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W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck-Engelke
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Metabolism of Anabolic Androgenic Steroids: 5α- and 5ß-Reduction of 3-Keto-4-ene Steroids

Institute of Biochemistry, German Sports University,
Carl-Diem-Weg 6, D-50933 Köln.

INTRODUCTION

To detect and monitor anabolic steroid misuse by athletes, urine samples are analyzed by gas chromatography/mass spectrometry (GC/MS). Determination of anabolic steroid misuse is based on comparision of the electron impact (EI) mass spectrum and retention time of the isolated steroid and/or its metabolite(s) with the mass spectrum and GC retention time of authentic reference material or an unambigously identified metabolite obtained from an excretion study with the anabolic steroid in question [**1**]. For this reason the metabolism of anabolic androgenic steroids (AAS) has to be known [**2**].

The importance of anabolic steroid metabolism is based on the fact that most steroids undergo extensive metabolism and the parent steroid cannot be detected in urine, or only in the first few hours after application. In this case, an urinary excreted metabolite (or metabolites) is much easier to detect and misuse of AAS can be confirmed for a much longer time period after the last application. With this paper we would like to summerize results regarding the 5α- and 5ß-reduction of 3-keto-4-ene steroids which were obtained in the Cologne laboratory during the last ten years.

The initial and rate-limiting step in the A-ring metabolism of 3-keto-4-ene steroids, such as testosterone, is the reduction of the double bond between C_4 and C_5. The reaction is catalyzed by two different enzymes, 5α- and 5ß-reductase [**3**, **4**], and yields an assymetric center at C-5 and two isomers with 5α- (hydrogen at C-5 below the planar molecule) and 5ß-configuration (hydrogen at C-5 above the planar molecule) are formed (Fig.1 and 2). The 5α-reductase is mainly located in the endoplasmic reticulum, whereas the 5ß-reductase is located in the cytoplasm. Both enzymes require NADPH as a co-factor. Once the double bond is reduced, the 3-keto group is immediately transformed, mainly to a 3α-hydroxy structure (for a few steroids, such as testosterone and 19-nortestosterone, 3ß-hydroxy steroids are produced to a low extent).

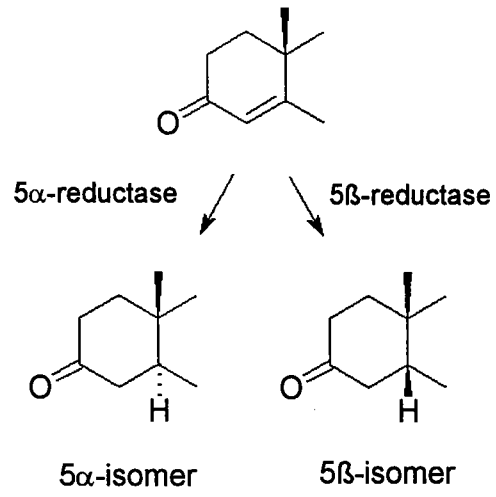

Fig.1 A-ring metabolism: 5α- and 5ß-Reduction of 3-keto-4-ene steroids

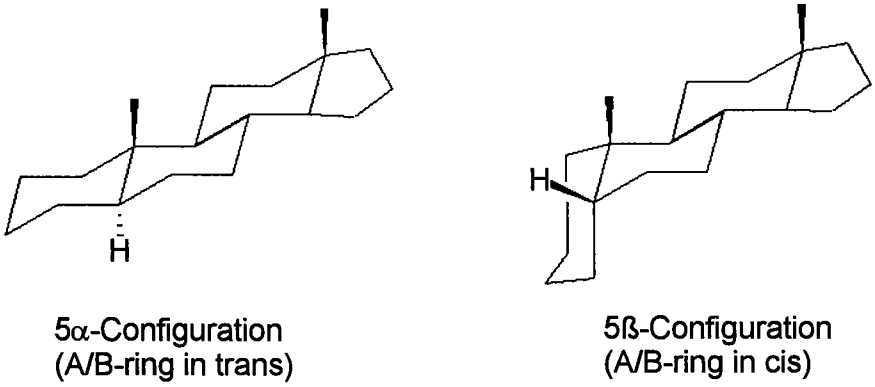

Fig.2 A/B-ring configuration of 5α- and 5ß-isomers

RESULTS

The current status of 5α/5ß-reduction for steroids with a 3-keto-4-ene structure is summarized in Table 1 [2]. The amount of 5α- and 5ß-isomers formed depends on the structure of the anabolic androgenic steroid (Table 2) and even the D-ring has a strong influence on the enzymatic activity of the C-4,5 double bond reducing enzymes.

Testosterone

Testosterone is the major AAS which is produced in man mainly in the testis. The main metabolic pathways of testosterone are summarized in Fig.3, where urinary excreted metabolites are underlined. All the excreted metabolites are conjugated

Table 1 AAS with a 3-keto-4-ene structure and reduction of the C-4,5 double bond in the metabolic pathway

Anabolic Androgenic Steroid	5α/5ß-Reduced Metabolites
Bolasterone	5ß-Isomer
Boldenone	5ß-Isomer
Calusterone	5α/5ß-Isomer
4-Chloro-1,2-dehydro 17α-methyltestosterone	Detected, only 5ß-isomer is proposed
Clostebol	Detected, both isomers are proposed
Fluoxymesterone	5α/5ß-Isomer
Formebolone	Not Detected
Metandienone	5ß-Isomer
Methyltestosterone	5α/5ß-Isomer
Mibolerone	Detected, only 5ß-isomer is proposed
19-Nortestosterone	5α/5ß-Isomer
Norclostebol	Detected, both isomers are proposed
Norethandrolone	Literature, both isomers are reported
Oxymesterone	Not detected
Testosterone	5α/5ß-Isomer
Trenbolone	Not detected

Table 2 Stereo specific metabolism of 3-keto-4-ene steroids to 5α- and 5ß-steroids (in relation to the D-ring structure of the metabolite) for the same male volunteer.

		D-ring strucutre			
		17ß-Hydroxy		17-Keto	
Substance	Applied amount	5α*	5ß*	5α*	5ß*
D3-Testosterone	20 mg	13	87	53	47
D3-Testosterone	2 mg	9	91	47	53
D7-11ß-Hydroxyandrost-4-en-3,17-dione, 20mg		NE	NE	94	6
19-Nortestosterone	20 mg	15	85	72	28
Methyltestosterone	100 mg	14	86	-	-
Methyltestosterone	10 mg	17	83	-	-
Bolasterone	20 mg	0	100	-	-
Calusterone	40 mg	22	78	-	-
Boldenone	22 mg	0	100	0	100
Boldenone	80 mg	0	100	0	100
Metandienone	22 mg	0	100	-	-
Metandienone	40 mg	0	100	-	-

* Results expressed in % of 5α- and 5ß-isomer, NE Not estimated,
D3 Deuteration at C-16,16,17, D7 Deuteration at C-2,2,4,6,6,16,16

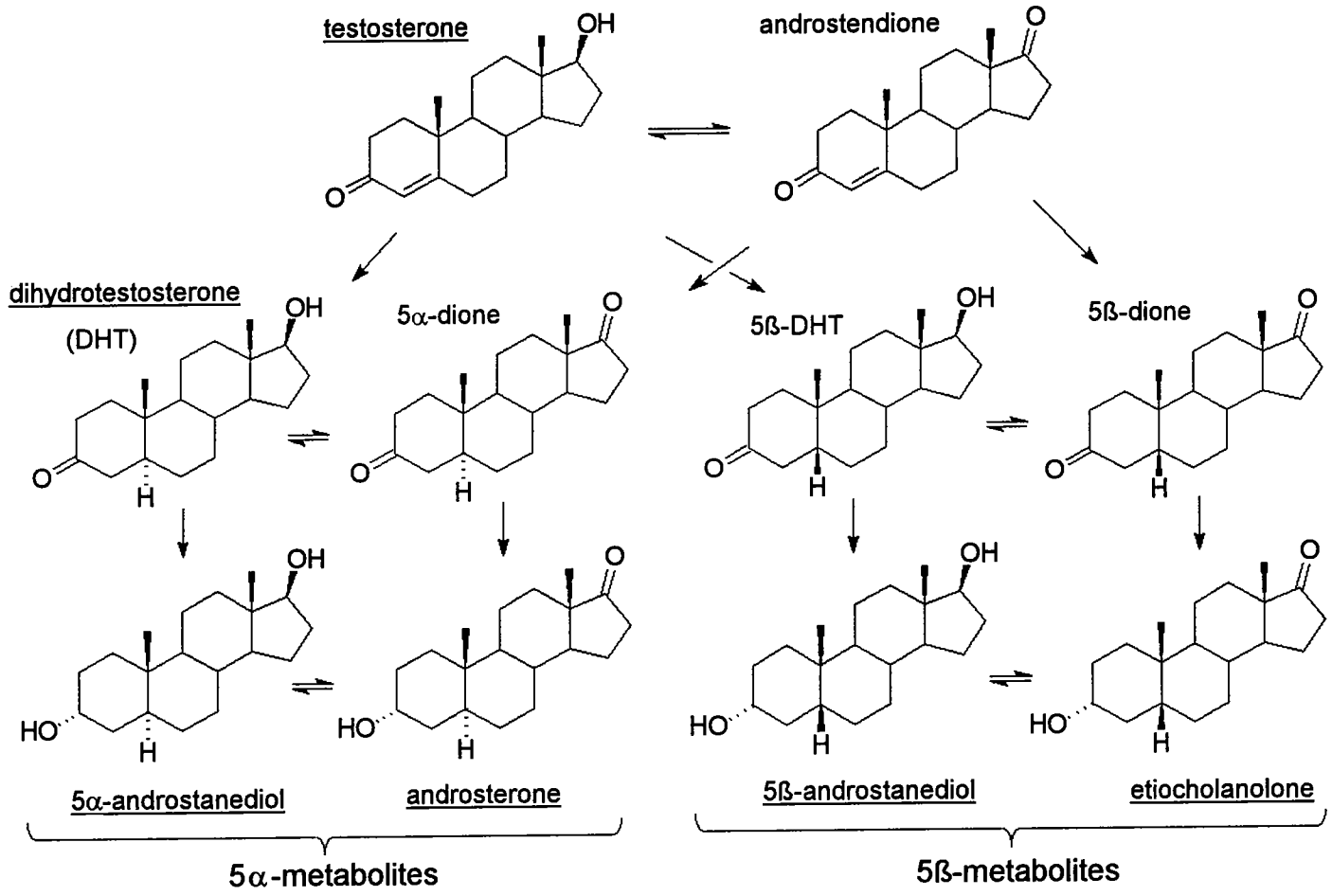

Fig.3 Metabolism of testosterone

in a 2nd phase metabolism, mainly with ß-glucuronic acid. Testosterone itself is excreted as a 17ß-glucuronide, whereas all other excreted metabolites have a 5α- or 5ß-structure.

After oral administration of 20 mg of [16,16,17-$^2H_3$]-testosterone to a male volunteer two deuterated diol isomers were confirmed, 5α-androstane-3α,17ß-diol and 5ß-androstane-3α,17ß-diol in a ratio of 13:87 (Table 2). A similar 5α/5ß-ratio of diol metabolites was observed when the same person applied 19-nortestosterone* (5α/5ß-ratio of 15:85 for 5α-estrane-3α,17ß-diol and 5ß-estrane-3α,17ß-diol) and methyltestosterone (Fig.4) (5α/5ß-ratio of 17:83 for 17α-methyl-5α-androstane-3α,17ß-diol and 17α-methyl-5ß-androstane-3α,17ß-diol for 10 mg application and a 5α/5ß-ratio of 14:86 after 100 mg application).

Influence of a 17-keto group

In contrast to the diol metabolites of testosterone with a 17ß-hydroxy structure (D-ring), the main metabolites androsterone (3α-hydroxy-5α-androstan-17-one) and etiocholanolone (3α-hydroxy-5ß-androstan-17-one) have a 17-keto D-ring structure. Compared to the above discussed 5α/5ß-ratio (13:87) of the diol metabolites of testosterone excretion, the 17-keto metabolites deuterated androsterone and etiocholanolone, show a different 5α/5ß-ratio of 53:47. These results can be explained through the metabolism of testosterone. The 17-keto metabolites are mainly produced via androstenedione (see Fig.3) which then is reduced by the 5α- and 5ß-reductase. In this case, the substrate for both reductases is not testosterone, rather androst-4-ene-3,7-dione, which has a 17-keto configuration instead of a 17ß-hydroxy group. The influence of the D-ring can be explained by a higher substrate specifity of the 5α-reductase to steroids with a 17-keto group with the result that the 5α-isomers are formed to a higher extent.

A similar result is observed in the metabolism of 19-nortestosterone (Table 2 and Fig.5) where the 5α- and 5ß-isomers with a 17-keto function are produced in a 5α/5ß-ratio of 72:28. Tho the corresponding diols, see above, are produced with a 5α/5ß-ratio of 15:85.

This difference in the rate of formation of 5α/5ß-isomers in relation to the D-ring structure can also be seen in steroid profiling of greater populations (Table 3).

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* The diol metabolites (5α-estrane-3α,17ß-diol and 5ß-estrane-3α,17ß-diol) of 19-nortestosterone are minor metabolites. The main metabolites are 17-keto metabolites as shown in Fig.5.

Table 3 5α/5ß-ratios of endogenous excreted metabolites of testosterone and cortisol (routine control samples of male athletes 1996)

	androsterone/ etiocholanolone	5α-androstanediol/ 5ß-androstanediol	allo-tetrahydrocorti-sol (THC)/THC
n	4542	4542	4524
mean	1.37 (58:42)	0.59 (37:63)	0.76 (43:57)
stdv	0.60	0.41	0.38
cv (%)	43.8	70.3	50.7

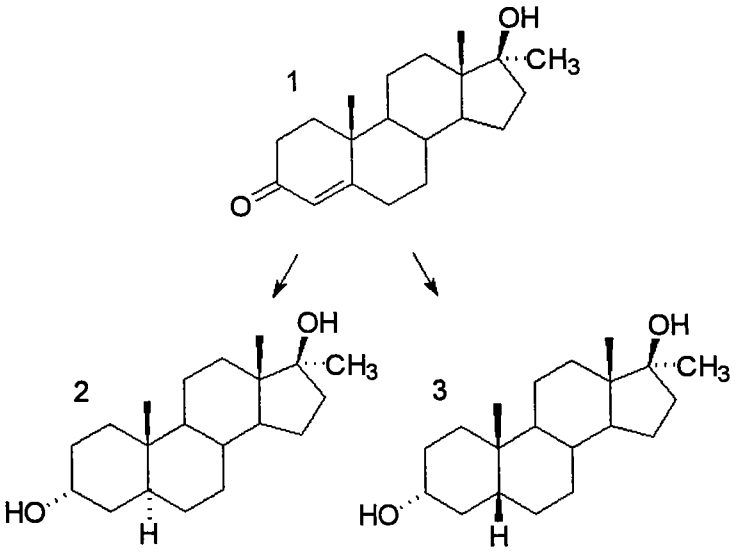

Fig. 4 Metabolism of methyltestosterone (1): main excreted metabolites: 17α-methyl-5α-androstane-3α,17ß-diol (2), 17α-methyl-5ß-androstane-3α,17ß-diol (3)

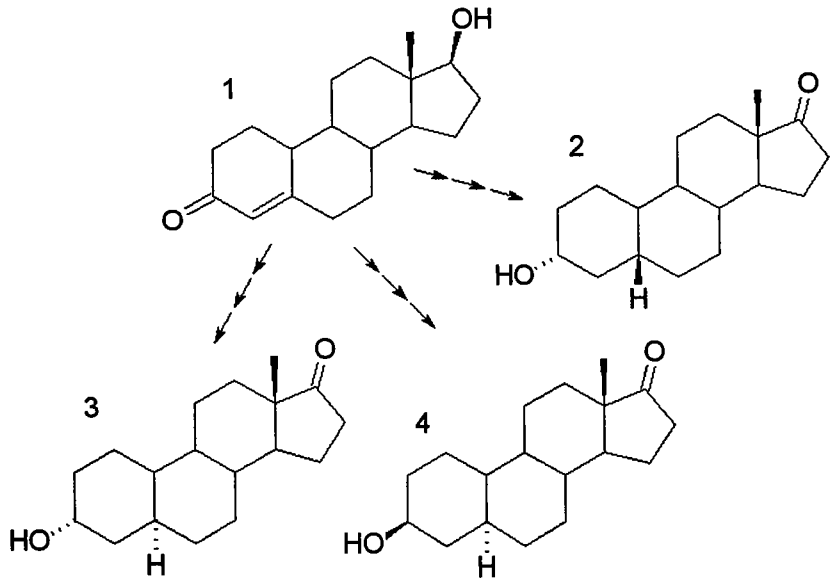

Fig.5 Metabolism of 19-nortestosterone (1): 3α-hydroxy-5ß-estran-17-one (2), 3α-hydroxy-5α-estran-17-one (3), 3ß-hydroxy-5α-estran-17-one (4)

Influence of a 11ß-hydroxy group

3-Keto-4-ene reduction of a steroid with a 11ß-hydroxy group and a 17-keto group was investigated with C-2,2,4,6,6,16,16 deuterated 11ß-hydroxyandrost-4-ene-3,17-dione. This steroid, which is produced by the adrenal gland, was mainly metabolized to the deuterated 5α-isomer, 3α,11ß-dihydroxy-5α-androstan-17-one (11ß-hydroxy-androsterone) (Fig.6). The 5α/5ß ratio was 95:5. The 5ß-reductase has a very low specifity for the 11ß-hydroxy structure But this result is strongly related to the 17-keto D-ring structure. In the metabolism of cortisol, a steroid which also has a 11ß-hydroxy structure, the 5ß-isomer is formed with a higher extent (Fig.7). The male volunteer peforming the excretion atudy shows in the metabolism of cortisol a 5α/5ß-ratio (allo-tetrahydrocortisol/tetrahydrocortisol) of 35:65. Results from 4524 male athletes controlled in 1996 show a 5α/5ß-ratio of 43:57 (Table 3).

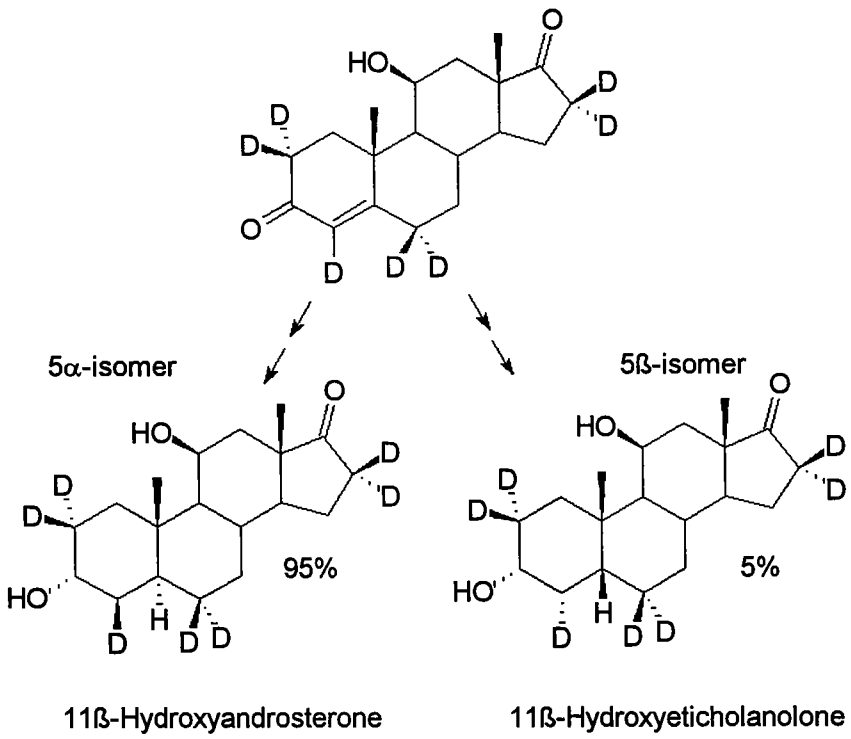

Fig. 6 Metabolism of deuterated [2,2,4,6,6,16,16,-$^{2}H_{7}$]-11ß-hydroxy-androst-4-ene-3,17-dione after oral application of 20 mg.

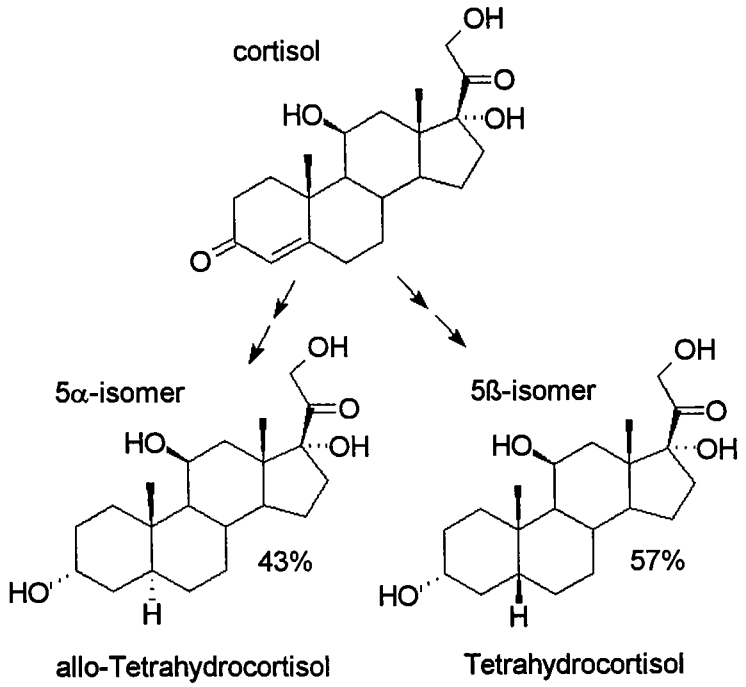

Fig. 7 Metabolism of cortisol (% values from Table 3)

Influence of a methyl group at C-7

Introduction of a methyl group at C-7 to methyltestosterone (C-7α, bolasterone and C-7ß, calusterone) has an influence on the activity of the 5α- and 5ß-reductase. An excretion study with 20 mg bolasterone to a male volunteer showed that no 5α-metabolite was produced (Fig.8). For 20 mg calusterone applied to the same volunteer the 5α/5ß-diol metabolites were produced in a ratio of 20:80 (Table 2 and Figure 9). Based on these results, one can conclude that introduction of a methyl group at C-7α totally hinders the activity of the 5α-reductase to reduce the C-4,5 double bond.

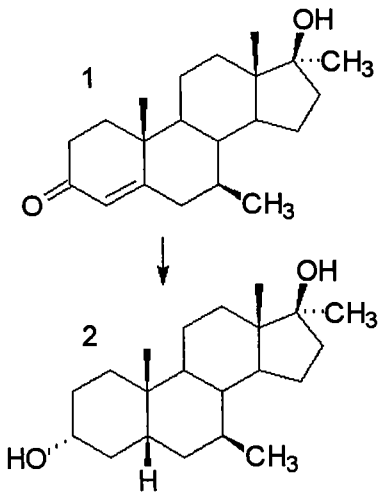

Fig.8 Metabolism of bolasterone (1): 7α,17α-dimethyl-5ß-androstane-3α,17ß-diol (2)

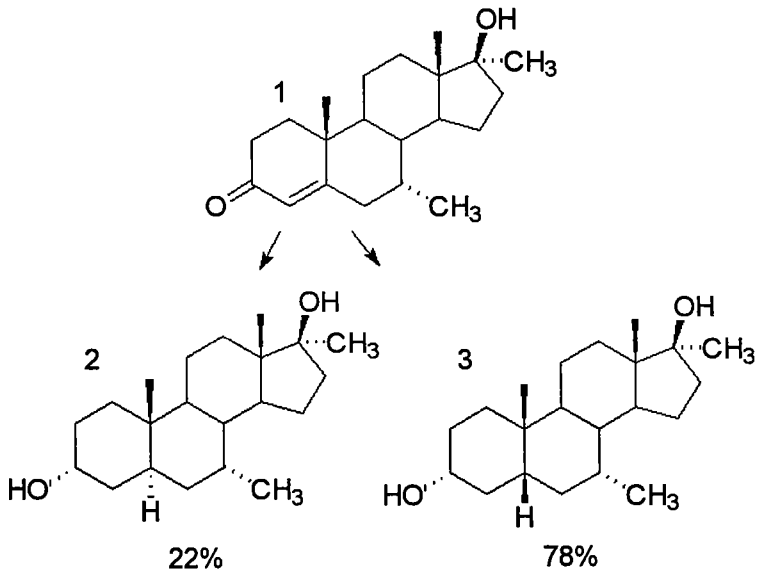

Fig. 9 Metabolism of calusterone (1): 7ß,17α-dimethyl-5α-androstane-3α,17ß-diol (2), 7ß,17α-dimethyl-5ß-androstane-3α,17ß-diol (3)

Influence of a double bond at C-1,2

The introduction of a double bond at C-1,2 completely inhibits the reduction of the C-4,5 double bond by 5α-reductase (Fig.10). Only 5ß-isomers were obtained in metabolism studies with boldenone (17ß-hydroxyandrosta-1,4-dien-3-one) (Fig.11) and metandienone (17ß-hydroxy-17α-methylandrosta-1,4-dien-3-one) (Fig.12) **[5, 6]**.

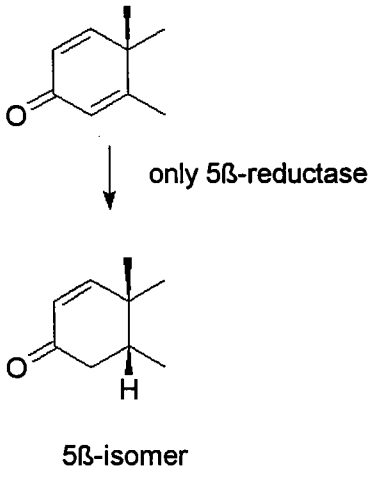

Fig. 10 A-Ring reduction of 3-ketoandrosta-1,4-diene such as boldenone and metandienone

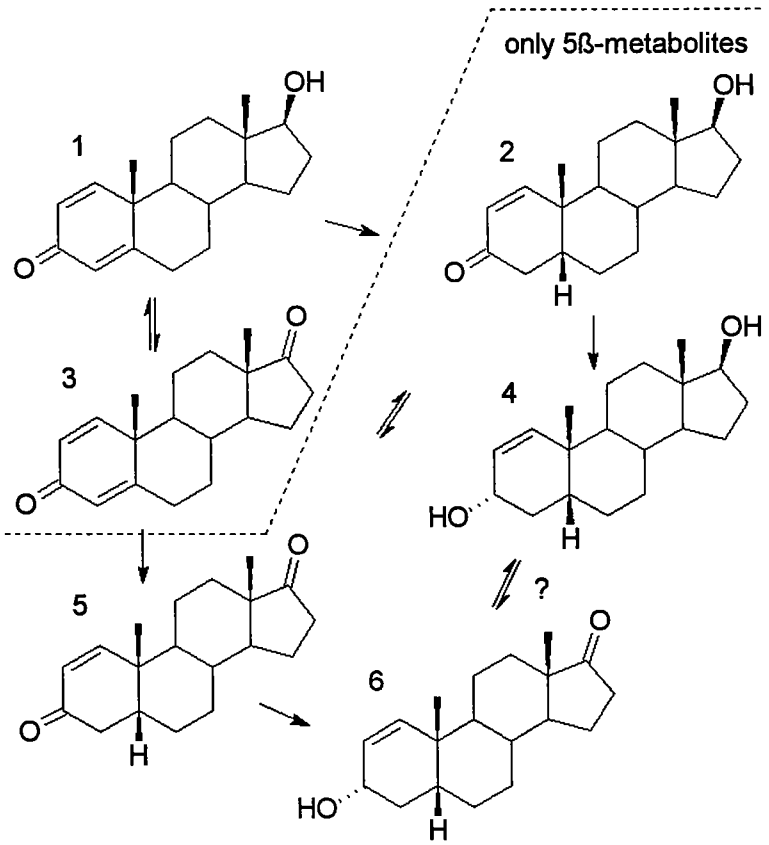

Fig.11 Metabolism of boldenone (1): 17ß-hydroxy-5ß-androst-1-en-3-one (2), androsta-1,4-diene-3,17-dione (3) (intermediate, not excreted into urine), 5ß-androst-1-ene-3α,17ß-diol (4), 5ß-androst-1-ene-3,17-dione (5) (intermediate, not excreted into urine), 3α-hydroxy-5ß-androst-1-en-17-one (6).

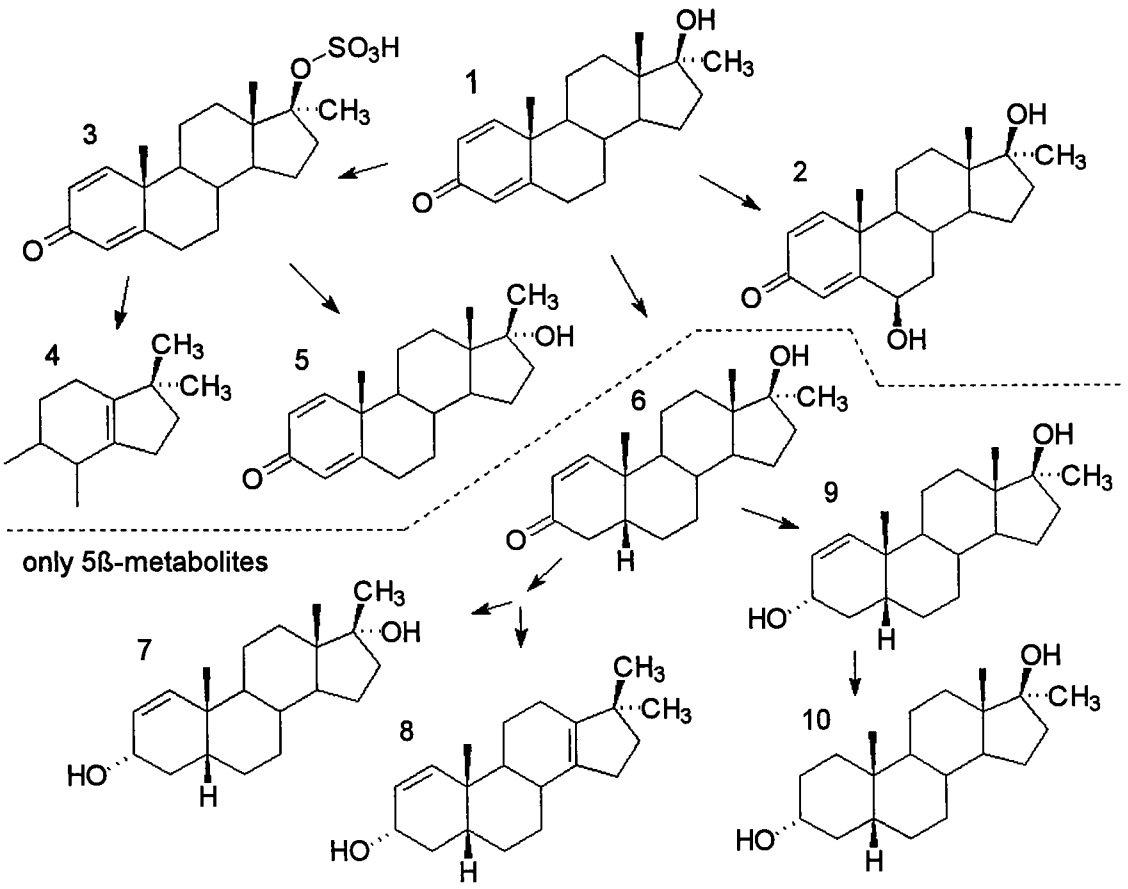

Fig.12 Metabolism of metandienone (1): 6ß-hydroxymetandienone (2) , metandienone 17ß-sulphate (3), 18-nor-17,17-dimethylandrosta-1,4,13-trien-3-one (4), 17-epimetandienone (5), 17ß-hydroxy-17α-methyl-5ß-androst-1-en-3-one (6), 17ß-methyl-5ß-androst-1-ene-3α,17α-diol (7), 18-nor-17,17-dimethyl-5ß-androsta-1,13-dien-3α-ol (8), 17α-methyl-5ß-androst-1-ene-3α, 17ß-diol (9), 17α-methyl-5ß-androstane-3α,17ß-diol (10)

Pharmacokinetics of 5α- and 5ß-reduced metabolites

In the metabolism of methyltestosterone (Fig.4) two tetrahydro metabolites, one with a 5α- and the other with a 5ß-structure, are detected. The ratio of these metabolites is not constant. The reason for the change in the ratio in one individual is the pharmacokinetic of methyltestosterone with different elimination times (half-lifes) of the 5α- and 5ß-metabolites. The scientific reason for this is yet not clear.

In Fig.13 and Fig.14 the urinary elimination ratios of the 5α- and 5ß-metabolites of methyltestosterone after oral administration of 10 and 100 mg to a male volunteer are presented. In both studies the 5α-metabolite is elimated at a smaller urinary

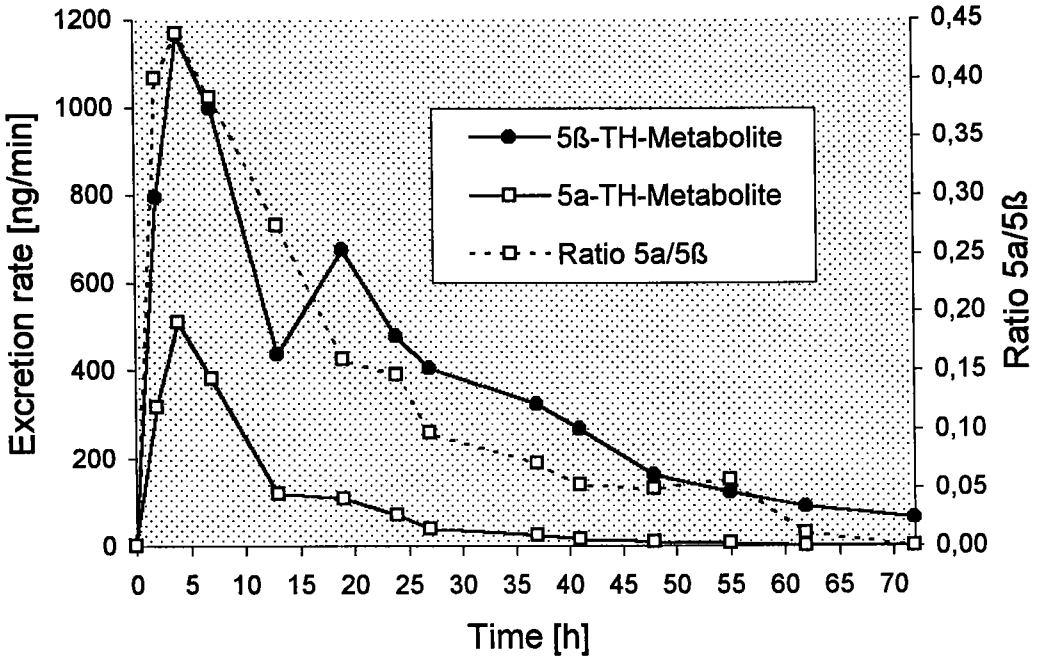

Fig. 13 Methyltestosterone: Excretion of 5α- and 5ß-metabolites (17α-methyl-5α-androstane-3α,17ß-diol and 17α-methyl-5ß-androstane-3α,17ß-diol) and the 5α/5ß-ratio after oral administration of 10 mg of methyltestosterone to a male volunteer.

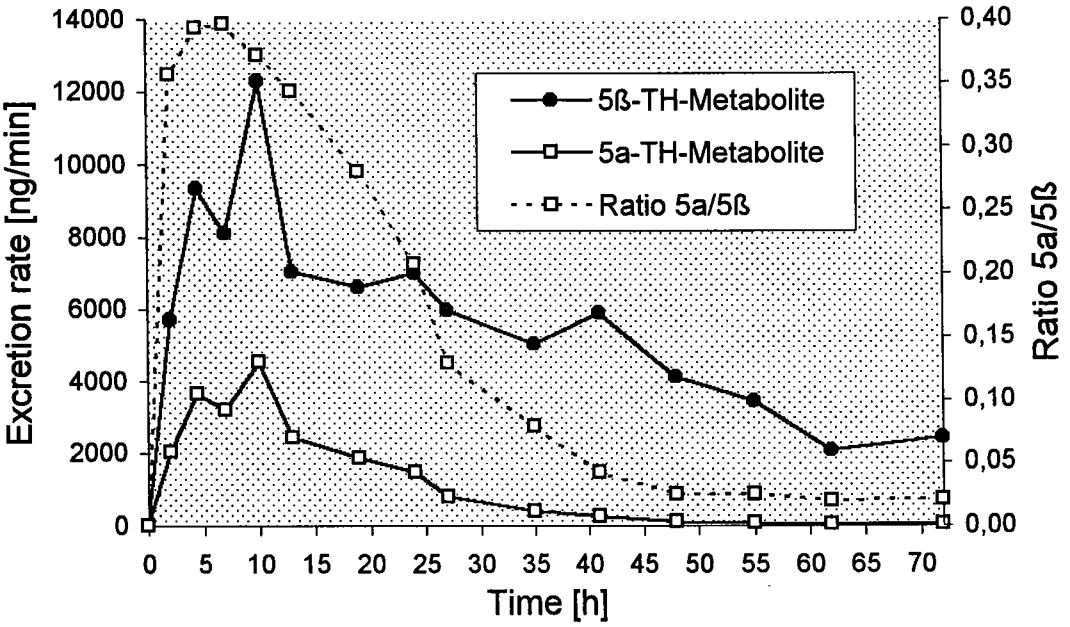

Fig. 14 Methyltestosterone: Excretion of 5α- and 5ß-metabolites (17α-methyl-5α-androstane-3α,17ß-diol and 17α-methyl-5ß-androstane-3α,17ß-diol) and the 5α/5ß-ratio after oral administration of 100 mg of methyltestosterone to a male volunteer.

excretion time and the 5α/5ß-ratio decreases from 0.4 shortly after administration to less than 0.05 after 40 hours. This indicates that after 40 hours the excretion of the 5ß-isomer is greater by a factor of more than 20.

In casesof methyltestosterone misuse, the 5α/5ß-ratio of the metabolites can be considered for additional discussion. When only the 5ß-isomer is detected in low concentration it can be expected that the concentration of the 5α-isomer is under the detection limit of the used analytical method. In this case, it cannot be concluded that methyltestosterone was not used but metandienone. The 5ß-isomer is also a metabolite of metandienone (Fig.12) and in the metabolism of metandienone no 5α-isomer is produced. To verify that metandienone was used, additional 5ß-metabolites, epimetendiol (17ß-methyl-5ß-androst-1-ene-3α,17α-diol) and 18-nor-epimetendiol (17,17-dimethyl-18-nor-5ß-androsta-1,13-dien-3α-ol) should be confirmed **[6, 7]**.

Different excretion profiles of 5α- and 5ß-metabolites were also observed in the metabolism of calusterone (Fig.9 and Fig.15), but the differences were not as clear as in the metabolism of methyltestosterone. Results of an excretion study with 20 mg of calusterone orally applied to a male volunteer are shown in Fig.15. The 5ß-tetrahydro metabolite is the dominant excreted steroid and the 5α/5ß-ratio diminishes within the first 40 hours after application, but after 40 hours it remains constant (in contrast to the methyltestosterone metabolites)

The excretion of 5α-and 5ß-metabolites was also investigated for 19-nortestosterone (Fig.5) which was administered orally to a male volunteer. The excretion of the main metabolites, 3α-hydroxy-5α-estran-17-one and 3α-hydroxy-5ß-estran-17-one, was very rapid (therefore the eliminaton curve in Fig.16 is shown on a logarithmic scale) and the 5α/5ß-ratio varied between 3 and 1 (Fig.16) and did not show the same decrease with time as compared to the methyltestosterone metabolites.

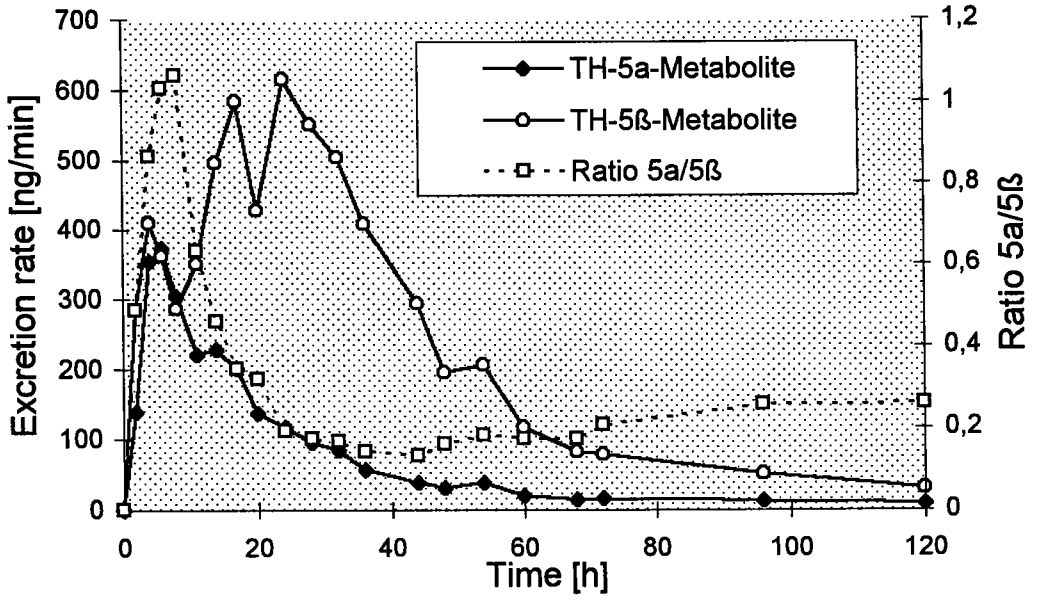

Fig. 15 Calusterone: Excretion of 5α- and 5ß-metabolites (7ß,17α-dimethyl-5α-androstane-3α,17ß-diol and 7ß,17α-dimethyl-5ß-androstane-3α,17ß-diol) and the 5α/5ß-ratio after oral administration of 20 mg of calusterone.

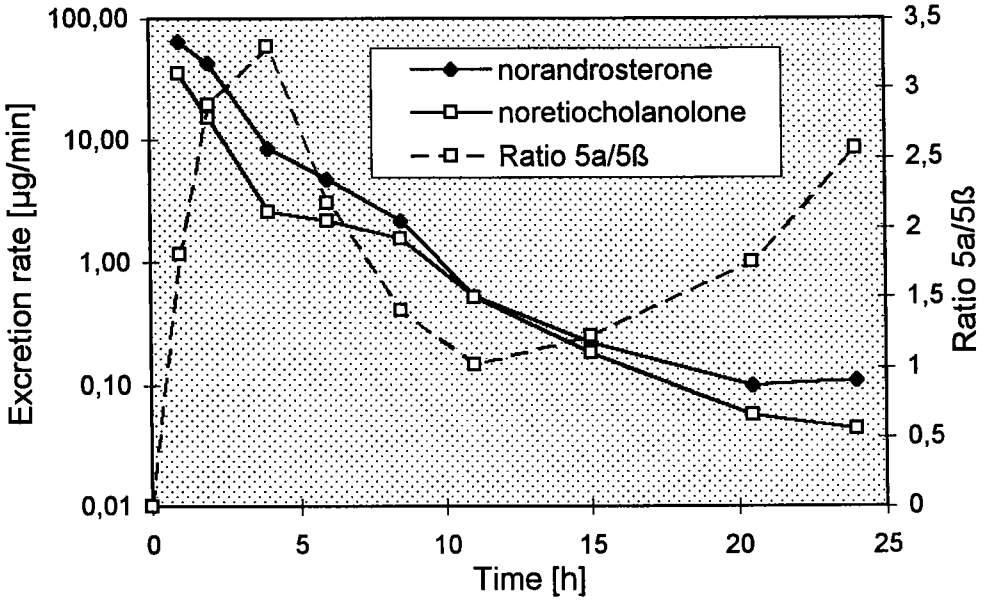

Fig. 16 19-Nortestosterone: Excretion of 5α- and 5ß-metabolites (3α-hydroxy-5α-estran-17-one and 3α-hydroxy-5ß-estran-17-one) and the 5α/5ß-ratio after oral administration of 20 mg of 19-nortestosterone.

Finally the pharmacokinetics of endogenous 5α-and 5ß-steroid metabolites of testosterone and 11ß-hydroxyandrost-4-ene-3,17-dione were investigated. Excretion studies with [16,16,17-$^2H_3$]-testosterone and [2,2,4,6,6,16,16-$^2H_7$]-11ß-hydroxyandrost-4-ene-3,17-dione were prepared. The pharmacokinetics of these endogenously produced, urinary excreted metabolites can only be estimated using lablled steroids, as the unlablled steroids are continueously produced in the body and excreted by the kidney. Fig. 17 shows that the excretion rates of the main metabolites of testosterone, androsterone (5α) and etiocholanolone (5ß), show similar behavior to the methyltestosterone metabolites. The 5ß-isomer is excreted with a longer elimination time, and, based on this, the 5α/5ß-ratio decreased with time. The 5α/5ß-ratio of the 17-keto metabolites show a maximum of 2.5 at 3 to 5 hours after oral administration of deuterated testosterone and decreased rapidly to a ratio of about 0.2.

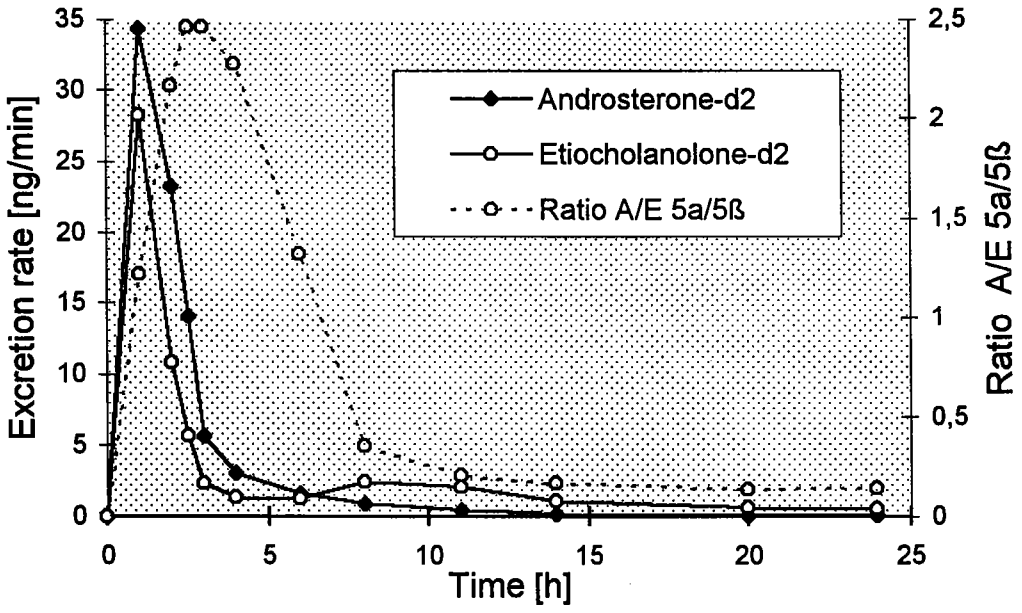

Fig. 17 Testosterone: Excretion of 5α- and 5ß-metabolites (d_2-androsterone - [16,16-$^2H_2$]-3α-hydroxy-5α-androstan-17-one - and d_2-etiocholanolone - [16,16-$^2H_2$]-3α-hydroxy-5ß-androstan-17-one) and the 5α/5ß-ratio after oral administration of 20 mg of [16,16,17-$^2H_3$]-testosterone.

This result show the importance of the excretion behavior of androsterone and etiocholanolone for the judgement of the urinary steroid profile. In general the androsterone/etiocholanolone ratio is very stable. This reflects the fact that androsterone and etiocholanolone are constantly produced in the body. Both steroids originate not only from testosterone, they are also metabolites of dehydroepiandrosterone (DHEA)

which is mainly produced in the adrenal gland. When production rates of androgens are high (which occurs in situations of stress) and androsterone and eticholanolone are excreted in high amounts, the 5α/5ß-ratio can increase since androsterone is eliminated more rapidly. Whether the change of the 5α/5ß-ratio is a possible indicator for high stress situations is under investigation.

Another steroid produced by the adrenal gland is 11ß-hydroxyandrost-4-ene-3,17-dione. The main metabolites of this steroid are 11ß-hydroxyandrosterone (5α-isomer) and 11ß-hydroxyetiocholanolone (5ß-isomer). Both steroids are also indirect metabolites of cortisol. They are produced via conversion of cortisol by a side-chain cleavage to 11-hydroxy-androst-4-ene-3,17-dione followed by the above discussed metabolism to the 5α- and 5ß-isomers, or by a side chain-cleavage of the main metabolites of cortisol, which are tetrahydrocortisol and allo-tetrahydrocortisol. The pharmacokinetics of the 11ß-hydroxy metabolites of 11ß-hydroxyandrost-4-ene-3,17-dione were studied after oral application of the deuterated compound (Fig.7). The excretion curve of both metabolites and the 5α/5ß-ratio is shown in Fig.18.

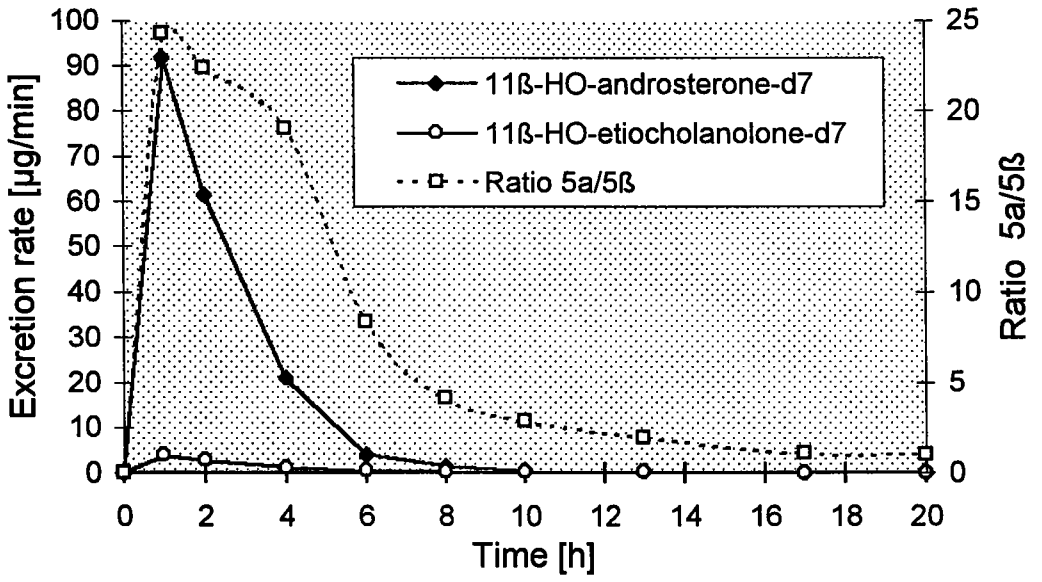

Fig. 18 11ß-Hydroxyandrost-4-ene-3,17-dione: Excretion of 5α- and 5ß-metabolites (d7-11ß-hydroxy-androsterone and d7-11ß-hydroxy-etiocholanolone) and the 5α/5ß-ratio after oral administration of 20 mg of [2,2,4,6,6,16,16,-$^{2}H_{7}$]-11ß-hydroxyandrost-4-ene-3,17-dione.

The excretion curves of both metabolites show that the 5α-metabolite is dominant and that the urinary elimination time is shorter, as compared to the 5ß-isomer.

The production rate of the precursors of the 11ß-hydroxy isomers varies by a factor of 10 depending on the daily activity of the adrenal gland. This implies that after high adrenal activity, the 5α-isomer, 11ß-hydroxyandrosterone, is predominately excreted as compared to the 5ß-isomer. The 5α/5ß-ratio can change and decrease in the time period after high adrenal activity, as shown in Fig.18.

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