oxymesterone	Oxymesterone (XXIII)	Parent	Conjugated
Oxymetholone	17α-Methyl-5α-androstane-3α,17β-diol (X)	Synthesized	Conjugated
	2z-Hydroxymethyl-17α-methyl-5α-androstane-3z,z,17β-triol	Urine ex study	Conjugated
Stanozolol	3'-Hydroxystanozolol (XXIV)	Synthesized	Conjugated and "free"
	3'-Hydroxy-17-epistanozolol (XXV)	Synthesized	"Free"
	4β-Hydroxystanozolol (XXVI)	Synthesized	Conjugated
	16β-Hydroxystanozolol (XXVII)	Synthesized	Conjugated

[a] z = Configuration not identified [b] Urine ex study = metabolite obtained from an excretion study [c] "Free" = unconjugated

Reduction of 3-keto groups with K-Selectride

The use of K-Selectride [17,18] instead of lithium aluminium hydride shows group selectivity, e g, the 3-keto group in 5α-estrane-3,17-dione will be reduced first and then with excess of reagent the 17-keto group reacts Moreover, the reduction of 3-keto groups is stereoselective, but different to lithium aluminium hydride reduction The 3-keto group reacts in over 85% yield to give 3α-hydroxy when the A-ring has a 5α configuration (Table 1) and it is reduced in more than 85% yield to 3β-hydroxy when the A-ring has a 5β configuration (Table 1), in both instances the reaction yields the opposite configuration to reduction with lithium aluminium hydride When a 3-keto-4-ene configuration is reduced with K-Selektride the 3-keto group reacts mainly to give 3β-hydroxy similarly to the reaction with lithium aluminium hydride (Table 1), but the yield of the 3α-isomer is much higher than in the reduction

TABLE 3

Temperature programme Kováts indices of anabolic steroids and their metabolites [a]

Steroid	Column				
	A OV-1 0 11 μm	B OV-1 0 33 μm	C SE-54 0 25 μm	D SE-54 0 25 μm	E SE-54 0 33 μm
5α-Estran-3α-ol-17-one bis-TMS (XVIII)	2440	2453	2451	2454	2468
5β-Androst-1-en-17β-ol-3-one bis-TMS (III)	2452	2467	2469	2471	2488
17β-Methyl-5β-androst-1-ene-3α,17α-diol bis-TMS (XV)	2454	2468	2472	2474	2492
5βEstran-3α-ol-17-one bis-TMS (XIX)	2490	2505	2502	2504	2520
2α-Methyl-5α-androstan-3α-ol-17-one bis-TMS (VI)	2555	2574	2563	2565	5686
1-Methylen-5α-androstan-3α-ol-17-one bis-TMS (XVII)	2583	2604	2599	2602	2623
9α-Fluoro-18-nor-17,17-dimethyl-4,13-dien-11β-ol-3-one bis-TMS (VII)	2600	2621	2619	2621	2641
17α-Methyl-5β-androst-1-ene-3α,17β-diol bis-TMS (epi-XV)	2607	2628	2626	2630	2649
1α-Methyl-5α-androstan-3α-ol-17-one bis-TMS (XI)	2607	2630	2619	2623	2644
17α-Methyl-5α-androstane-3α,17β-diol bis-TMS (X)	2611	2632	2625	2628	2650
17α-Methyl-5β-androstane-3α,17β-diol bis-TMS (XIV)	2617	2636	2628	2631	2654
17-Epimetandienone TMS (XII)	2625	2657	2674	2680	2713
Boldenone bis-TMS (II)	2648	2671	2671	2674	2698
17-Epioxandrolone TMS (XXII)	2673	2707	2735	2741	2777
7α,17α-Dimethyl-5β-androstane-3α,17β-diol bis-TMS (I)	2692	2713	2706	2710	2730
4-Chloroandrost-4-en-3α-ol-17-one bis-TMS (V)	2693	2712	2720	2724	2746
Metenolone bis-TMS (XVI)	2694	2718	2716	2721	2743
17α-Ethyl-5β-estrane-3α,17β-diol bis-TMS (XX)	2695	2715	2710	2714	2733
Methyltestosterone bis-TMS (internal standard)	2754	2778	2775	2779	2803
Oxandrolone TMS (XXI)	2778	2816	2845	2851	2893
6β-Hydroxymetandienone bis-TMS (XIII)	2846	2873	2877	2882	2911
9α-Fluoro-17α-methylandrost-4-ene-3α,6β,11β,17β-tetrol tetra-TMS (VIII)	2854	2855	2855	2854	2860
Oxymesterone tris-TMS (XXIII)	2952	2977	2968	2972	2993
6β-Hydroxy-4-chlorodehydromethyl-testosterone bis-TMS (IV)	3007	3039	3044	3048	3081
3'-Hydroxy-17-epistanozolol tris-TMS (XXV)	3100	3113	3119	3122	3137
2z-Hydroxymethyl-17α-methyl-androsta-1,4-diene-11α-,17β-diol-3-one tris-TMS (IX)	3163	3187	3203	3206	3232
3'-Hydroxystanozolol tris-TMS (XXIV)	3219	3238	3242	3245	3266
4β-Hydroxystanozolol tris-TMS (XXVI)	3219	3245	3244	[b]	3277
4α-Hydroxystanozolol tris-TMS (internal standard)	3238	3262	3268	[b]	3296
16β-Hydroxystanozolol tris-TMS (XXVII)	3334	3360	3368	3402	3397

[a] Indices were determined on columns A–E (see Experimental) Temperature programme from 180°C at 5°C/min to 320°C

[b] Substance showed strong tailing

with lithium aluminium hydride, in particular, fluoxymesterone and 6β-hydroxyfluoxymesterone react to give the 3α-hydroxy isomers with 90 and 72% yields (Table 1)

Reduction of 3-keto groups with hydrogen

The catalytic reduction of 3-keto groups was group selective because the 3-keto group reacts much faster than the 17-keto group A stereoselective reduction yielding preferentially a 3α- or 3β-hydroxy configuration was not obtained

6β-Hydroxylation

6β-Hydroxylation of steroids was first described for cortisol in 1954 by Burstein et al [19] and in 1956 by Nadel et al [20] for cortisol In 1967 Cardi and Lusignani [21] published a very simple procedure for the synthesis of 6β-hydroxy steroids They converted androst-4-ene-3-ones into their corresponding *n*-alkyl-3,5-diene enol ethers, especially ethyl enol ethers, and exposed them, dissolved in ethanol, to direct sunlight The autooxidation yielded 6β-hydroxyandrost-4-en-3-one steroids in high yield This reaction was chosen here to obtain 6β-hydroxy derivatives of testosterone, metandienone, 4-chlorodehydromethyltestosterone and fluoxymesterone As the formation of 3-enol alkyl ethers of 17α-methyl-17β-hydroxy steroids by commonly described methods led to dehydration of the acidic lable 17β-hydroxy group, trimethylsilyl enol ethers were used and these reactions were easily performed using MSTFA–ammonium iodide The androsta-3,5-dien-3-enol and androsta-1,3,5-trien-3-enol TMS-ethers were stable in ethanol and were autooxidized when exposed to sunlight, yielding 6β-hydroxy-androst-4-en-3-one and 6β-hydroxy-androsta-1,4-dien-3-one steroids Testosterone was used as a reference and was autooxidized to 6β-hydroxytestosterone, which was confirmed using authentic reference substance

17-Epimerization of 17α-methyl-17β-hydroxy steroids

17-Epimers were synthesized for the first time by Macdonald et al [5] in 1971, confirming 17-epimetandienone as a metabolite of metandienone Edlund et al [22] in 1989 published a simple method for the synthesis of 17-epimetandienone They dissolved metandienone-17-sulphate in water The sulphate decomposed in water, yielding several dehydration products and 17-epimetandienone In this work, 17-sulphates of 17α-methyl-17β-hydroxy steroids were synthesized using sulphur trioxide–pyridine complex in dimethylformamide, and then dissolved in water, yielding the 17-epimers

Screening of anabolic steroids

Routinely 20 anabolic steroids were controlled by a screening method for 29 steroids (4 parent steroids and 25 metabolites, see Table 2), 27 of which (**I–XXVII**) were available as pure reference substances (4 parent compounds and 23 synthesized metabolites) Two metabolites (furazabol and oxymetholone) were obtained from urinary excretion studies Table 2 shows further which anabolic steroids and metabolites are excreted unconjugated or conjugated The hydrolysis of conjugates was performed with β-glucuronidase from *E coli* The screening samples were analysed by GC–MS (see Experimental) with selected ion monitoring (SIM) using two or three of the most abundant ions for each substance

Kováts indices (methylene units)

Temperature-programmed Kováts indices (methylene units) were determined for the trimethylsilylated anabolic steroids and synthesized metabolites to be screened (Table 3) A column temperature programme from 180°C at 5°C/min to 320°C was used This programme was chosen to determine all the steroids within a 25-min programme Five different columns were tested, namely OV-1 and SE-54 with different film thicknesses and from different companies All the columns had a length of 17 m The anabolic steroids and their metabolites were determined as TMS derivatives (the same derivatives as used in routine control), the conjugated excreted steroids and fluoxymesterone metabolites were trimethylsilylated to per-TMS derivatives whereas the unconjugated steroids were derivatized with MSTFA–Imi, which does not derivatize androsta-1,4-diene-3-ones to enol TMS derivatives

The results for column B (OV-1, 0 33 μm) and column C (SE-54, 0 25 μm) show for almost all the steroids almost identical indices (differences less than 10 methylene units) Oxandrolone (**XXI**) and epioxandrolone (**XXII**) both show a difference of about 30 methylene units and the metabolite of formebolone a difference of 16 methylene units

Mass spectrometry

All anabolic steroids and their metabolites were analysed as TMS derivatives by mass spectrometry with EI ionization at 70 eV General EI fragmentation patterns were registered for 17α-methyl-17β-hydroxy steroids and their 17-epimers which show as TMS derivatives an abundant D-ring fragment at *m/z* 143 and with less intensity an ion at *m/z* 130 [5,23]

16-Hydroxylated 17α-methyl-17β-hydroxy steroids display the corresponding fragment ions (+88 u) when they are trimethylsilylated, at *m/z* 231 and 218, respectively

17-Keto steroids show a typical C/D-ring fragment at *m/z* 169 when the 17-keto group is trimethylsilylated to an enol TMS derivative The fragment at *m/z* 169 is still unexplained and the only information is that deuteration at C-16 gave a fragment at *m/z* 170 (results not shown)

Metabolism of anabolic steroids and synthesis of metabolites

The metabolism of 20 anabolic steroids after oral application was investigated (Table 2) For the following discussion the anabolic steroids were divided according to their structure into five classes androst-4-en-3-ones, androsta-1,4-dien-3-ones, 5α-androstan-3-ones, 5α-androstanes with special structure and methandriol

For the investigated anabolic steroids a brief overview of the main excreted metabolites and their synthesis is presented

Androst-4-en-3-ones

Methyltestosterone Methyltestosterone (17α-methylandrost-4-en-17β-ol-3-one) was first synthesized in 1935 by Ruzieka et al [24] and in 1937 by Oppenhauer [25] The metabolism of methyltestosterone in man was investigated by Rongone

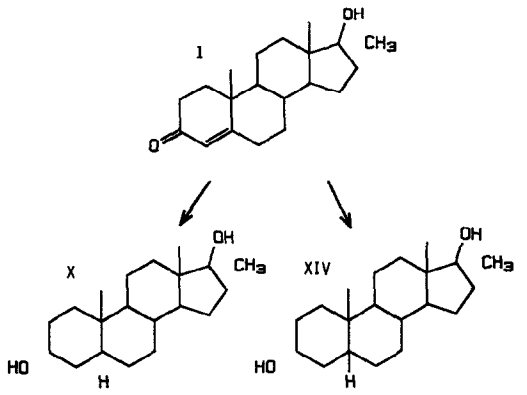

Fig 1 Metabolism of methyltestosterone (1) to 17α-methyl-5α-androstane-3α,17β-diol (X) and 17α-methyl-5β-androstane-3α,17β-diol (XIV)

and Segaloff in 1962 [6], identifying 17α-methyl-5α-androstane-3α,17β-diol (**X**) and 17α-methyl-5β-androstane-3α,17β-diol (**XIV**) as the main metabolites (Fig 1), a similar A-ring metabolism to that for testosterone [26].

Compared with the metabolism of testosterone, the ratio of the urinary excreted 17α-methyl 5α/5β-isomers is different to the ratio of androsterone to etiocholanolone (5α/5β), the main metabolites of testosterone Excretion studies with a male volunteer showed a 5α/5β (**X/XIX**) ratio of 1 6 4 after oral application of 100 mg of testosterone (0–60 h after application) and a ratio of 1 5 1 when 10 mg of methyltestosterone were applied orally The same subject showed in both excretion studies an androsterone/etiocholanolone ratio of 1·1 1

Both **X** and **XIV** were synthesized by a reaction of the Grignard reagent methylmagnesium iodide with 5α-androstan-3α-ol-17-one (androsterone) and 5β-androstan-3α-ol-17β-one (etiocholanolone) The 17-keto group reacts in about 95% yield to give a 17α-methyl-17β-hydroxy structure

The EI mass spectrum of **X** bis-TMS is shown in Fig 2 and that of **XIV** bis-TMS in Fig 3 Both derivatives show an abundant D-ring fragment at *m/z* 143 with 100% relative intensity (base peak)

Nandrolone Nandrolone (estr-4-en-17β-ol-3-one) was first synthesized in 1950 by Birch [27] and by Wilds and Nelson in 1953 [28] The

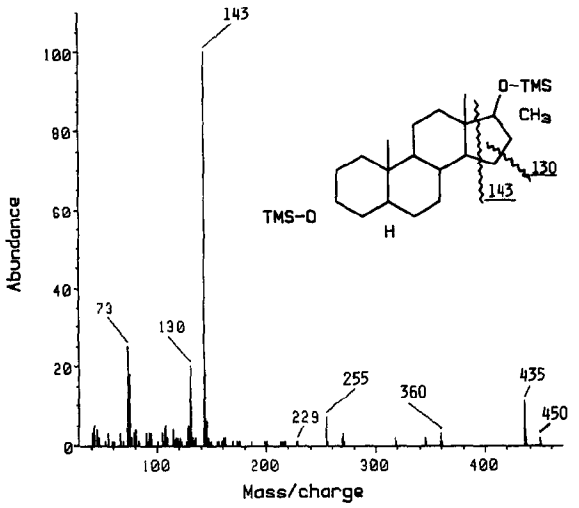

Fig 2 EI mass spectrum of 17α-methyl-5α-androstane-3α,17β-diol bis-TMS (X), molecular ion at m/z 450

metabolism was investigated by Engel et al in 1958 [7], who isolated two metabolites 5α-estran-3α-ol-17-one (**XVIII**) and 5β-estran-3α-ol-17-one (**XIX**) (Fig 4) Both metabolites were synthesized in 1960 by Kupfer et al [29] and in 1961 by Counsell [30]

Both metabolites were synthesized here starting with nortestosterone which was oxidized with

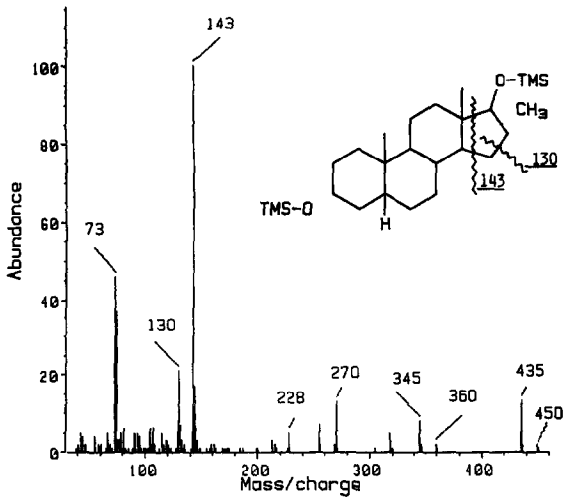

Fig 3 EI mass spectrum of 17α-methyl-5β-androstane-3α,17β-diol bis-TMS (**XIV**), molecular ion at m/z 450

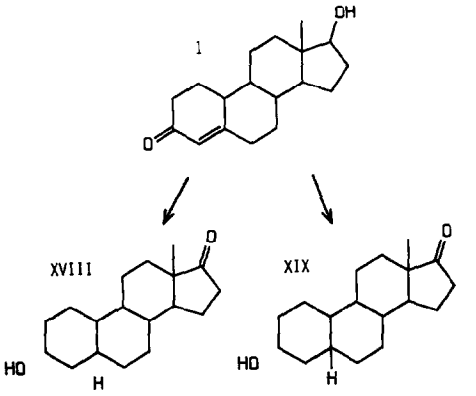

Fig 4 Metabolism of nandrolone (1) to 5α-estran-3α-ol-17-one (**XVIII**) and 5β-estran-3α-ol-17-one (**XIX**)

chromium trioxide to yield estr-4-ene-3,17-dione Subsequent reduction with hydrogen in methanol using platinum dioxide as catalyst yielded 35% of 5α-estrane-3,17-dione and 65% of 5β-estrane-3,17-dione Both isomers were separated by repeated crystallization and could be easily distinguished by their melting points, which are 73–75°C for 5α-estrane-3,17-dione [30] and 179–181°C for 5β-estrane-3,17-dione [31] Reduction of 5α-androstane-3,17-dione with K-Selektride yielded **XVIII** in about 93% yield (Table 1) Reduction of 5β-estrane-3,17-dione with hydrogen using platinum dioxide as catalyst yielded about 64% of **XIX** and 36% of the 3β-isomer, whereas the reduction with K-Selektride yielded **XIX** in about 6% and the 3β-isomer in 94% yield (Table 1)

The EI mass spectrum of **XVIII** bis-TMS is shown in Fig 5 and that of **XIX** bis-TMS in Fig 6

Norethandrolone Norethandrolone (17α-ethyl-estr-4-en-17β-ol-3-one) was first synthesized in 1957 by Colton et al [32]

17α-Ethyl-5α-estrane-3α,17β-diol and 17α-ethyl-5β-estrane-3α,17β-diol (**XX**) were reported as main metabolites by Brooks et al [8] in 1971

When an excretion study was made of norethandrolone, only **XX** could be identified and no 5α-isomer of **XX** was detected (Fig 7) Both isomers were synthesized and are well separated in GC as bis-TMS derivatives [difference of 40 methylene units on an OV-1 capillary column

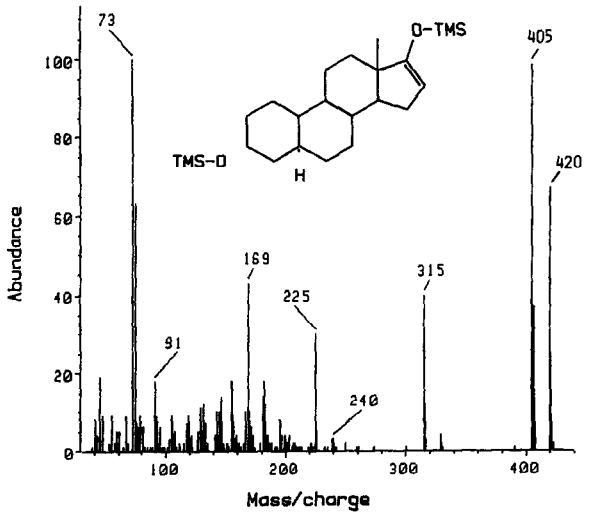

Fig 5 EI mass spectrum of 5α-estran-3α-ol-17-one bis-TMS (XVIII), molecular ion at m/z 420

(column A, see Experimental)] A further metabolite was detected which is suggested to be the hydroxy metabolite of XX, hydroxylated at the 17α-ethyl group (Fig. 7)

The synthesis of XX started with norethandrolone, which was reduced with hydrogen in methanol–6 M sodium hydroxide (10 1, v/v) using 10% palladium on charcoal as catalyst, yielding 82% of 17α-ethyl-5β-estrane-17β-ol-3-one, which was further reduced with lithium aluminium hydride to 17α-ethyl-5β-estrane-3α,17β-diol (XX) in 90% and the 3β-isomer in 10% yield (Table 1)

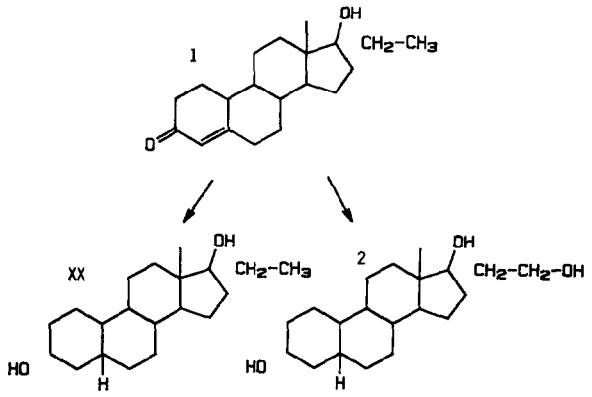

Fig 7 Metabolism of norethandrolone (1) to 17α-ethyl-5β-estrane-3α,17β-diol (XX) and 17α-ethyl-5β-estrane-3α,17β, 20-triol (2)

Figure 8 shows the EI mass spectrum of XX bis-TMS The spectrum shows no molecular ion (M^+, m/z 450) but the highest ion at m/z 435 ($M^+ - 15$) and 421 ($M^+ - 29$) Abundant fragments are the basic ion at m/z 157 and a fragment ion at m/z 144, both D-ring fragments The

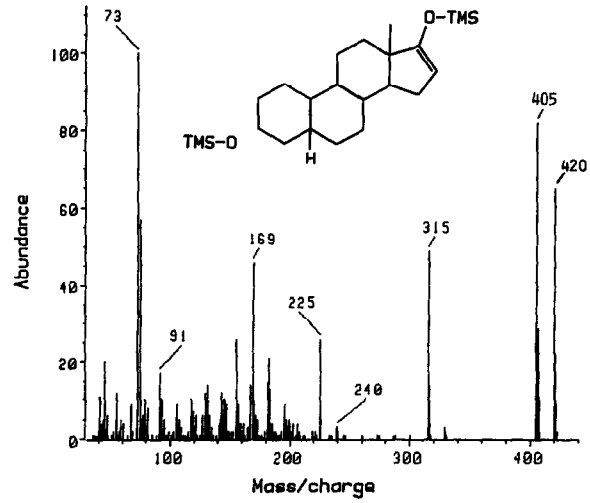

Fig 6 EI mass spectrum of 5β-estran-3α-ol-17-one bis-TMS (XIX), molecular ion at m/z 420

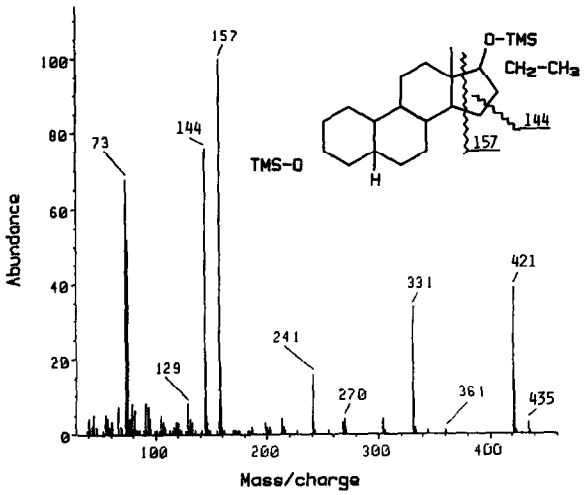

Fig 8 EI mass spectrum of 17α-ethyl-5β-estrane-3α,17β-diol bis-TMS (XX), molecular ion at m/z 450

origin of these ions can be explained similarly to the D-ring fragmentation of 17α-methyl-17β-hydroxy trimethylsilylated steroids (*m/z* 130 and 143, as shown in Figs 2 and 3), both with an additional 14 u

Bolasterone Bolasterone (7α,17α-dimethyl-androst-4-en-17β-ol-3-one) was synthesized by Campell and Babcock [33] There appears to be no report of the metabolism of bolasterone in man

Excretion studies with oral administration of 20 mg of bolasterone to two male volunteers show two metabolites excreted conjugated into urine Both metabolites show a molecular ion 4 u higher than bolasterone Synthesis of 7α,17α-dimethyl-5β-androstane-3α,17β-diol (I) elucidated that this steroid was the main metabolite 17-Epimerization of I [15] confirmed the second metabolite as 7α,17β-dimethyl-5β-androstane-3α,17α-diol (Fig 9)

The synthesis of I started with bolasterone, which was reduced with hydrogen in methanol using 10% palladium on charcoal The reaction yielded only one isomer, 7α,17α-dimethyl-5β-androstan-17β-ol-3-one This configuration could be confirmed by ^{13}C NMR and synthesis of the 5α-isomer, which will be published elsewhere The 5β-isomer obtained was reduced with lithium aluminium hydride, giving I in about 80% and the 3β-hydroxy isomer in 20% yield (Table 1)

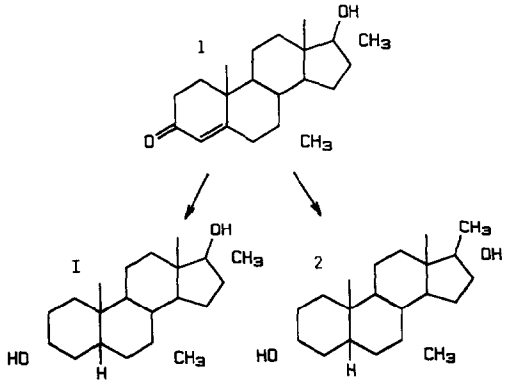

Fig 9 Metabolism of bolasterone (1) to 7α,17α-dimethyl-5β-androstane-3α,17β-diol (I) and 7α,17β-dimethyl-5β-androstane-3α,17α-diol (2)

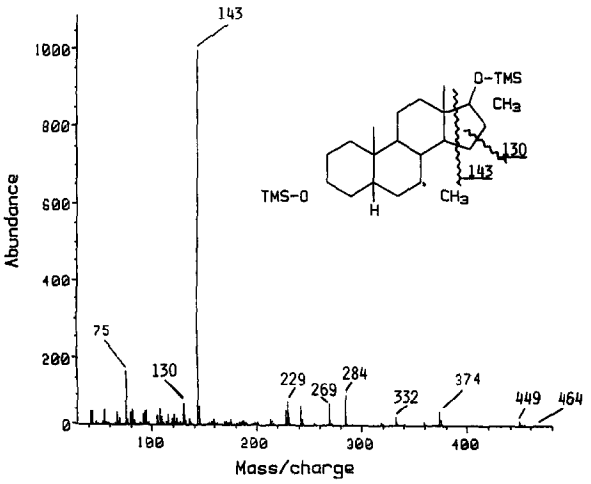

Fig 10 EI mass spectrum of 7α,17α-dimethyl-5β-androstane-3α,17β-diol bis-TMS (I), molecular ion at *m/z* 464

The EI mass spectrum of I bis-TMS (Fig 10) displays a strong D-ring fragment at *m/z* 143 (100%) and a less intense molecular ion at *m/z* 464 (1%)

Clostebol Clostebol (4-chloroandrost-4-en-17β-ol-3-one or 4-chlorotestosterone) was synthesized in 1956 by Camerino and co-workers [34,35] and by Ringold et al [36] In 1969 Starka et al [37] investigated the metabolism of clostebol by liver homogenates of man Oxidation of the 17β-hydroxy group to 17-keto and reduction of the A-ring to dihydro and tetrahydro metabolites were the main metabolic pathways, but the exact identification of the A-ring configuration of the reduced metabolites was not possible

The present investigations showed after oral administration of 20 mg of clostebol acetate four main metabolites The main metabolite identified and synthesized was 4-chloroandrost-4-en-3α-ol-17-one (V) (Fig 11) Two further metabolites showed complete reduction of the A-ring and a 17-keto group It is assumed that the 4-chloro analogues of androsterone and etiocholonolone are produced, but the exact configuration of the A-ring has not yet been elucidated The fourth metabolite is a hydroxylation product of which the exact configuration also was not confirmed

The results show that the chlorine atom at C-4 inhibits the 5α- and 5β-reductase to a great ex-

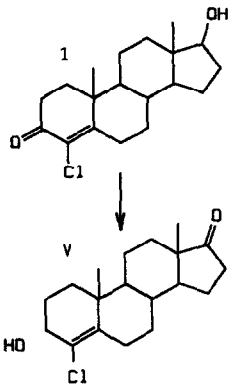

Fig 11 Metabolism of clostebol (1) to 4-chloroandrost-4-en-3α-ol-17-one (V)

tent and the 3α-hydroxydehydrogenase can reduce clostebol-17-one in the presence of the 4,5-double bond to yield V, which is conjugated and excreted as the main metabolite

The synthesis of V started with clostebol, which was oxidized with chromium trioxide to 4-chloroandrost-4-ene-3,17-dione followed by reduction with K-Selektride to yield a mixture of 19% of V and 81% of the corresponding 3β-isomer (Table 1)

The EI mass spectrum of V bis-TMS is shown in Fig 12

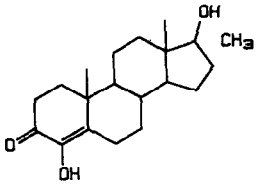

Fig 13 Structure of oxymesterone (XXIII)

Oxymesterone (XXIII) Oxymesterone (XXIII) (17α-methylandrost-4-ene-4,17β-diol-3-one, Fig 13), which was synthesized in 1965 by Camerino [38], is less metabolized and mainly excreted unchanged as a conjugate The hydroxy group at C-4 may hinder the reduction of the 4,5-double bond and/or allow a very rapid conjugation of XXIII at the 4-hydroxy group and excretion into urine

The EI mass spectrum of XXIII tris-TMS (Fig 14) is dominated by an abundant molecular ion at m/z 534

Fluoxymesterone Fluoxymesterone (9α-fluoro-17α-methylandrost-4-ene-11β,17β-diol-3-one) was synthesized in 1956 by Herr et al [39] In 1990 Kammerer et al [40] published a GC–MS investigation of fluoxymesterone metabolism in man They excluded excretion of A-ring reduced metabolites by using reference substances

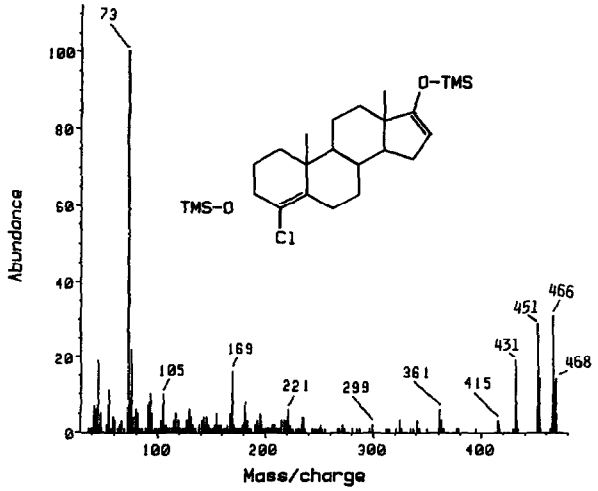

Fig 12 EI mass spectrum of 4-chloroandrost-4-en-3α-ol-17-one bis-TMS (V), molecular ion at m/z 466

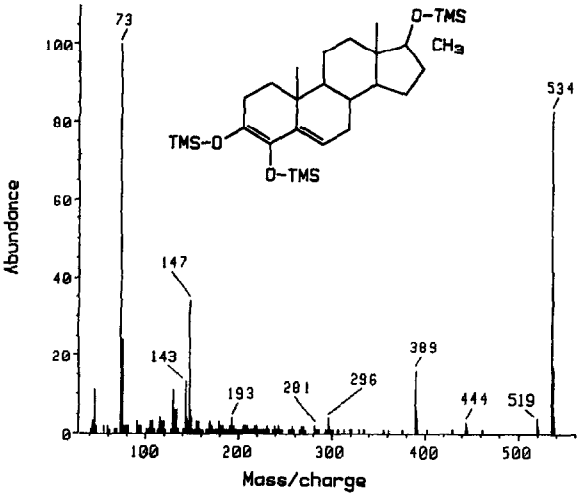

Fig 14 EI mass spectrum of oxymesterone tris-TMS (XXIII), molecular ion at m/z 534

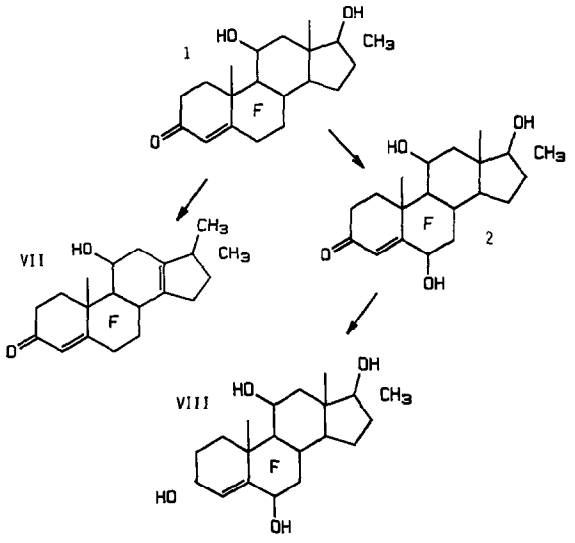

Fig 15 Metabolism of fluoxymesterone (1), 6β-hydroxyfluoxymesterone (2), 9α-fluoro-18-nor-17,17-dimethyl-4,13-diene-11β-ol-3-one (**VII**) and 9α-fluoro-17α-methylandrost-4-ene-3α,6β,11β,17β-tetrol (**VIII**)

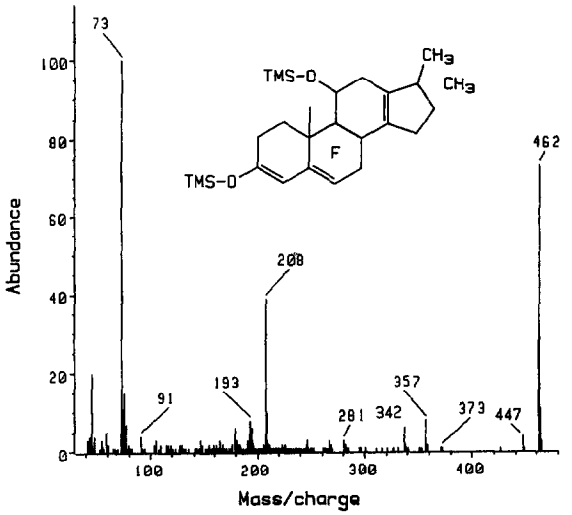

Fig 16 EI mass spectrum of 9α-fluoro-18-nor-17,17-dimethyl-4,13-diene-11β-ol-3-one bis-TMS (**VII**), molecular ion at m/z 462

The present investigations confirmed 9α-fluoro-18-nor-17,17-dimethyl-4,13-diene-11β-ol-3-one (**VII**), 6β-hydroxyfluoxymesterone and 9α-fluoro-17α-methylandrost-4-ene-3α,6β,11β,17β-tetrol (**VIII**) as the main metabolites (Fig 15) All three metabolites were synthesized A detailed publication is in preparation Compound **VII** was obtained by refluxing fluoxymesterone in acetonitrile–trifluoroacetic acid (10 1, v/v) 6β-Hydroxyfluoxymesterone was synthesized via direct oxidation of the enol-TMS derivative of fluoxymesterone in ethanol catalysed by sunlight The reduction of 6β-hydroxyfluoxymesterone with K-Selektride yielded **VIII** in about 72% and the 3β-isomer in 28% yield (Table 1)

The EI mass spectrum of **VII** bis-TMS is shown in Fig 16 and that of **VIII** tetra-TMS in Fig 17

The derivatization of **VII** and **VIII** is satisfactory with MSTFA–ammonium iodide as described above, **VII** reacts within 5 min at 80°C completely to a bis-TMS derivative whereas trimethylsilylation of the 11β-hydroxy group in **VIII** is sterically hindered by the fluorine atom at C-9α and the reaction needs 2 h at 80°C, yielding a tetra-TMS derivative Derivatization with MSTFA–Imi (100 1, v/w) is unsatisfactory This should be considered when fluoxymesterone metabolites are screened in the unconjugated urine fraction

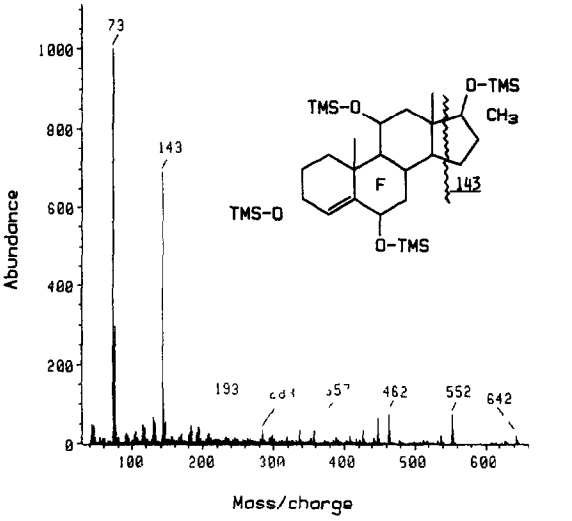

Fig 17 EI mass spectrum of 9α-fluoro-17α-methylandrost-4-ene-3α,6β,11β,17β-tetrol tetra-TMS (**VIII**), molecular ion at m/z 642

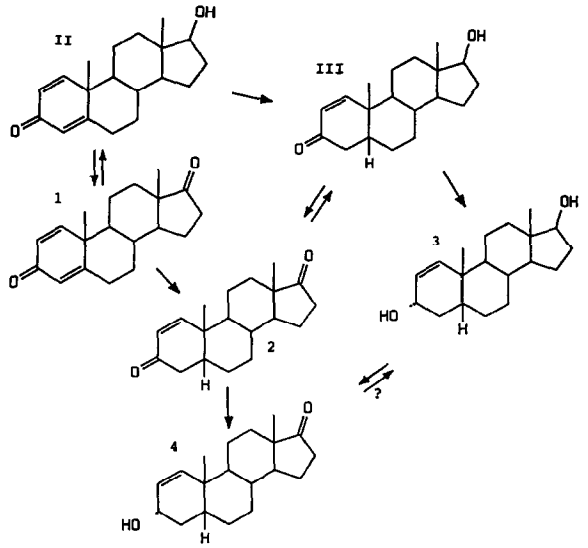

Fig 18 Metabolism of boldenone (II) to 5β-androst-1-en-17β-ol-3-one (III), androsta-1,4-diene-3,17-dione (1), 5β-androst-1-ene-3,17-dione (2), 5β-androst-1-ene-3α,17β-diol (3) and 5β-androst-1-en-3α-ol-17-one (4)

Androsta-1,4-dien-3-ones

Androsta-1,4-dien-3-ones are reduced by the 5β-reductase only, but to a smaller extent than testosterone. In this instance 6β-hydroxylation is favoured for metandienone, 4-chlorodehydromethyltestosterone and fluoxymesterone

Boldenone Boldenone (II) (androsta-1,4-dien-17β-ol-3-one) was synthesized in 1956 by Meystre et al [41]

The metabolism of II was investigated in 1971 by Galetti and Gardi [1] GC–MS of boldenone and its main metabolites was reported by Schanzer and Donike in 1992 [12]

Boldenone itself is excreted as a conjugate in urine The main metabolites are 5β-androst-1-en-17β-ol-3-one (III), 5β-androst-1-en-3α-ol-17-one and 5β-androst-1-ene-3α,17β-diol (Fig 18) In the proposed screening procedure II and III are used to detect boldenone misuse

The synthesis of III started with II, which was selectively reduced with hydrogen in methanol–potassium hydroxide using 10% palladium on charcoal as catalyst [12]

The EI mass spectrum of II bis-TMS is shown in Fig 19 and that of III bis-TMS in Fig 20.

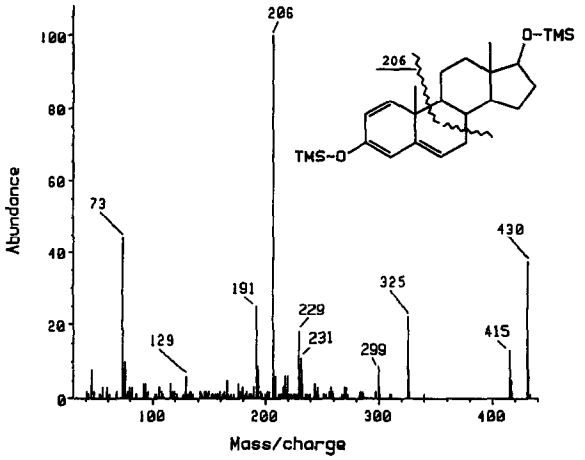

Fig 19 EI mass spectrum of boldenone bis-TMS (II), molecular ion at m/z 430

Metandienone Metandienone (17α-methylandrosta-1,4-dien-17β-ol-3-one) was synthesized in 1955 by Vischer et al [42] by microbiological dehydrogenation of methyltestosterone In 1956 Meystere et al [41] reported the dehydrogenation of methyltestosterone with selenium dioxide 6β-Hydroxymetandienone (XIII) was identified in 1963 by Rongone and Segaloff as the main metabolite [4], which was excreted unconjugated in urine A further metabolite is 17-epimetan-

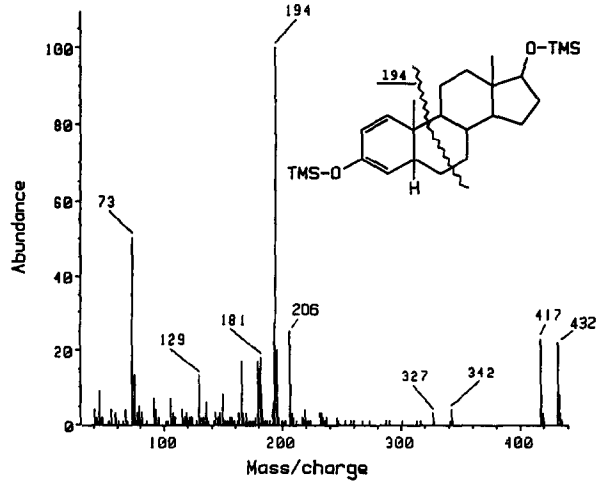

Fig 20 EI mass spectrum of 5β-androst-1-en-17β-ol-3-one bis-TMS (III), molecular ion at m/z 432

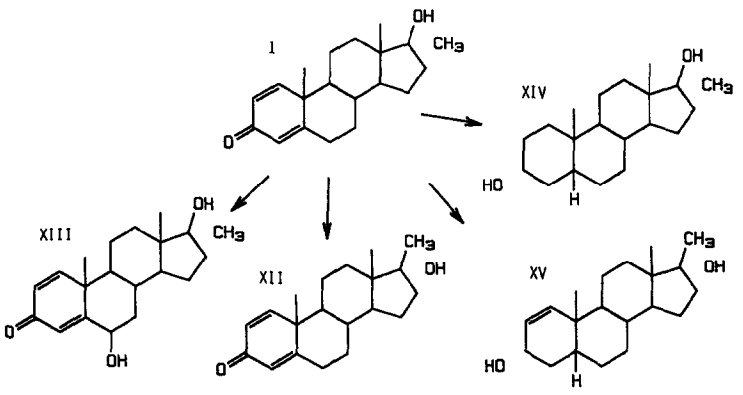

Fig 21 Metabolism of metandienone (1) to 17-epimetandienone (XII), 6β-hydroxymetandienone (XIII), 17α-methyl-5β-androstane-3α,17β-diol (XIV) and 17β-methyl-5β-androst-1-ene-3α,17α-diol (XV)

dienone (XII) obtained from the unconjugated fraction which was identified and synthesized in 1971 by Macdonald et al [5] In 1980 Durbeck and Bucker [23] investigated the unconjugated urine fraction after application of metandienone by GC–MS and discussed the EI fragmentation of the trimethylsilylated metabolites In 1991 Schanzer et al [13] published the identification of A-ring reduced metabolites excreted as conjugates in urine, 17α-methyl-5β-androst-1-ene-3α,17β-diol, its 17-epimer 17β-methyl-5β-androst-1-ene-3α,17α-diol (XV) and 17α-methyl-5β-androstane-3α,17β-diol (XIV) (Fig 21); XIV is also a metabolite of methyltestosterone and methandriol

The synthesis of 6β-hydroxymetandienone (XIII) was performed as described above by autooxidation of the enol-TMS derivative in ethanol catalysed by sunlight The synthesis of XII was performed via the 17β-sulphate of metandienone which spontaneously decomposed in water giving several dehydration products and XII in about 30% yield Synthesis of XV started with 17-epimetandienone (XII), which was selectively reduced with hydrogen in methanol–potassium hydroxide using 10% palladium on charcoal giving 17β-methyl-5β-androst-1-en-17α-ol-3-one in about 20% yield The reduction of this 3-ketone with lithium aluminium hydride gave XV in more than 85% yield For the synthesis of XIV, see the discussion of methyltestosterone

EI mass spectra are shown for XII TMS in Fig 22, XIII bis-TMS in Fig 23, XIV bis-TMS in Fig 3 and XV bis-TMS in Fig 24 All these EI mass spectra are dominated by the D-ring fragment at m/z 143

4-Chlorodehydromethyltestosterone. 4-Chlorodehydromethyltestosterone (4-chloro-17α-methylandrosta-1,4-dien-17β-ol-3-one) was synthesized in 1960 by Schubert et al [43] The metabolism of 4-chlorodehydromethyltestosterone in man was reported by Schubert and co-workers [2,3] In addition to the excretion of 4-chlorodehydromethyltestosterone itself they also identified 6β-hydroxy-4-chlorodehydromethyltestosterone (IV), 16β-hydroxy-4-chlorodehydromethyltestosterone

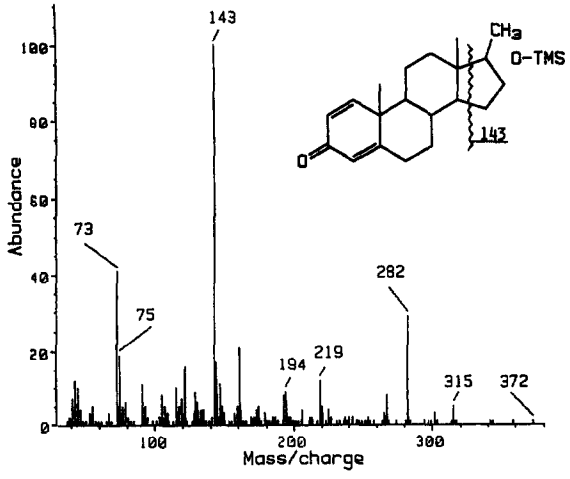

Fig 22 EI mass spectrum of 17-epimetandienone TMS (XII), molecular ion at m/z 372

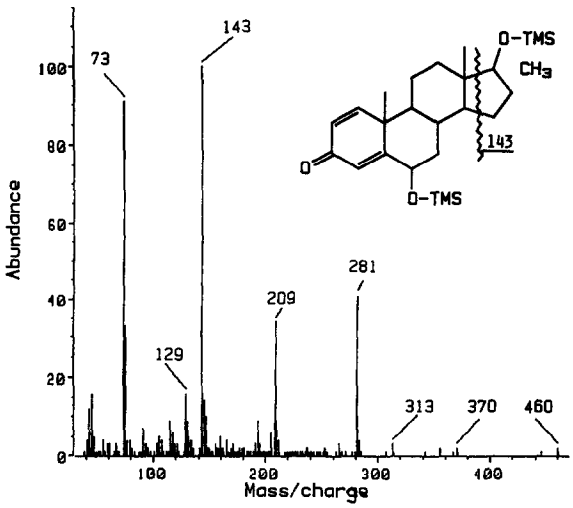

Fig 23 EI mass spectrum of 6β-hydroxymetandienone bis-TMS (XIII), molecular ion at m/z 460

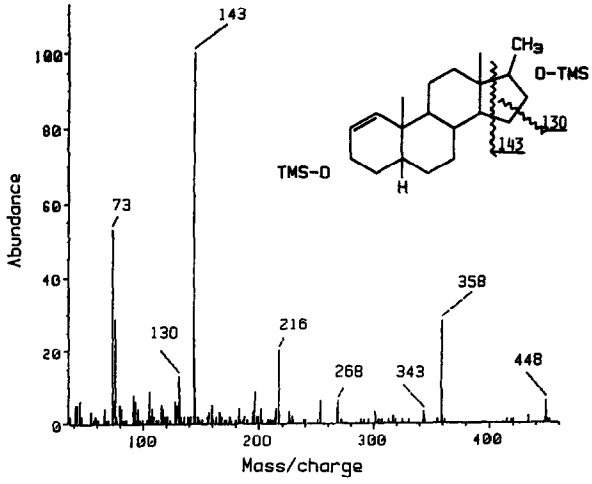

Fig 24 EI mass spectrum of 17β-methyl-5β-androst-1-ene-3α,17α-diol bis-TMS (XV), molecular ion at m/z 448

and 6β,16β-dihydroxy-4-chlorodehydromethyltestosterone as the main metabolites In 1983 Durbeck et al [44] studied the metabolism of 4-chlorodehydromethyltestosterone by GC–MS They were able to confirm 6β-hydroxy-4-chlorodehydromethyltestosterone (IV) and the 6β,16β-dihydroxy metabolite They did not detect 4-chlorodehydromethyltestosterone and the 16β-hydroxy metabolite, but a substance showing the same EI mass spectrum as the parent steroid and it was therefore assumed to be a 17-epimer They further recorded a bishydroxy metabolite which was considered to be a 6β,12-dihydroxy metabolite (Fig 25)

In an excretion study with 22 mg of orally administered 4-chlorodehydromethyltestosterone, it was possible to identify in the unconjugated urine fraction a 17-epimer, which was synthesized [15], 6β-hydroxy-4-chlorodehydromethyltestos-

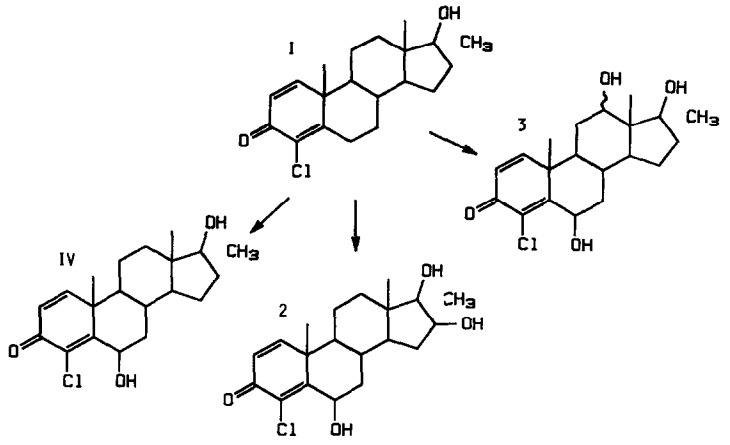

Fig 25 Metabolism of 4-chlorodehydromethyltestosterone (1) to 6β-hydroxy-4-chlorodehydromethyltestosterone (IV), 6β,16β-dihydroxy-4-chlorodehydromethyltestosterone (2) and 6β,12z-dihydroxy-4-chlorodehydromethyltestosterone (3) (z = unidentified configuration)

terone (IV), which was also synthesized, a 6β,16-dihydroxy metabolite and a 6β,12-dihydroxy metabolite, as discussed by Durbeck et al [44] The 12-hydroxy group could be confirmed using the EI mass spectra of the tris-TMS derivative of this metabolite, displaying an abundant fragment at m/z 170 This fragment was previously reported [13] to originate from a 12,17-dihydroxy-17-methyl-bis-TMS structure

Compound IV was synthesized by the above-described synthesis The enol-TMS ether was autooxidized when dissolved in isopropanol and exposed to sunlight, yielding IV A detailed description of this synthesis is in preparation

The EI mass spectrum of IV bis-TMS is shown in Fig 26 The D-ring fragment at m/z 143 is the most abundant ion The fragments at m/z 243 and 315 are the 4-chloro analogue fragments of the ions at m/z 209 and 281 of 6β-hydroxy-metandienone bis-TMS (Fig 23)

Formebolone Formebolone (2-formyl-17α-methylandrosta-1,4-diene-11α,17β-diol-3-one) was synthesized in 1965 by Canonica et al [45]

A GC–MS investigation of formebolone metabolism in man was published by Masse et al [46]

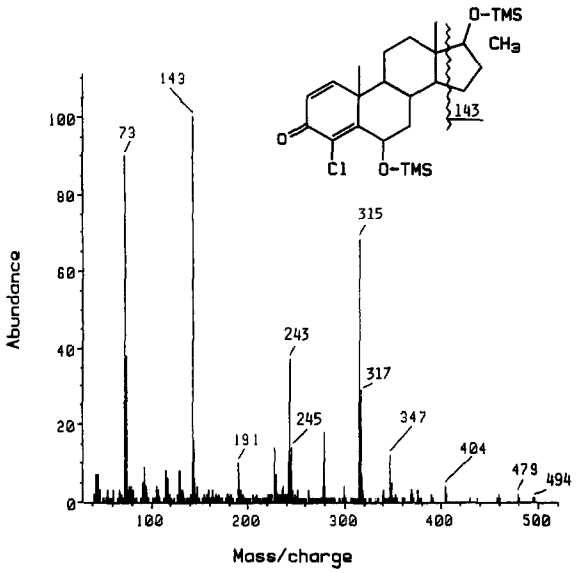

Fig 26 EI mass spectrum of 6β-hydroxy-4-chlorodehydromethyltestosterone bis-TMS (IV), molecular ion at m/z 494

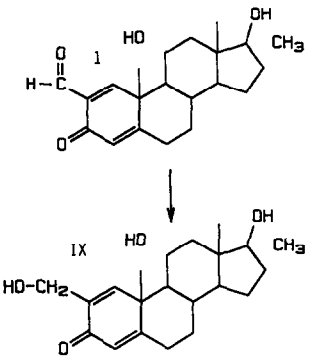

Fig 27 Metabolism of formebolone (1) to 2-hydroxymethyl-17α-methylandrosta-1,4-diene-11α,17β-diol-3-one (IX)

In the present investigation with 20 mg of formebolone applied orally to a male volunteer, a reduced metabolite was identified in the unconjugated fraction (Fig 27) after basic extraction This polar metabolite was identified by synthesis as 2-hydroxymethyl-17α-methylandrosta-1,4-diene-11α,17β-diol-3-one (IX)

Compound IX was synthesized by reduction of formebolone with K-Selectride in tetrahydrofuran The K-Selectride showed a selectivity to the formyl group which was first reduced and the 3-keto group remained intact when the reagent was not used in excess Isolation of IX by preparative HPLC yielded a pure reference substance

The EI mass spectrum of IX tris-TMS is displayed in Fig 28

5α-Androstan-3-ones

Drostanolone Drostanolone (2α-methyl-5α-androstan-17β-ol-3-one) was synthesized in 1959 by Ringold et al [47] Drostanolone is orally applied as propionate

After oral application of 20 mg of drostanolone propionate to a male volunteer, 2α-methyl-5α-androstan-3α-ol-17-one (VI) was identified as the main metabolite (Fig 29), which is excreted as a conjugate in urine No drostanolone or drostanolone propionate was excreted

The synthesis of VI started with drostanolone, which was oxidized with chromium trioxide to 2α-methyl-5α-androstane-3,17-dione and then reduced with K-Selektride yielding about 99% of 2α-methyl-5α-androstan-3α-ol-17-one (VI) and

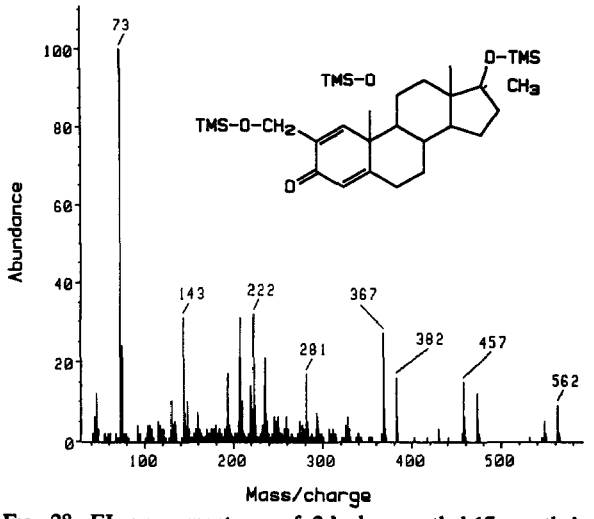

Fig 28 EI mass spectrum of 2-hydroxymethyl-17α-methyl-androsta-1,4-diene-11α,17β-diol-3-one tris-TMS (IX), molecular ion at m/z 562

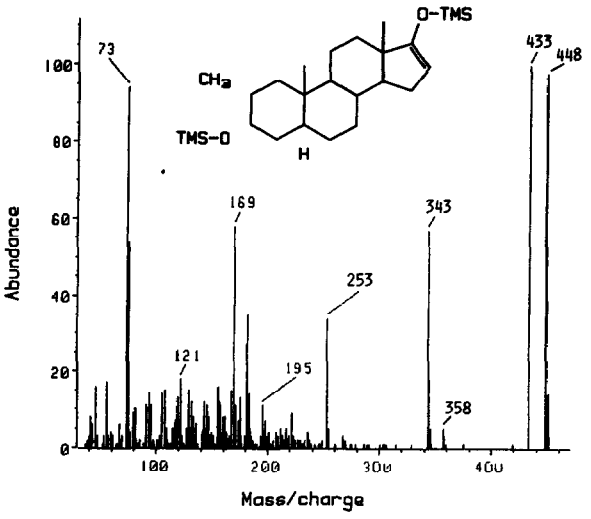

Fig 30 EI mass spectrum of 2α-methyl-5α-androstan-3α-ol-17-one bis-TMS (VI), molecular ion at m/z 448

1% of the 3β-isomer (Table 1)

The EI mass spectrum of VI bis-TMS is presented in Fig 30

Mesterolone Mesterolone (1α-methyl-5α-androstan-17β-ol-3-one was synthesized by Wiechert [48]

It is metabolized in the same way as drostanolone After oral application of 20 mg of mesterolone the main metabolite was identified as 1α-methyl-5α-androstan-3α-ol-17-one (XI), excreted conjugated in urine (Fig 31) No parent drug was detected

The synthesis of XI was carried out in the same way as that of the drostanolone metabolite VI Starting with the chromium trioxide oxidation of mesterolone 1α-methyl-5α-androstane-3,17-dione was obtained, which was reduced with K-Selektride to 99% of 1α-methyl-5α-androstan-3α-ol-17-one (XI) and 1% of the 3β-isomer (Table 1)

The EI mass spectrum of XI bis-TMS is shown in Fig 32

Mestanolone Mestanolone (17α-methyl-5α-androstan-17β-ol-3-one) was synthesized in 1935 by Ruziecka et al [24]

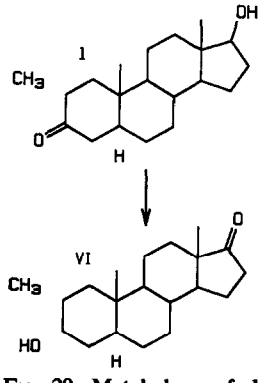

Fig 29 Metabolism of drostanolone (1) to 2α-methyl-5α-androstan-3α-ol-17-one (VI)

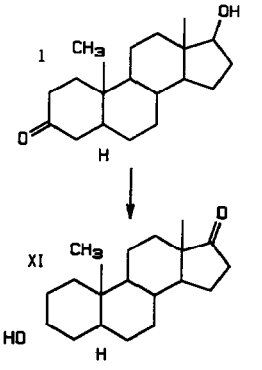

Fig 31 Metabolism of mesterolone (1) to 1α-methyl-5α-androstan-3α-ol-17-one (XI)

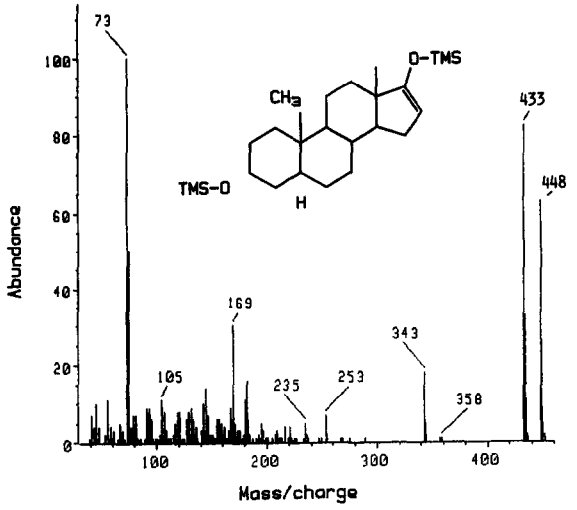

Fig 32 EI mass spectrum of 1α-methyl-5α-androstan-3α-ol-17-one bis-TMS (XI), molecular ion at m/z 448

After oral administration of 20 mg of mestanolone to a male volunteer, 17α-methyl-5α-androstane-3α,17β-diol (X) was identified as the main metabolite, excreted as a conjugate in urine (Fig 33) No parent drug was detected The same metabolite was obtained in the metabolism of methyltestosterone and oxymetholone

For the synthesis of X see the discussion of methyltestosterone and for the EI mass spectrum of X bis-TMS see Fig 2

Metenolone (XVI) Metenolone (1-methyl-5α-androst-1-en-17β-ol-3-one) (XVI) was synthesized

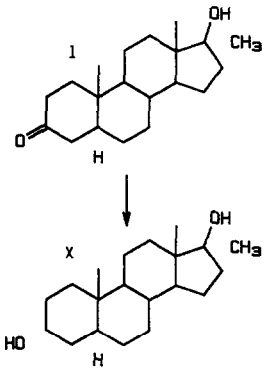

Fig 33 Metabolism of mestanolone (1) to 17α-methyl-5α-androstane-3α,17β-diol (X)

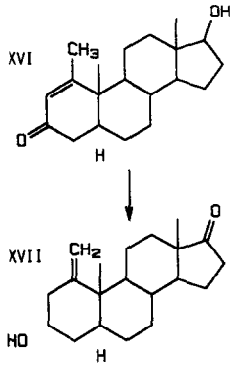

Fig 34 Metabolism of metenolone (XVI) to 1-methylen-5α-androstan-3α-ol-17-one (XVII)

in 1960 by Wiechert and Kaspar [49] Metenolone is applied orally as the acetate or by intramolecular injection as the enanthate The metabolism of XVI was investigated by Gerhards et al in 1965 [50], identifying 1-methylen-5α-androstan-3α-ol-17-one (XVII) as the main metabolite and unchanged metenolone (XVI), both excreted as conjugates (Fig. 34) Recently Goudreault and Masse [51] described a GC–MS investigation of XVI and its metabolites

In this work the synthesis of XVII started with metenolone (XVI), which was oxidized to 1-methyl-5α-androst-1-ene-3,17-dione The latter was isomerized to 1-methylen-5α-androstane-3,17-dione as described by Wiechert et al [52] using the method of Ringold and Malhotra [53], who converted α,β-unsaturated ketones into β,γ-unsaturated ketones with potassium *tert*-butoxide in *tert*-butanol

The reduction of the isomerized product with K-Selektride yielded 80% of XVII, 10% of the corresponding 3β-isomer and about 10% of 1-methyl-5α-androst-1-ene-3β,17β-diol (Table 1)

The EI mass spectrum of XVI bis-TMS is presented in Fig 35 and that of XVII bis-TMS in Fig 36

Oxymetholone Oxymetholone (2-hydroxymethylene-17α-methyl-5α-androstan-17β-ol-3-one) was first prepared by Ringold et al in 1959 [47] Oxymetholone is substantially metabolized and several metabolites can be detected by GC–MS In the neutral and basic fraction used for screen-

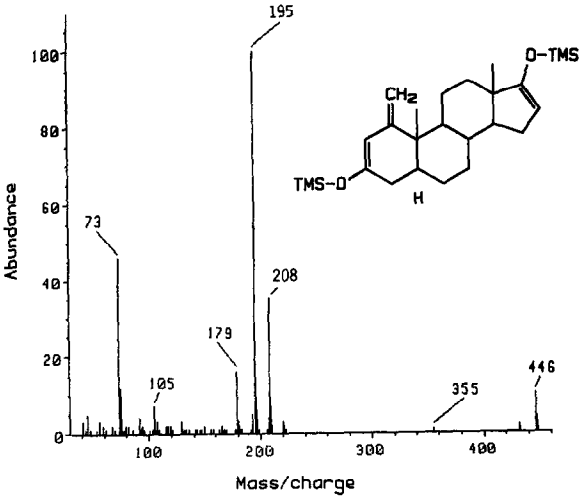

Fig 35 EI mass spectrum of metenolone bis-TMS (XVI), molecular ion at m/z 446

ing of anabolic steroids, 17α-methyl-5α-androstane-3α,17β-diol (X) was identified as a metabolite The origin of this metabolite can be explained by oxidation of the 2-hydroxymethylene group to a 2-carbonic acid This oxidation product is a β-ketocarbonic acid, which is less stable and decomposes to 17α-methyl-5α-androstan-17β-ol-3-one Subsequent reduction of the 3-keto

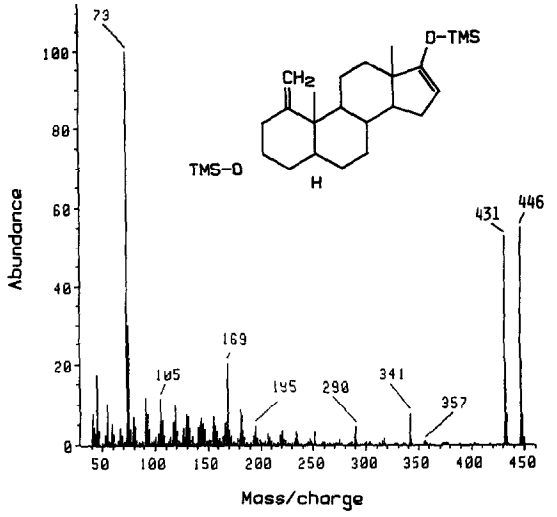

Fig 36 EI mass spectrum of 1-methylen-5α-androstan-3α-ol-17-one bis-TMS (XVII), molecular ion at m/z 446

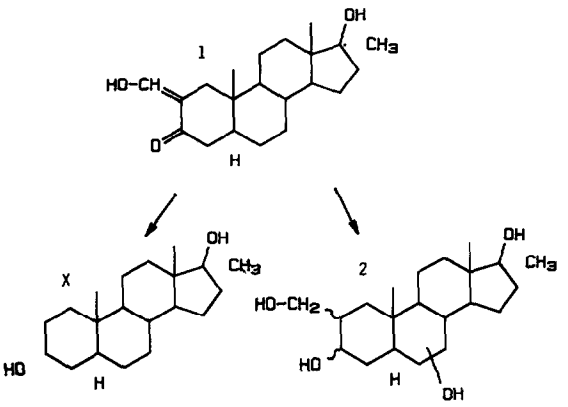

Fig 37 Metabolism of oxymetholone (1) to 17α-methyl-5α-androstane-3α,17β-diol (X) and 2z-hydroxymethyl-17α-methyl-5α-androstane-3z,z,17β-triol (2) (z = unidentified configuration)

group yields X with a 3α-hydroxy configuration A further metabolite is detected in excretion studies with oxymetholone with a molecular ion at m/z 640, which is explained as a hydroxy derivative of the completely reduced oxymetholone (Fig 37) This metabolite is further used for screening of an oxymetholone abuse even though its structure is not completely known

The synthesis of X is described in the discussion of methyltestosterone and the EI mass spectrum of X bis-TMS is shown in Fig 2

5α-Androstanes with special structure

Oxandrolone (XXI) Oxandrolone (XXI) (17α-methyl-2-oxa-5α-androstan-17β-ol-3-one) was synthesized in 1962 by Pappo and Jung [54] In 1989 Masse et al [55] published a GC–MS investigation of oxandrolone metabolism in man Compound XXI is excreted unchanged to a great extent As the main metabolite a 17-epimer (XXII) of oxandrolone (XXI) was described (Fig 38) Further metabolites hydroxylated at C-16 were detected by GC–MS but in low concentration

In this work XXII was synthesized by the above described method for 17-epimerization [15]

The EI-mass spectra of XXI TMS in Fig 39 and XXII TMS in Fig 40 show that these spectra are identical and dominated by the abundant D-ring fragment at m/z 143

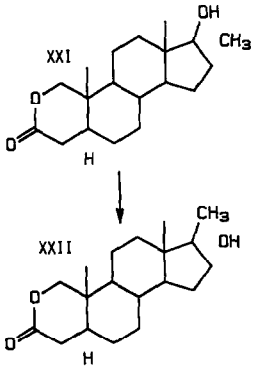

Fig 38 Metabolism of oxandrolone (**XXI**) to 17-epioxandrolone (**XXII**)

Furazabol Furazabol was synthesized in 1965 by Ohta et al [56] Metabolism studies of furazabol with rats were published by Takegoshi et al [57], but no study of the metabolism of furazabol in man has been reported

After oral administration of 20 mg of furazabol in man furazabol itself and a metabolite hydroxylated at C-16 were detected in the conjugated urine fraction (Fig 41) The EI mass spectrum of the TMS derivative of this metabolite in Fig 42 show fragments at m/z 218 and 231, confirming a D-ring with a 17-methyl-16,17-dihydroxy-bis-TMS structure

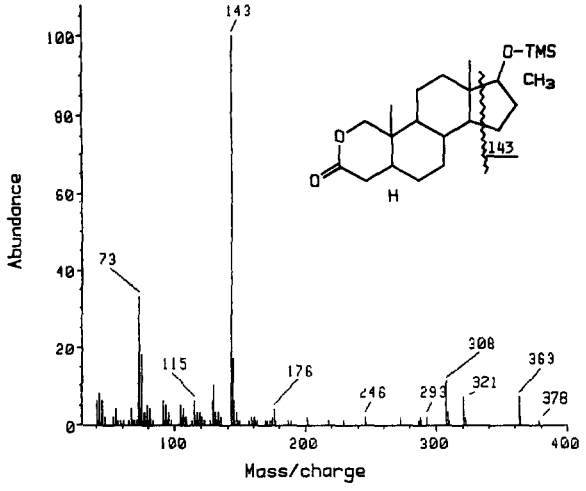

Fig 39 EI mass spectrum of oxandrolone TMS (**XXI**), molecular ion at m/z 378

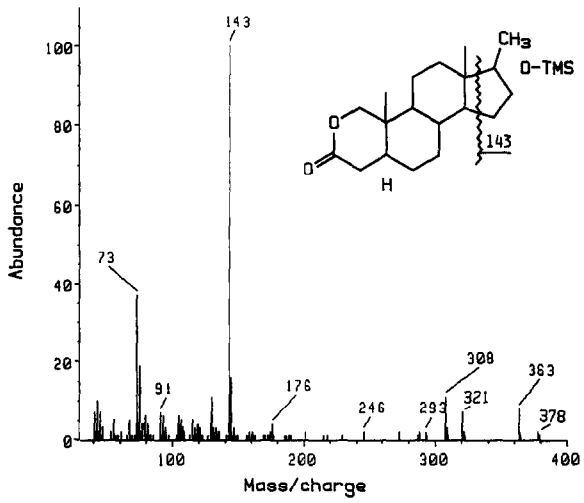

Fig 40 EI mass spectrum of 17-epioxandrolone TMS (**XXII**), molecular ion at m/z 378

Because no misuse of furazabol has been reported by any IOC accredited laboratory, this metabolite was not synthesized but a reference obtained from an excretion study with furazabol was used for screening purposes

Stanozolol Stanozolol (17α-methyl-17β-hydroxy-5α-androst-2-eno[3,2-*c*]pyrazole) was synthesized in 1959 by Clinton and co-workers [58,59] The metabolism of stanozolol in man was investigated in this laboratory and published in 1990 [14]

About eleven metabolites were confirmed and the main metabolites were synthesized, namely

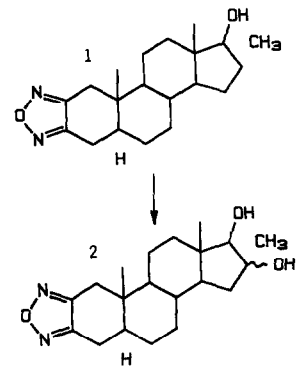

Fig 41 Metabolism of furazabol (1) to 16z-hydroxyfurazabol (2) (z = unidentified configuration)

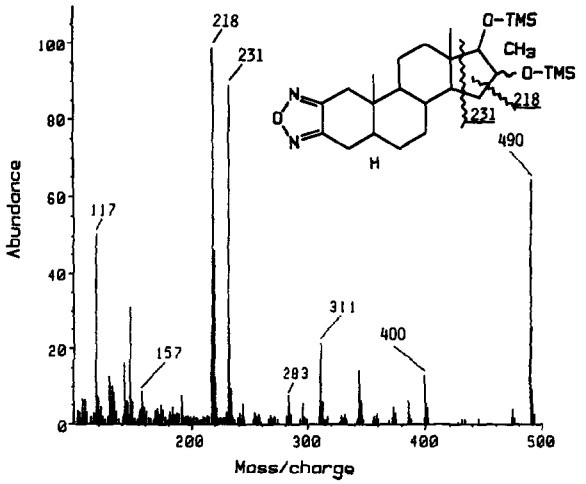

Fig 42 EI mass spectrum of 16*z*-hydroxyfurazabol bis-TMS (*z* = unidentified configuration), molecular ion at *m/z* 490

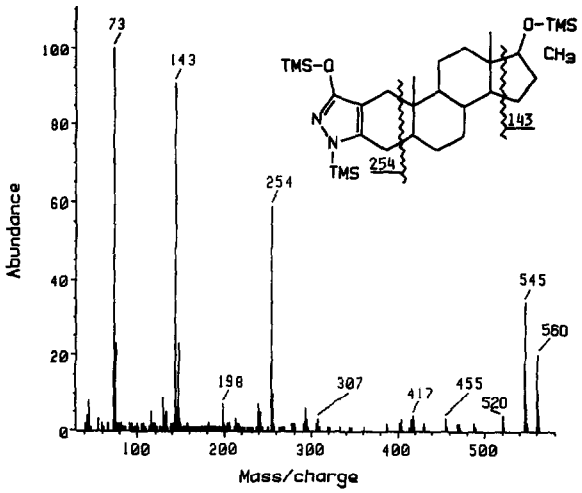

Fig 44 EI mass spectrum of 3′-hydroxystanozolol tris-TMS (XXIV), molecular ion at *m/z* 560

3′-hydroxystanozolol (**XXIV**), 3′-hydroxy-17-epistanozolol (**XXV**) [15], 4β-hydroxystanozolol (**XXVI**) and 16β-hydroxystanozolol (**XXVII**) (Fig 43)

EI mass spectra are displayed for **XXIV** tris-TMS in Fig 44, **XXV** tris-TMS in Fig. 45, **XXVI** tris-TMS in Fig 46 and **XXVII** tris-TMS in Fig 47

Methandriol. Methandriol (17α-methylandrost-5-ene-3β,17β-diol) was synthesized in 1935 by Ruziecka et al. [24]. It is applied orally as the dipropionate

The metabolism of methandriol and methandriol dipropionate was investigated and after 30 mg of orally applied methandriol dipropionate excreted methandriol was identified in very low concentration in the sulphate fraction which is routinely not controlled The main metabolite was identified as 17α-methyl-5β-androstane-3α,17β-diol (**XIV**) (Fig 48) in the conjugated urine

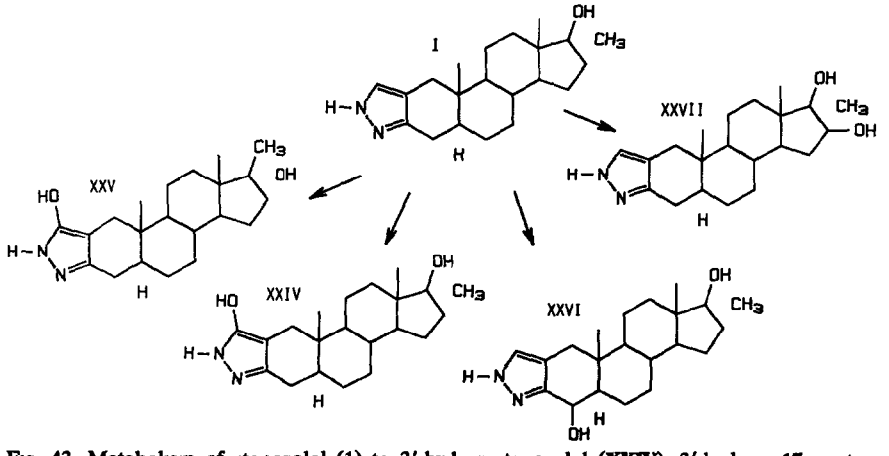

Fig 43 Metabolism of stanozolol (1) to 3′-hydroxystanozolol (**XXIV**), 3′-hydroxy-17-epistanozolol (**XXV**), 4β-hydroxystanozolol (**XXVI**) and 16β-hydroxystanozolol (**XXVII**)

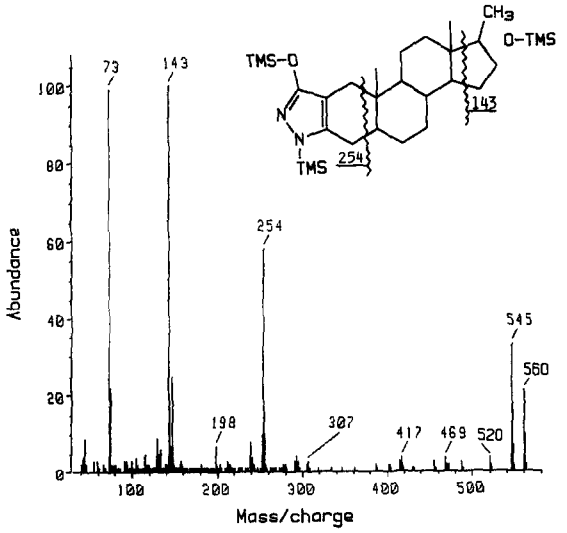

Fig 45 EI mass spectrum of 3'-hydroxy-17-epistanozolol tris-TMS (XXV), molecular ion at m/z 560

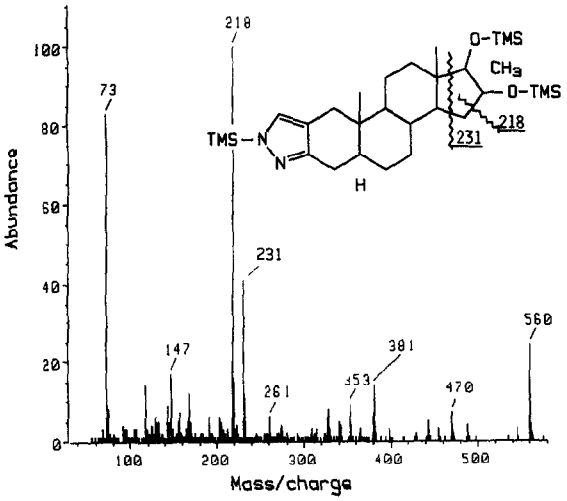

Fig 47 EI mass spectrum of 16β-hydroxystanozolol tris-TMS (XXVII), molecular ion at m/z 560

fraction, but in low concentration An excretion study with 20 mg of methandriol confirmed metabolite **XIV** as the main metabolite and a possible 5α-isomer was not detected

This result can only be explained by a metabolic pathway of methandriol in man including 3-oxidation and isomerization of the 5,6-double bond to C-4,5, similarly as the metabolism of dehydroepiandrosterone (androst-5-en-3β-ol-17-one). The oxidation and isomerization product is methyltestosterone following the above-described metabolic pathway with the excretion of mainly **XIV**, a metabolite with a 3α-hydroxy configuration

For the synthesis of **XIV** see the discussion of methyltestosterone and for the EI mass spectrum of **XIV** bis-TMS see Fig 3.

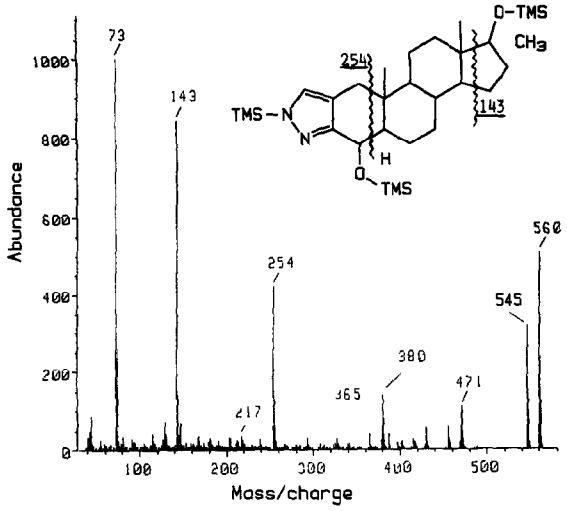

Fig 46 EI mass spectrum of 4β-hydroxystanozolol tris-TMS (XXVI), molecular ion at m/z 560

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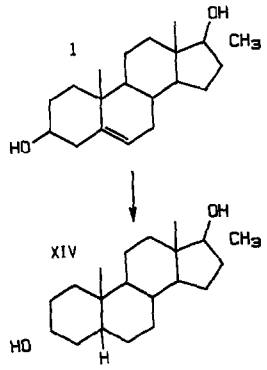

Fig 48 Metabolism of methandriol (1) to 17α-methyl-5β-androstane-3α,17β-diol (XIV)

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