Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (8)

W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck-Engelke
(Editors)

Sport und Buch Strauß, Köln, 2000

W.SCHÄNZER, A.BREIDBACH, H.GEYER, C.V.KUK, E.NOLTEERNSTING, M.THEVIS:
Metabolism of Nortestosterone, Norandrostenedione and Norandrostenediol: Identification of 3a-hydroxyestr-4-en-17-one glucuronide and 3a,16a-dihydroxy-5a-estran-17-one glucuronide and sulphate
In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (8). Sport und Buch Strauß, Köln, (2000) 155-174

Wilhelm Schänzer, Andreas Breidbach, Hans Geyer, Carsten van Kuk, Eckhard Nolteernsting† and Mario Thevis

Metabolism of Nortestosterone, Norandrostenedione and Norandrostenediol
Identification of 3α-hydroxyestr-4-en-17-one glucuronide and 3α,16α-dihydroxy-5α-estran-17-one glucuronide and sulphate

Institute of Biochemistry, German Sport University Cologne

Introduction

Nortestosterone (19-nortestosterone; nandrolone; 17ß-hydroxyestr-4-en-17-on) is an anabolic androgenic steroid and has been marketed for medical treatment since 40 years. Because the oral administration of nortestosterone will result in an extensive metabolism of nortestosterone by the liver (first pass effect) yielding no or less therapeutical effects nortestosterone is applied as an ester, e.g. nortestosterone decanoate, by intra muscular injection .

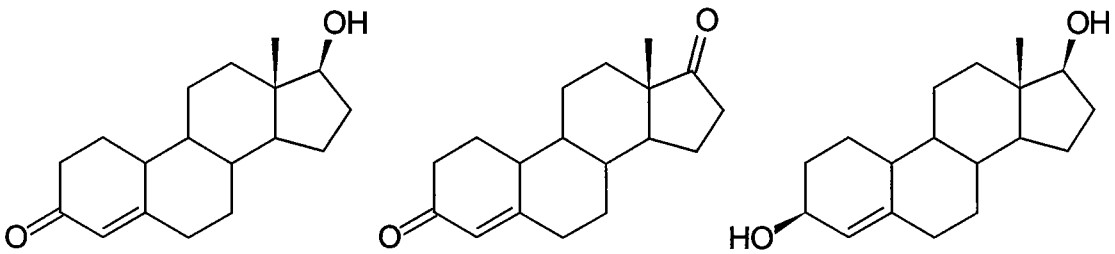
Nortestosterone (Fig.1) was synthesised in 1950 by Birch [1] and by Wilds and Nelson [2] in 1953. The metabolism was investigated by Engel et al. in 1958 [3]. The metabolism follows strongly the testosterone pathway and the main metabolites were confirmed as 3α-hydroxy-5α-estran-17-one (norandrosterone) and 3α-hydroxy-5α-estran-17-one (noretiocholanolone) (Fig.2). The structure of both metabolites was elucidated by synthesis in 1960 [4]. Beside of these metabolites also 3ß-hydroxy-5α-estran-17-one is excreted into urine as a 3ß-sulphate in a comparable amount to the 3α-hydroxy metabolites [5].

Since 1998 prohormones of nortestosterone such as 4-norandrostenedione (estr-4-ene-3,17-dione), 4-norandrostenediol (estr-4-ene-3ß,17ß-diol and 5-norandrostenediol (estr-5-ene-3ß,17ß-diol) (Fig.1) are marketed in the United States and other countries as nutritional supplements and offered as oral and sublingual preparations.

All these so-called pro hormones are banned by the International Olympic Committee. The misuse of nortestosterone is controlled by detection of norandrosterone and noretiocholanolone, the main metabolites of nortestosterone. As the prohormones of nortestosterone are following the same metabolic pathway as nortestosterone (Fig.2) norandrosterone is the target substance in doping control for all these 19-norsteroids. The identification of norandrosterone is achieved using gas chromatographic/mass spectrometric (GC/MS) analysis.

The actual determination does not allow to distinguish and estimate which 19-norsteroid was misused. Nevertheless all the 19-norsteroids are banned and an offence against the

doping rule can by proved by the identification of norandrosterone when exceeding an urinary concentration of 2ng/ml of urine for male and 5 ng/ml for female athletes.



Nortestosterone 4-Norandrostenedione 4-Norandrostenediol

Fig 1. Structure formular of 19-norsteroids

Following a recent report of the metabolism of 4-norandrostenedione [6] an unknown metabolite has been described as dehydro-19-norandrosterone but the structure of the metabolite could not be elucidated. The possibiliy of a 1,2-dehydro structure was speculated by the authors.
This work presents the identification of new metabolites of nortestosterone and its pro hormones. The metabolites were identified after oral administration of nortestosterone, 4-norandrostendione and 4 norandrostenediol.

Results and Discussion

Synthesis of metabolites

3α-Hydroxyestr-4-en-17-one

The metabolite 3α-hydroxyestr-4-en-17-one was synthesised in mg amounts by reduction of 4-norandrostenedione with K-Selektride (Fig.3) [7]. The reaction yielded mainly the 3α-hydroxyestr-4-en-17-one (3α/3ß, 93%/7%. The reaction with K-Selektride is selective for the 3-keto group and the 17-keto group will only be reduced when the reagent is used in excess.

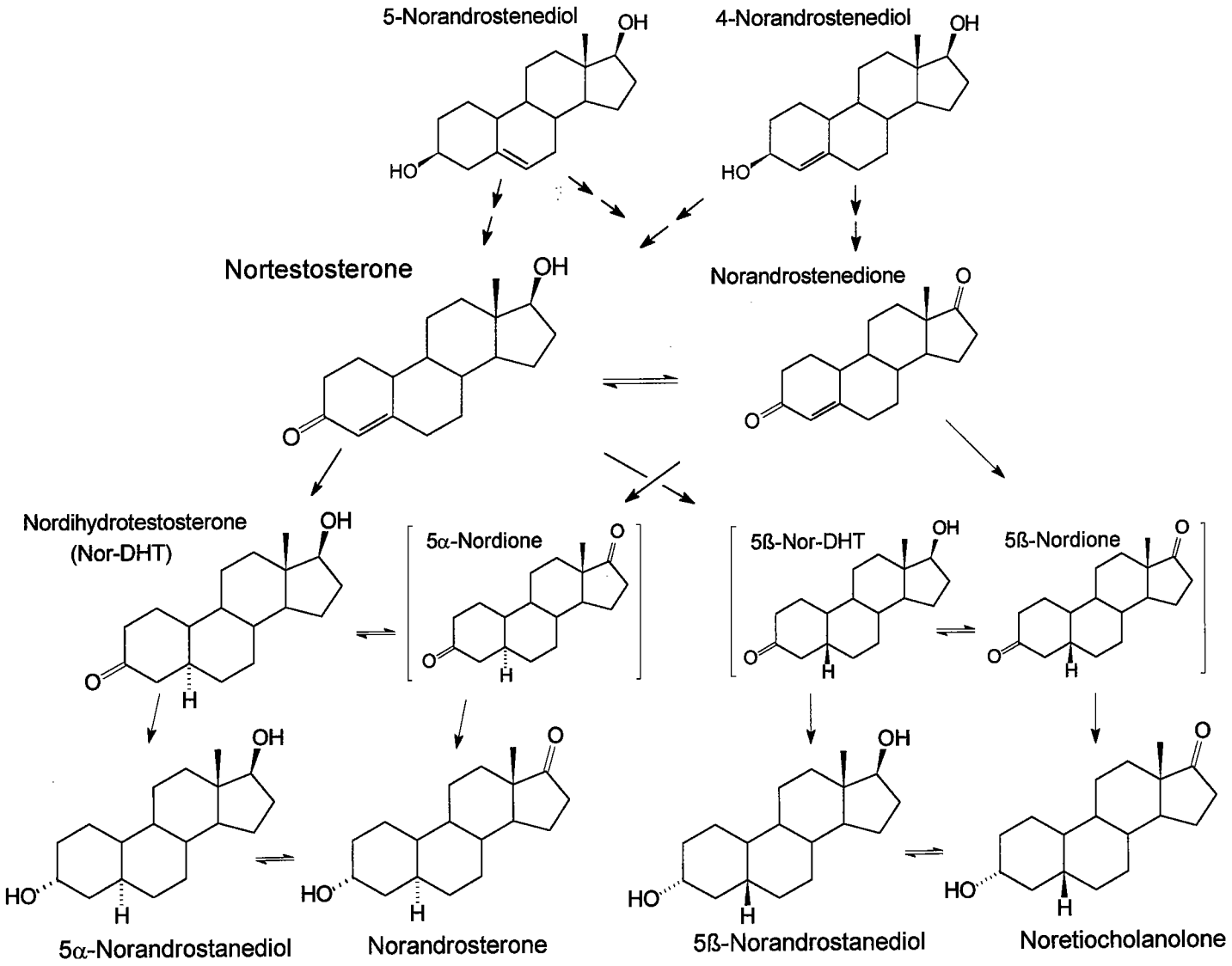


Fig.2 Principle metabolism of nortestosterone and its pro hormones yielding norandrosterone and noretiocholanolone

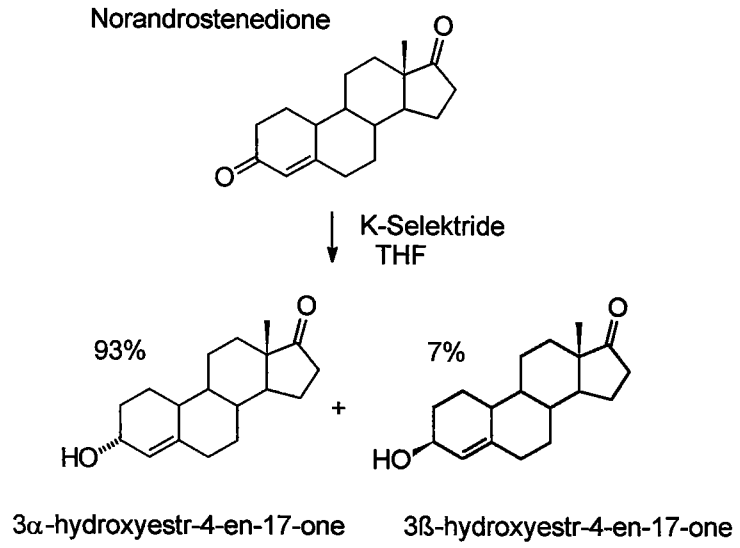


Fig.3 Synthesis of 3α-hydroxyestr-4-en-17-one

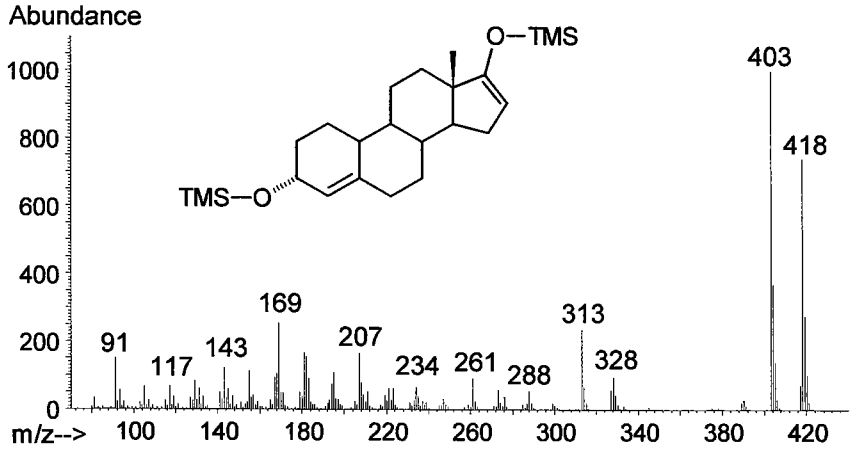


Fig.4 EI mass spectrum of 3α-hydroxyestr-4-en-17-one bis-TMS (M^+ 418)

The EI mass spectrum of the 3α-hydroxyestr-4-en-17-one bis-TMS derivative is displayed in Fig.4 and is characterised by an abundant molecule ion 418 and the fragment ion m/z 403 (M^+ -15). The fragment ion m/z 169 is typical for trimethylsilylated 17-keto steroids and is assumed to be generated after cleavage of the C11-12, C13-14 and C14-15 bonds. The fragmentation is proposed as presented in Fig.5.

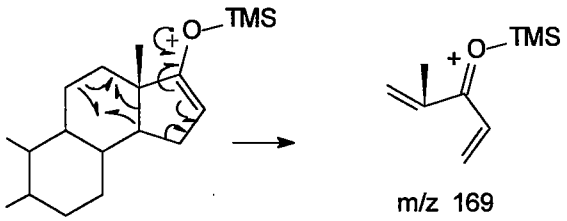


Fig.5. Proposed fragmentation for the C/D-ring fragment ion m/z 169.

3α,16α-Dihydroxy-5α-estran-17-one (16α-hydroxynorandrosterone)

The synthesis of 16ß-hydroxynorandrosterone started with 20 mg of norandrosterone followed by the same reaction pathways as described for the synthesis of 16ß-hydroxystanozolol [8], see Fig.6.

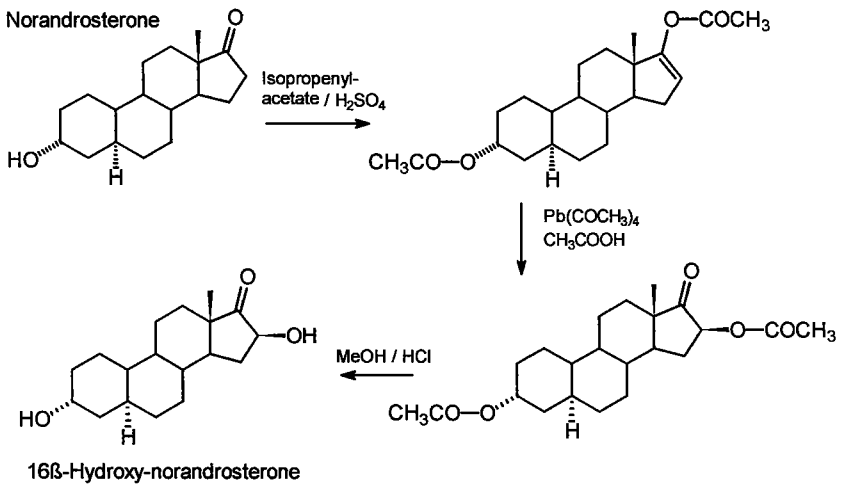


Fig.6 Synthesis of 16ß-hydroxynorandrosterone

The synthesis of 16α-hydroxynorandrosterone started with 20 mg of norandrosterone followed by the same reaction pathways as described for the synthesis of 16ß-hydroxystanozolol [8], see Fig.7.

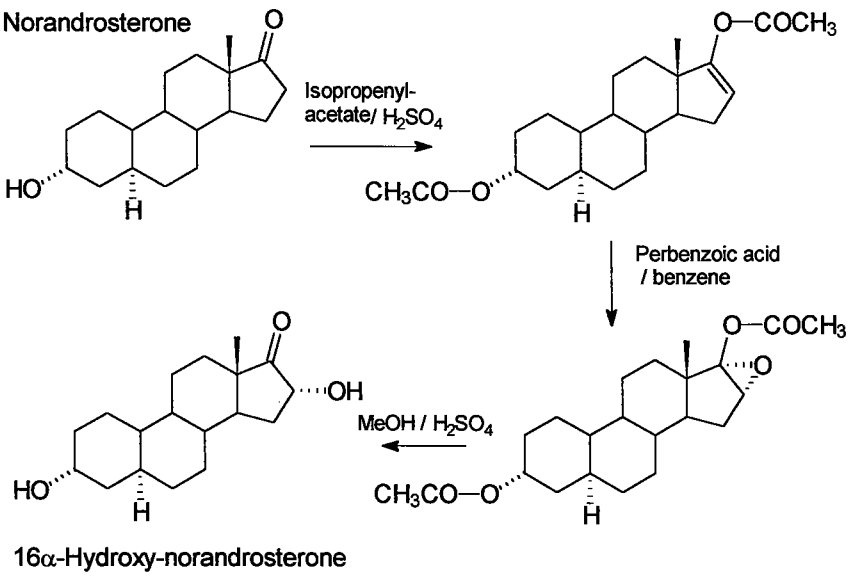


Fig.7 Synthesis of 16α-hydroxynorandrosterone

The EI-mass spectrum of 16α-hydroxynorandrosterone tris-TMS (Fig.11) was identical with the mass spectrum of the metabolite isolated from urine but it is also identical with the mass spectrum of 16ß-hydroxynorandrosterone tris-TMS.

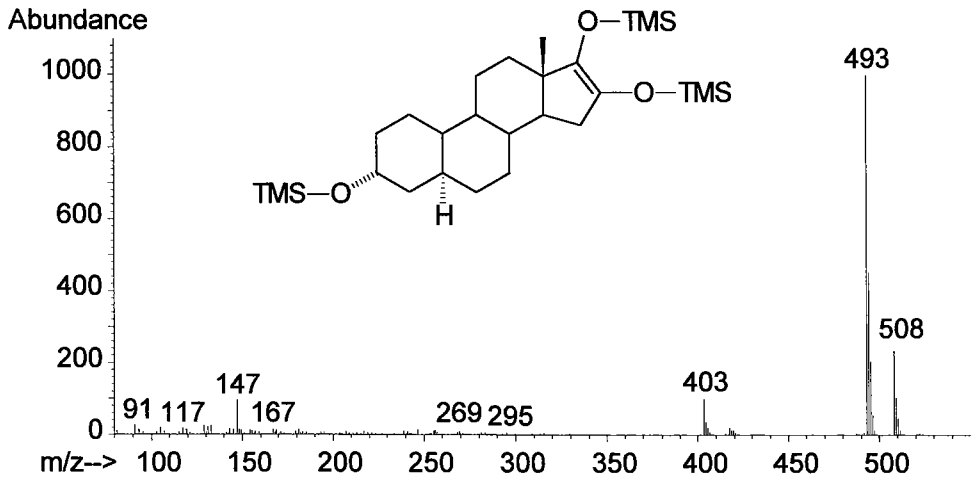


Fig.8 EI-mass Spectrum of 16α-hydroxynorandrosterone tris-TMS

Discussion: Syntehsis of 16α-hydroxynorandrosternone

An unknown metabolite was detected after oral administration of nortestosterone, 4-norandrostenedione and 4-norandrostenediol with a molecular weight of 292. Compared to norandrosterone with a molecular weight of 276 the difference of 16 mass units may be explained by the indroduction of a hydroxy group to norandrosterone. A hydroxylation of 4-norandrostenedione was assumed followed by the A-ring metabolism yielding a tetrahydro isomer with 5α-structure and a possible 3α-hydroxy group. The EI-mass spectrum of the per-TMS derivative of this metabolite showed a molecular ion of 508 corresponding to the introduction of three TMS-groups. A comparison of the mass spectra with a reference spectrum of 16ß-hydroxy-dehydroepiandrosterone (16ß-DHEA) shows high similarities in fragmentation pattern from which we conclude that the structure of the D-ring of the metabolite is based on a hydroxylation at C-16. The question remains whether this metabolite has the hydroxy-group at the C-16α or C-16ß-position. Both isomers yield the same D-ring structure, 16-ene-16,17-bis-O-TMS when derivatised using MSTFA/TMIS based on the enolisation of the 17-keto group.
Identification of the 16-hydroxy structure is based on the synthesis of 16ß- and 16 α-hydroxy-norandrosterone and characteristic reactions of both isomers.

The synthesised 16ß-hydroxynorandrosterone was identical with the urinary metabolite after per-TMS derivatisation, which confirmed the 16-hydroxy position but nevertheless the enol-TMS-derivative does not allow to establish the exact configuration of the 16-hydroxy position (see Fig.9)

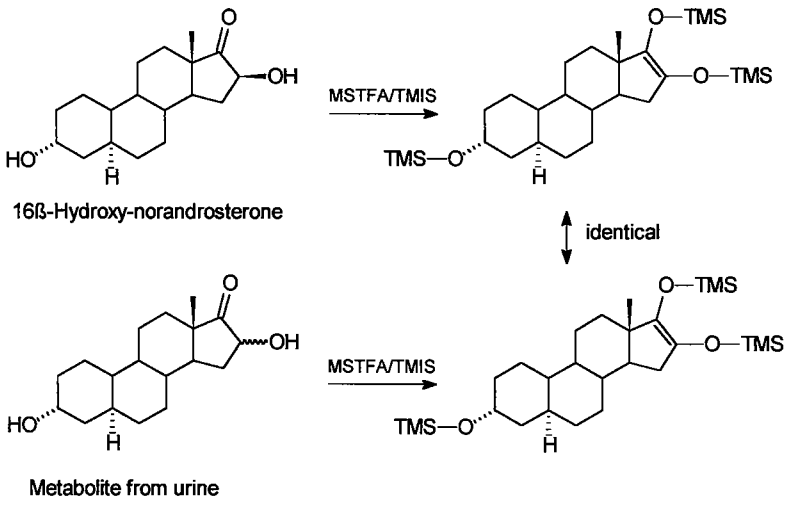


Fig.9 Comparison of per-TMS derivatives of the isolated metabolite and synthesised 16ß-hydroxynorandrosterone

To establish whether the 16-hydroxy configuration of the urinary metabolite is 16ß-hydroxy, both steroids (urinary metabolite and synthesis product) were reduced with lithium aluminium hydride yielding mainly (>90%) the 17ß-configuration. The derivatised reduction products (TMS-derivatives) of metabolite and synthesis product were not in agreement confirming that the structure of the metabolite is not 16ß-hydroxy (Fig.10).

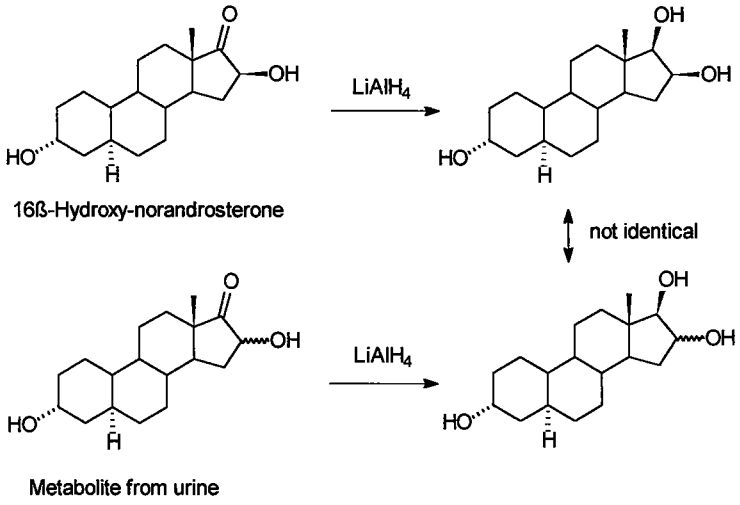


Fig.10 Comparison of per-TMS derivatives of lithium aluminium hydride reduced urinary metabolite and synthesised 16ß-hydroxy norandrosterone

Also the reaction of the reduced isolated metabolite and the reduced synthesized 16ß-hydroxy product with acetone catalysed by perchloric acid yielded only an acetonide with the synthesised 16ß-hydroxy-norandrosterone (16ß and 17ß-hydroxy-groups are in cis-configuration), confirming additionally that the structure of the metabolite was not 16ß-hydroxy (Fig.11).

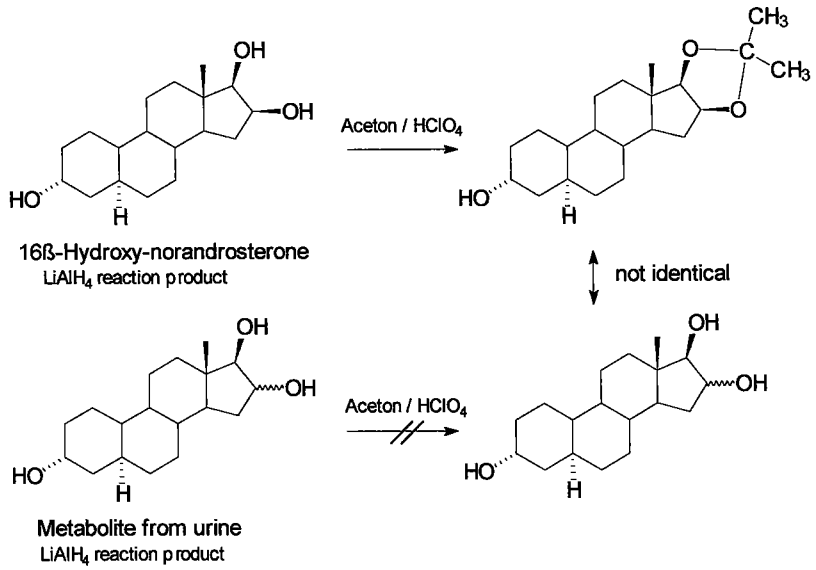


Fig.11 Acetonide formation of the lithium aluminium hydride reduction products of synthesised 16ß-hydroxy norandrosterone and the urinary metabolite

The conclusion of these investigations was that the structure of the metabolite is 16α-hydroxy which was proved by synthesis of 16α-hydroxynorandrosterone following the same reaction pathways as for the synthesis of 16α-hydroxystanozolol [8] in Fig.7.
The 16α-hydroxynorandrosterone product was identical with the metabolite when both steroids were reduced with lithium aluminium hydride and analysed by GC/MS after per-TMS derivatisation.

6ß-Hydroxy-4-norandrostenedione

6ß-Hydroxynorandrostenedione was synthesized following the light oxidation of the 3-enol TMS ether [9]. The reaction is highly stereo selective yielding mainly the 6ß-hydroxy derivative. When derivatised with MSTFA/NH_4I the 6ß-hydroxy-4-norandrostenedion yields a tris-TMS derivative. The EI-mass spectrum displays an intense molecular ion m/z 504 and less abundant A/B-ring fragement m/z 282 (Fig.13). A similar fragmentation has been reported for per-TMS enol-TMS derivatives of 6ß-hydroxy steroids of 3-keto-4ene steroids such as testostereone, methyltestosterone etc. [9]

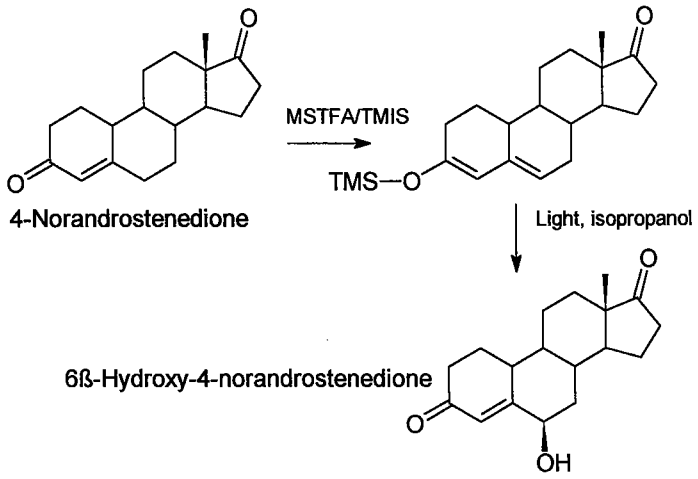
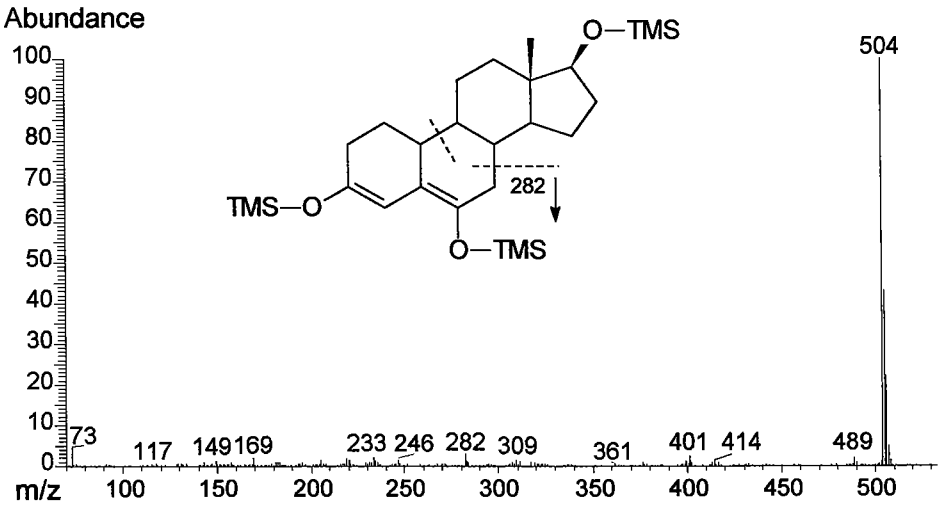


Fig.12 Synthesis of 6ß-hydroxy-4-norandrostenedione



Fig,13 EI mass spectrum of 6ß-hydroxy-4-norandrostenedione tris TMS

Metabolism studies

Metabolism studies were performed after a single oral application with 50 mg of nortestosterone (volunteer A), 100 mg of 4-norandrostenedione (volunteer A and B) and 100 mg of 4-norandrostenediol (Volunteer A). Vounteer A performed the excretion experiments with a time delay of 3-4 weeks considering that nortestosterone metabolites of the preceding administration were completely eliminated. Urine samples were collected after administration and stored at 4°C. It was obvious that after oral administration of nortestosterone, 4-norandrostenedione and 4-norandrostenediol the main metabolites norandrosterone, noretiocholanolone were excreted as

glucuronides but also as 3-sulphated conjugates. Further metabolites which could be confirmed in both fractions in all excretion studies was 16α-hydroxynorandrosterone, whereas the 3ß-hydroxy-5α-estrane (epinorandrosterone) metabolite was only identified in the sulphate fraction. On the other hand the identified metabolite 3α-hydroxyestr-4-en-17-one in all studies was only excreted in the glucuronic fraction. This metabolite was even compared to norandrosterone and noretiocholanolone less stable in urine. Further studies concerning the stability of this metabolite should be performed. As a result of this instability the conjugated metabolite was hydrolysed (possible isolation in the unconjugated fraction) and the 3α-hydroxy group was oxidised to 4-norandrostenedione. For precise estimation of 4-norandrostenedione the urine samples should be stored at -20°.

The following identified metabolites where excreted as glucuronide and/or as sulpfate or only as glucuronide (Table.1)

It was not investigated to what extent nortestosterone and 4-norandrostenedione are excreted into urine after oral application of 19-norsteroids. Both steroids were detected only in small amounts in the first hours after administration. Both steroids were analysed in the total fraction (unconjugated and glucuronide fraction after hydrolysis with ß-glucuronidase from E.coli). The excretion of 4-norandrostenedione in the unconjugated fraction was confirmed but the possibility of a 3-enol glucuronide has to be taken into consideration and should be further investigated. Neither nortestosterone nor 4-norandrostenendione were detected in the sulphate fraction. For both steroids the formation of a labile 3-enol sulphate should also be considered.

Table 1

Substance	Glucuronide	Sulphate
Nortestosterone	yes	no
4-Norandrostenedione	no ?	no
3α-Hydroxy-5α-estran-17-one*	yes	yes
3α-Hydroxy-5ß-estran-17-one**	yes	yes
3ß-Hydroxy-5α-estran-17-one***	no	yes
3α-Hydroxyestr-4-en-17-one	yes	no
3ß-Hydroxyestr-4-en-17-one	yes	no
5α-Estrane-3α,17ß-diol	yes	yes
5ß-Estrane-3α,17ß-diol	yes	yes
3α,16α-Dihydroxy-5α-estran-17-one	yes	yes

? possibility of a 3-enol glucuronide

* norandrosterone, ** noretiocholanolone, *** epinorandrosterone

The following Fig.14-16 show the excretion of the main metabolites after a single oral application at the beginning of the excretion (A) and after 60 to 70 hours (B). The chromatograms present also a comparision between the glucuronide and sulphate fractions.

4-Norandrostenedione (Estr-4-ene-3,17-dione)

After a single dose of 100 mg of 4-norandrostenedione norandrosterone was the dominating metabolite in the glucuronide fraction. The concnetration of norandrosterone and noretiocholanolone exceeds the concentration of the same metabolites in the sulphate fraction (Fig.14). Norandrosterone could be identified even 70 hours after application in both fractions, whereas noretiocholanolone could not be confirmed in the sulphate fraction (screening) after 70 hours. The 3α-hydroxyestr-4-en-17-one metabolite was easily determined in the glucuronide fraction in the first day and longer. In general it was 2-5% in its intensity compared to norandrosterone. In both fractions (glucuronic and sulphate) 16α-hydroxynorandrosterone was identified in the beginning of the excretion and also 70 hours after application. Compared to the excretion of noandrosterone the 16α-hydroxy metabolite showed a delayed excretion profile and the ratio of norandrosterone to 16α-hydroxynorandrosterone increased by time. As presented in Fig.14 a triplett of 16-hydroxymetabolites was detected at m/z 493 (M^+ -15) from which the first signal corresponds to synthesised 16α-hydroxynorandrosterone. The other both isomers show similar EI mass spectra and they are assumed to be the corresponding 16α-hydroxy metabolites of noretiocholanolone and epinorandrosterone.

4-Norandrostenediol (Estr-4-ene-3ß,17ß-diol)

After oral application of 100 mg of 4-norandrostenediol no parent compound was detected in the glucuronide and sulphate fraction. Compared to 4-norandrostenedione application the excretion of 3α-hydroxyestr-4-en-17-one metabolite was more intense in relation to norandrosterone and noretiocholanolone (Fig.15A). But only after 4-norandrostenediol administration 3ß-hydroxyestr-4-en-17-one, the 17-oxidation metabolite of the administered substance, could be identified in the glucuronide fraction. The ratio of norandrosterone to noretiocholanolone in the glucuronide fraction was lower than after 4-norandrostenedione application and even after about 70 hours the concentration of noretiocholanolone in urine exceeds that of norandrosterone (Fig.15B).

The differences in the ratio of norandrosterone to noretiocholanolone after 4-norandrostenediol application compared to 4-norandrostenedione and nortestosterone application may allow to distinguish in an individual between an application of 4-norandrostenediol and other 19-nor-steroids. 16α-Hydroxynorandrosterone was detected in both fractions and the elimination pattern

as glucuronide and sulphate was comparable to the 4-norandrostenedione application. In general the intensities of all metabolites were lower than after the 4-norandrostenedione administration.

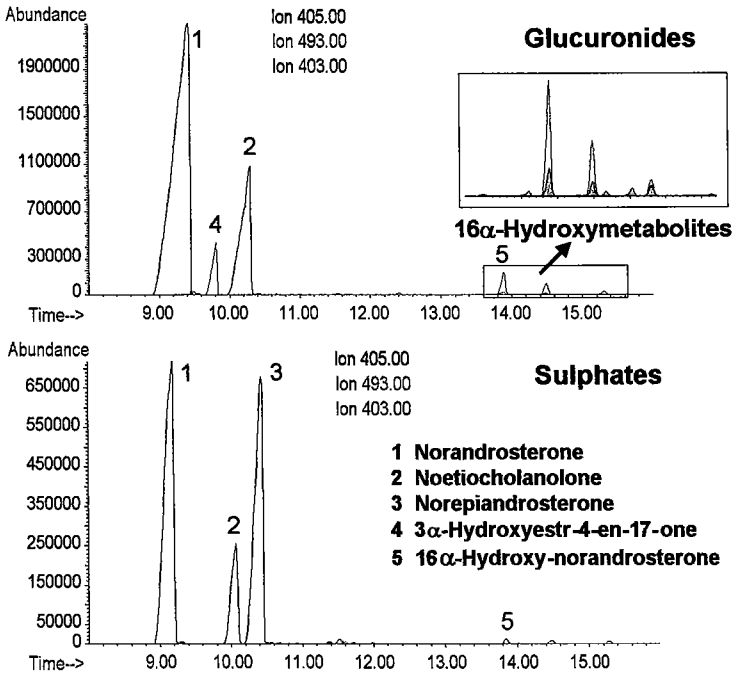


Fig.14A Profile of urinary excreted 19-normetabolites 2.7-5.3h after oral administration of 100 mg of 4-norandrostenedione (glucuronide and sulphate fraction)

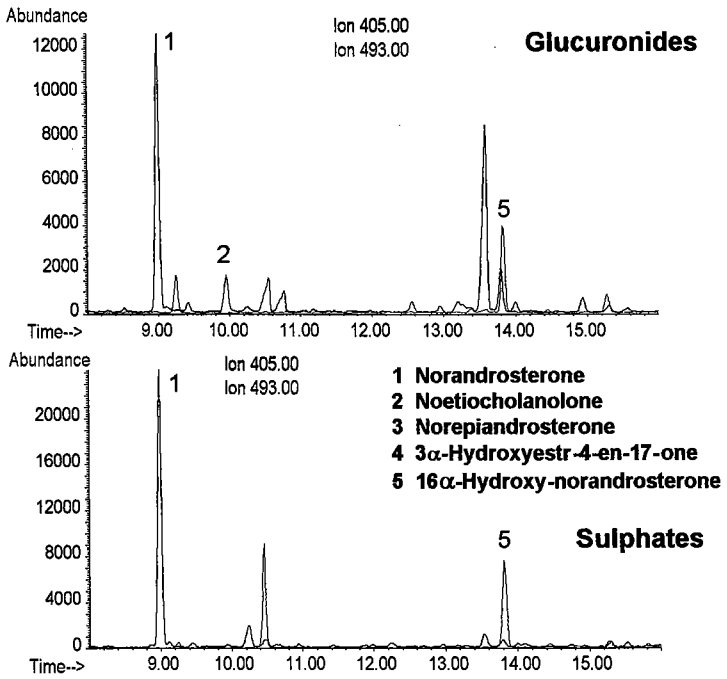


Fig.14B Profile of urinary excreted 19-normetabolites 64.5-70.5 h after oral administration of 100 mg of 4-norandrostenedione (glucuronide and sulphate fraction)

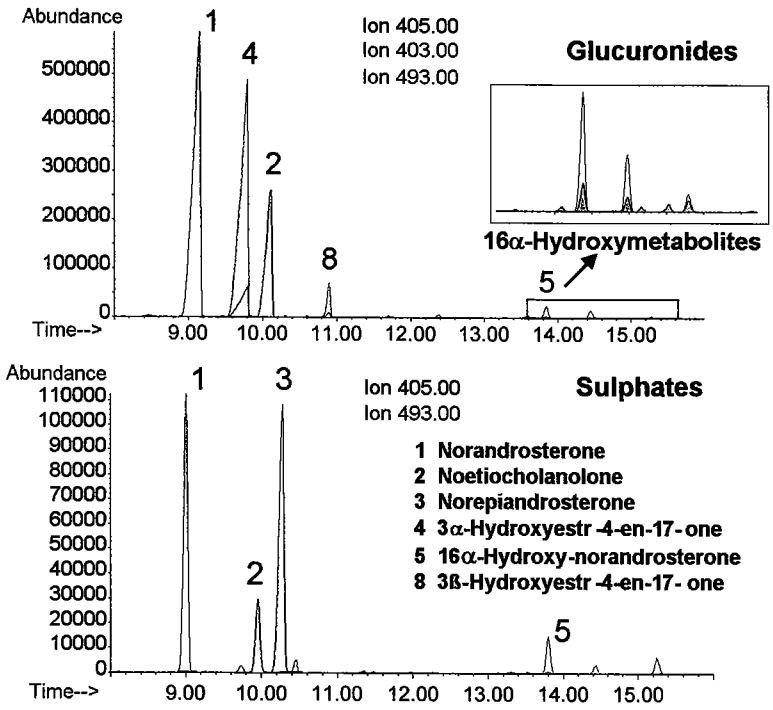


Fig.15A Profile of urinary excreted 19-normetabolites 2-4,5h after oral administration of 100 mg of 4-norandrostenediol (glucuronide and sulphate fraction)

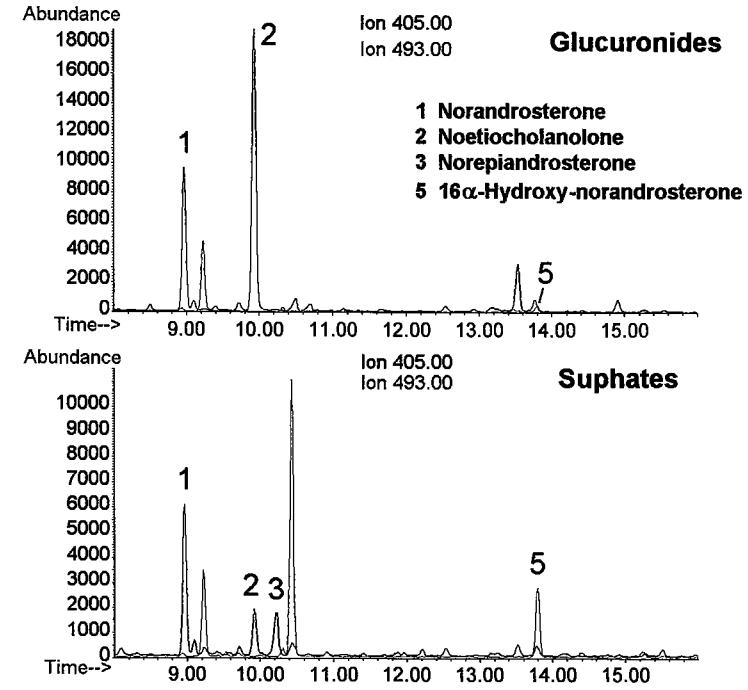


Fig.15B Profile of urinary excreted 19-normetabolites 66-72 h after oral administration of 100 mg of 4-norandrostenediole (glucuronide and sulphate fraction)

Nortestosterone (17ß-hydroxyestr-4-en-3-one)

Following oral nortestosterone application norandrosterone was the main metabolite excreted in the glucuronide and sulphate fraction over the complete excretion period. In the first hours after application besides the 3α-hydroxyestr-4-en-17-one metabolite also diol metabolites were identified as 5α-estrane-3α,17ß-diol and 5ß-estrane-3α,17ß-diol (Fig.16A). The metabolites were only detected after nortestosterone administration. The excretion of these metabolites is reasonable when compared to testosterone metabolism, where also the corresponding diols are formed and excreted. A possible explanation is that nortestosterone is metabolised during the first pass effect in the liver to a 17ß-glucuronide which possibly is further reduced to the corresponding 5α-estran-3α-ol and 5ß-estrane-3α-ol A-ring structure.

6ß-Hydroxy-4-norandrostenedione

6ß-Hydoxy-metabolites of anabolic androgenic steroids have been published for 3-keto-1,4-diene steroids such as metandienone, boldenone and 4-chloro-1,2-dehydromethyltestosterone [9]. 6ß-Hydroxylation also occurs in the metabolism of fluoxymesterone, a 3-keto-4-ene steroid [10]. Recently Ayotte et al. [11] published the identification of 6α-hydroxy-4-androstenedione as a metabolite of androstenedione allowing to determine the use of androstenedione or androstenediol. We therefore investigated the possible excretion of a 6-hydroxy metabolite after oral application of 4-norandrostenedione. Synthesis of 6ß-hydroxy-4-norandrostenedione was achieved as presented in Fig.12. As demonstrated in Fig.17 a possible 6-hydroxy-metabolite of 4-norandrosternedione could be detected in the glucuronide fraction only in the first hours after administration. As the metabolite was analysed as enol-TMS derivative no differentiation between 6ß-hydroxy and 6α-hydroxy structure was possible. To obtain data concerning the exact structure of the metabolite further research is necessary. Based on the small amount of excreted metabolite compared to all other metabolites the 6-hydroxy metabolite was of less interst.

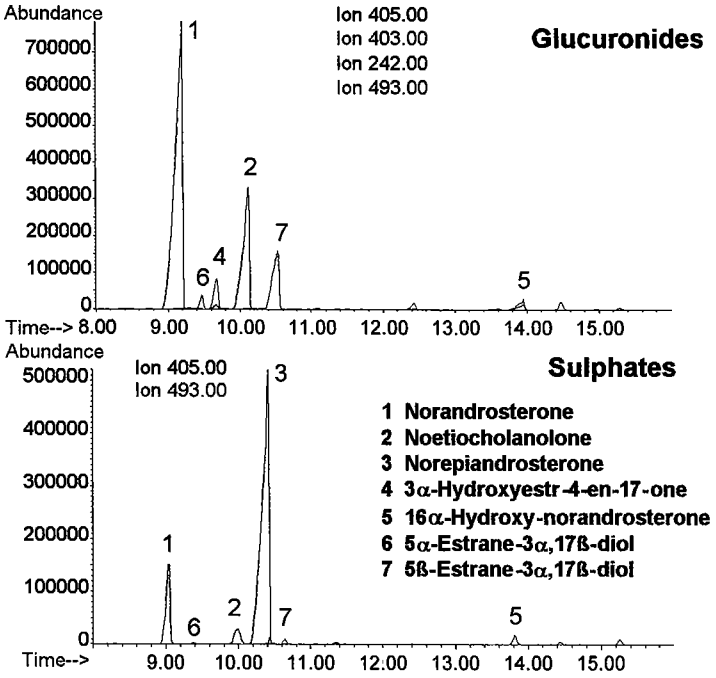


Fig.16A Profile of urinary excreted 19-normetabolites 1.5-3.5h after oral administration of 50 mg of nortestosterone (glucuronide and sulphate fraction)

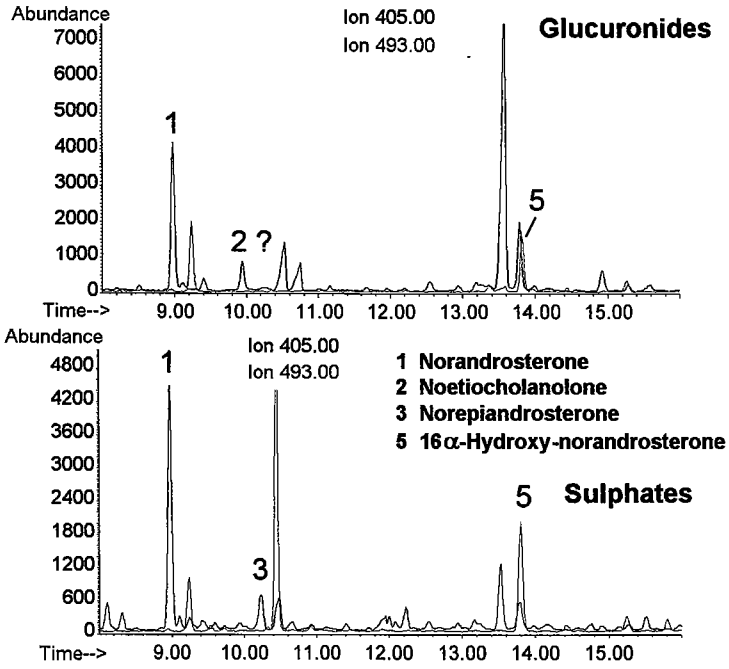


Fig.16B Profile of urinary excreted 19-normetabolites 66-72 h after oral administration of 50 mg of 4-nortestosterone (glucuronide and sulphate fraction)

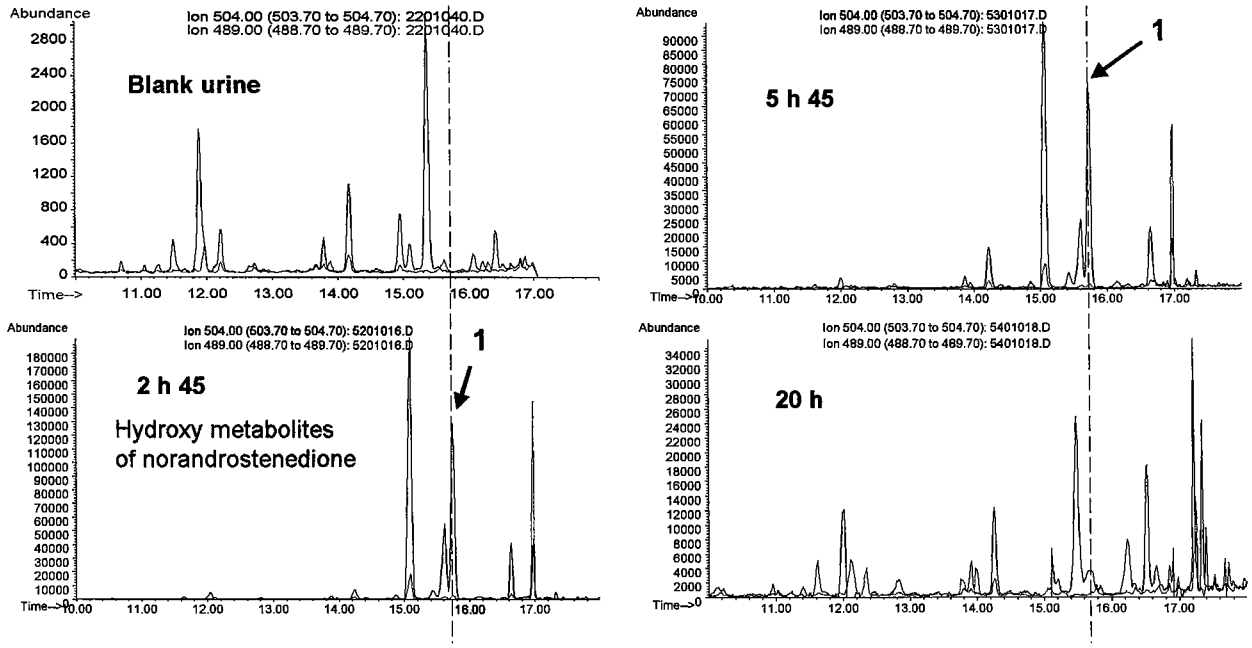


Fig.17 6ß-hydroxy-4-norandrostenedione (**1**) detection after oral administration of 100 mg of 4-norandrostenedione

Pharmacokinitic of 19-norsteroids

Following the pharmacokinetic of norandrosterone in the glucuronide and sulphate fractions after oral application of 4-norandrostenedione (100 mg, each to two male volunteers), 4-norandrostenediol (100mg) and nortestosterone (50mg) it was possible to detect norandrosterone higher than 20 ng/ml and 2 ng/ml urine (Table 2). The excretion profile for both metabolites are displayed in Fig.19-22 using a logarithmic scaling for the norandrosterone concentration.

Table 2 Detection time for norandrosterone (NA) in urine (glucuronide fraction; NA concentration > than 20 ng/ml und > 2 ng/ml following oral application of different 19-norsteroids

Steroid	Applied amount	Detection time in urine (NA > 2 ng/ml)	Detection time in urine (NA > 20 ng/ml)
4-Norandrostenedioe (A)	100 mg	111 h	89,5 h
4-Norandrostenedioe (B)	100 mg	210 h	80 h
4-Norandrostenediol (A)	100 mg	115* h	96 h
Nortestosterone (A)	50 mg	127 h	71,5 h

A) male volunteer 49, years; B) male volunteer, 44 years

* Urine samples only collected for 115h

The elimination kinetics of norandrosterone glucuronide and norandrosterone sulphate show obviously a similar elimination kinetic. Further research should be focused on the sulphated metabolites to obtain more data concerning the generation and elimination of the sulphated metabolites.

For norandrosterone glucuronide the following elimination half times were calculated: For 4-norandrostenedione in two elimination studies with oral application of 100 mg of substance a biphasic elimination was measured with a first elimination half life of 3.6 to 4.3 h and a second elimination half life of 13.6 to 20.7 h. On the contrary in case of 100mg of 4-norandrostenediol application a monophasic elimination with an elimination half life of 10.5 h was estimated. Similar to 4-norandrostenedione elimination the excretion study with 50 mg of orally applied nortestosterone allows to determine a biphasic elimination with a first erlimination halflife of 1.5 h and a second with 15.5 h. These data should be supported by further studies with 19-norsteroids including a higher number of volunteers.

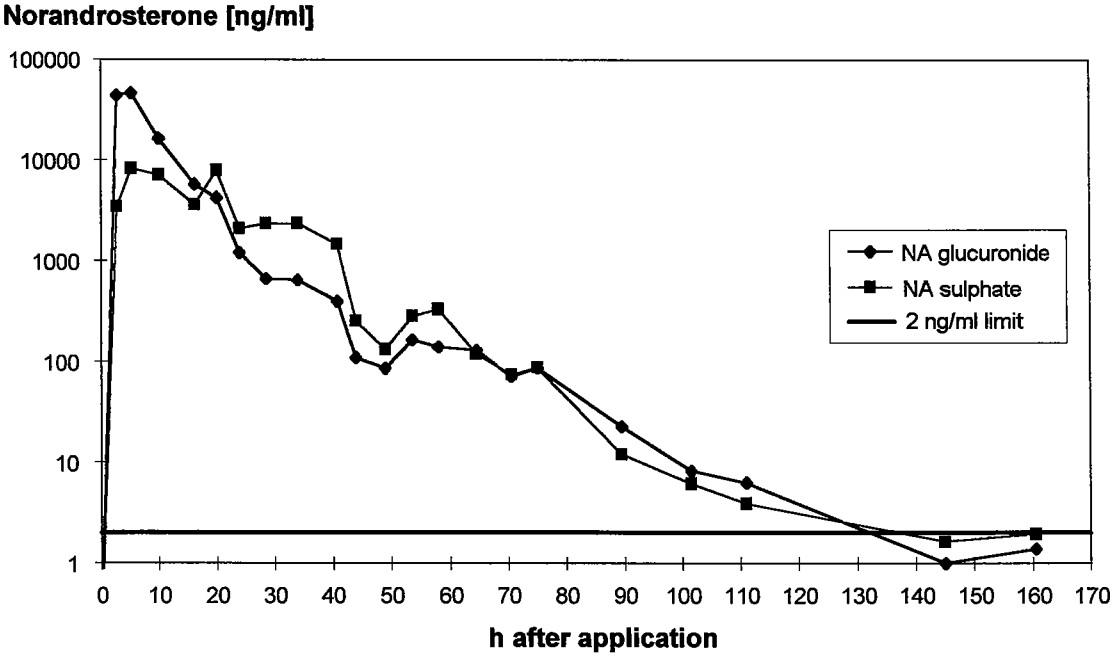


Fig.18 Norandrosterone glucuronide and sulphate excretion in urine following oral administrration of 100 mg of 4-norandrostenedione (volunteer A)

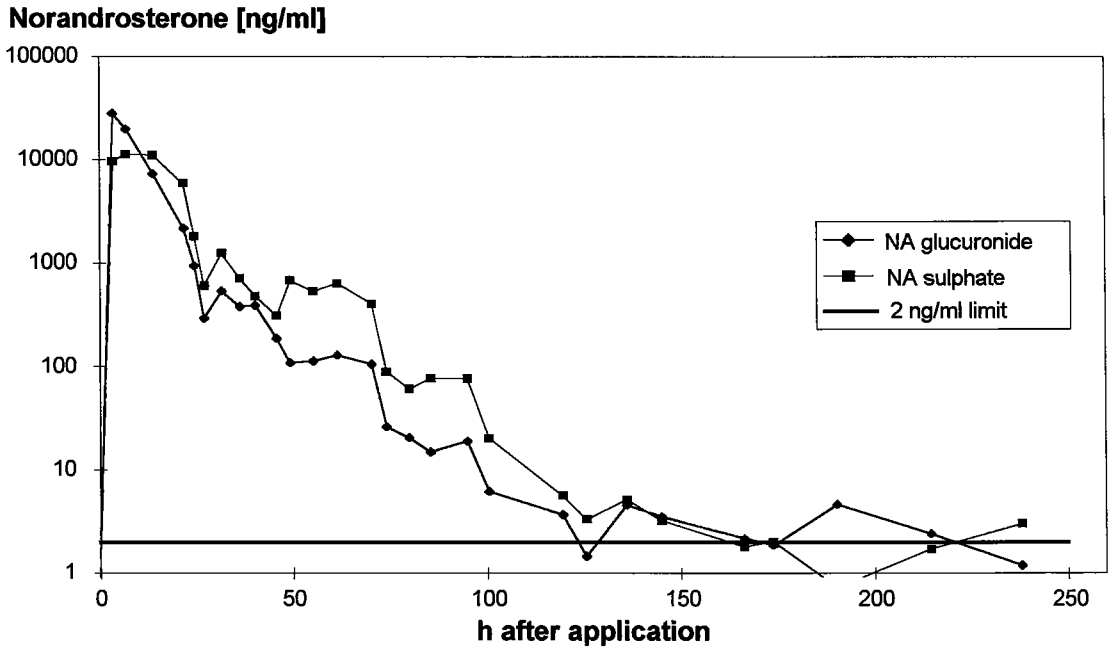


Fig.19 Norandrosterone glucuronide and sulphate excretion in urine following oral administrration of 100 mg of 4-norandrostenedione (volunteer B)

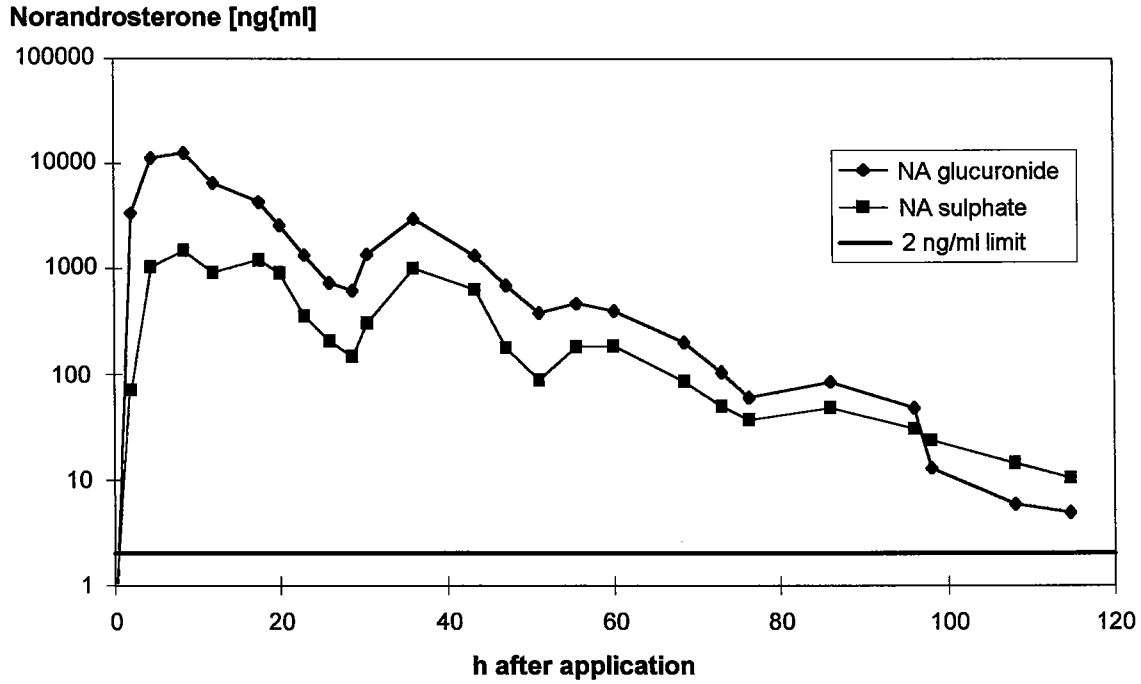


Fig.20 Norandrosterone glucuronide and sulphate excretion in urine following oral administrration of 100 mg of 4-norandrostenediol (volunteer A)

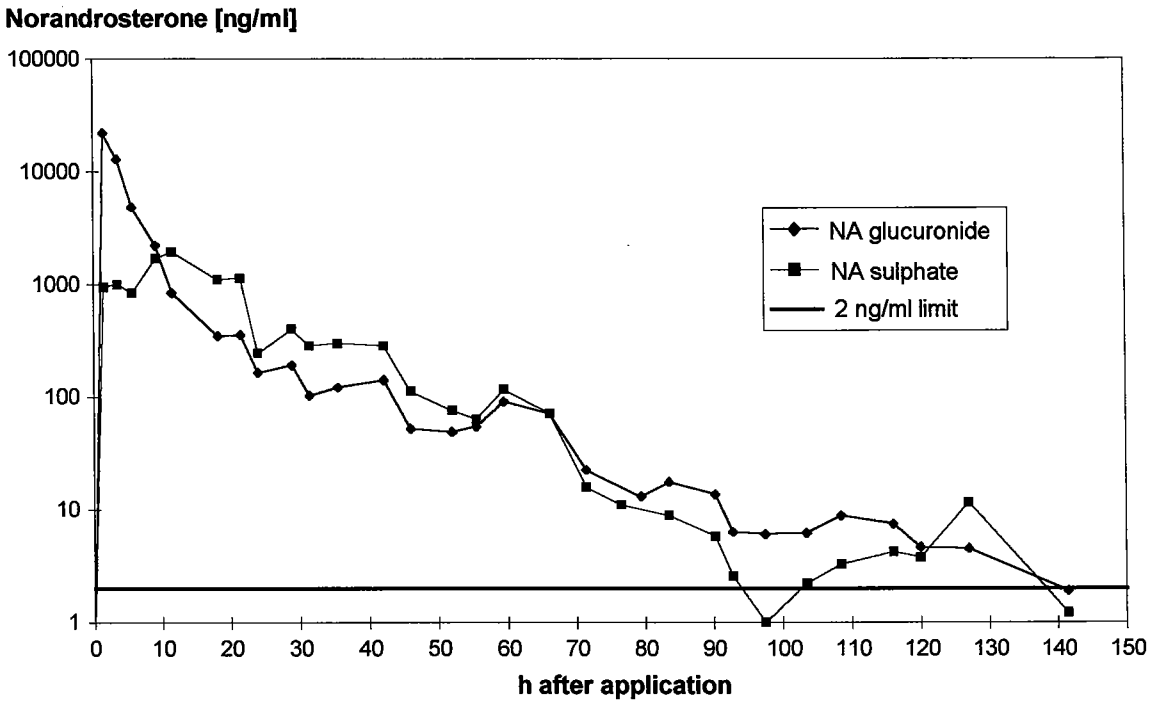


Fig.21 Norandrosterone glucuronide and sulphate excretion in urine following oral administrration of 50 mg of nortestosterone (volunteer A)

References

1 Birch JA. Hydroaromatic steroid hormones. I. 10-Nortestosterone. J Chem Soc, (1950) 367-8.

2 Wilds AL, Nelson NA. The facile synthesis of 19-nortestosterone and 19-norandrostendione from estrone. J Am Chem Soc, 75 (1953) 5366-9.

3 Engel LL, Alexander J, Wheeler M. Urinary metabolites of administered 19-nortestosterone. J Biol Chem, 231 (1958) 159-65.

4 Kupfer D, Forchielli E, Dorfmann RI. 3α-Hydroxy-19-nor-5α-androstan-17-on and 19-nor-5α-androstane 3α,17ß-diol. J Org Chem, 25 (1960) 1674-5.

5 Schänzer W, Donike M. unpublished data, (1988-93)

6 Uralets VP, Gilette PA, Latven RK: Over the counter anabolic steroids 4-androsten-3,17-dione, 4-androsten-3ß,17ß-diol, and 19-nor-4-androsten-3,17-dione; Excretion studies in man. In: Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U(eds.) Recent advances in doping analysis (6). Sport und Buch Strauß, Köln , (1999) 147-70.

7 Schänzer W, Donike M: Metabolism of anabolic steroids in man: Synthesis and use of reference substances for identification of anabolic steroid metabolites. Anal. Chim. Acta, 275 (1993) 23-48.

8 Schänzer W, Opfermann G, Donike M: Metabolism of stanozolol: Identification and synthesis of urinary metabolites. J. Steroid Biochem., 36 (1990) 153-174.

9 Schänzer W, Horning S, Donike M: Metabolism of anabolic steroids in humans: synthesis of 6ß-hydroxy metabolites of 4-chloro-1,2-dehydro-17α-methyltestosterone, fluoxymesterone and metandienone. Steroids, 60 (1995) 353-366.

10 Schänzer, W.: Metabolism of anabolic androgenic steroids. Review. Clinical Chemistry, 42 (1996) 1001-1020

11 Lèvesque JF, Ayotte Ch: Criteria for the detection of androstenedione oral administration. In: Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U(eds.) Recent advances in doping analysis (7). Sport und Buch Strauß, Köln , (1999) 169-179.