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Prescription Drugs and New Anabolic Steroids in Nutritional Supplements

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Introduction

During the last years anabolic androgenic steroids, mainly prohormones of testosterone and nandrolone, were found in nutritional supplements [1-4]. It was also shown that the labelling of prohormone supplements does not reflect their actual content. Many prohormone products contain concentrations as well as prohormones different from those declared on the labels [5-9]. Additionally supplements with dubious background contained synthetic anabolic steroids. Recently the occurrence of Metandienone in high amounts in different supplements of one supplier was reported [10-12]. Based on this knowledge other supplements from that distributor were analysed for their steroid content.

Experimental

Chemicals

Androst-4-en-17β–ol-3-one (Testosterone), Androst-4-en-17α-ol-3-one (Epitestosterone), 5α-Androstan-17β-ol-3-one (5α-Dihydrotestosterone), 5β-Androstan-17β-ol-3-one (5β-Dihydrotestosterone), 5α-Androstane-3α,17β-diol, 5α-Androstane-3β,17β-diol and 5β-Androstane-3α,17β-diol were purchased from Sigma (Steinheim, Germany). 5α-Androstane-3α,17α-diol, 5α-Androstane-3β-17α-diol, 5β-Androstane-3α,17α-diol, 5β-Androstane-3β,17β-diol, 5α-Androst-1-ene-3,17-dione (1-Androstenedione), 5α-Androst-1-en-17β-ol-3-one (1-Testosterone) and Androst-4-ene-3b,17b-diol were obtained from Steraloids (Wilton, USA). Androst-4-ene-3α,17β-diol, 5β-Androst-1-ene-3,17-dione and 5β-Androst-1-en-17β-ol-3-one were synthesised in our laboratory [13,14]. N-Methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) was purchased from Chem. Fabrik Karl Bucher (Waldstetten, Germany). Other reagents and solvents were obtained from Merck (Darmstadt, Germany).

Synthesis of reference material

Hydrogenation of Epitestosterone

Epitestosterone (Androst-4-en-17α-ol-3-one, 7 mg) was dissolved in 4 ml of methanol and reduction of the 4,5-double bond was performed with hydrogen and palladium on charcoal (10 %) as catalyst.

Reduction of 3- and/or 17-keto groups

5β-Androst-1-ene-3,17-dione, 5β-Androst-1-en-17β-ol-3-one, 5α-Androst-1-ene-3,17-dione (1-Androstenedione), 5α-Androst-1-en-17β-ol-3-one (1-Testosterone), Androst-4-en-17α-ol-3-one (Epitestosterone), Androst-4-en-17β–ol-3-one (Testosterone), 1 mg (~3 µmol), were dissolved in 1 ml of methanol each. After addition of 200 µl of H_2O and 1 mg of sodium borohydride (26 µmol) the mixture was kept at room temperature for one hour. The reduction was stopped by adding hydrochloric acid (~200 µl, 1 M). After neutralisation the mixture was evaporated, the residue re-dissolved in KOH (0.1 M in H_2O) and extracted with n-pentane. The n-pentane layer was evaporated to dryness.

Derivatisation

The reference compounds were derivatised with 100 µl of TMIS reagent (MSTFA/ ammonium iodide/ ethanethiol, 1000:2:3, v:w:v) within 20 min at 60°C and analysed by means of gas chromatography / mass spectrometry (GC-MS).

Supplements

Three supplements were ordered by telephone from a company called Sledgehammer and were sent by regular mail from a German address.

All these products seem to be prohormone supplements. The declared ingredients were

Parabolon - S:	17 Hydroxy-17-beta-1,4-dien-3-on Matrix, Nor19dion, 4-Adiol
Stanozolon - S:	4-Androstenediol, 1-A-diol, 19-Nor-4-a-dion, 5-alpha-androsteno-(3,2-c)-pyrazol-17-beta Matrix
1-Adiol:	Androst-1-ene-3β,17β-diol.

Sample preparation

The supplements were prepared according to the screening procedure for anabolic steroids in nutritional supplements [15] including methanolic extraction from the supplement matrix. The dried methanolic extract was re-dissolved in KOH (0.1 M) and extracted with n-pentane. After re-extraction of the n-pentane layer with MeOH/H_2O (95:5) the methanolic layer was evaporated to dryness and the steroids were analysed as per-TMS-derivatives with GC-MS in SCAN mode. Additionally dried methanolic extracts of the supplements were derivatised and injected into GC-MS.

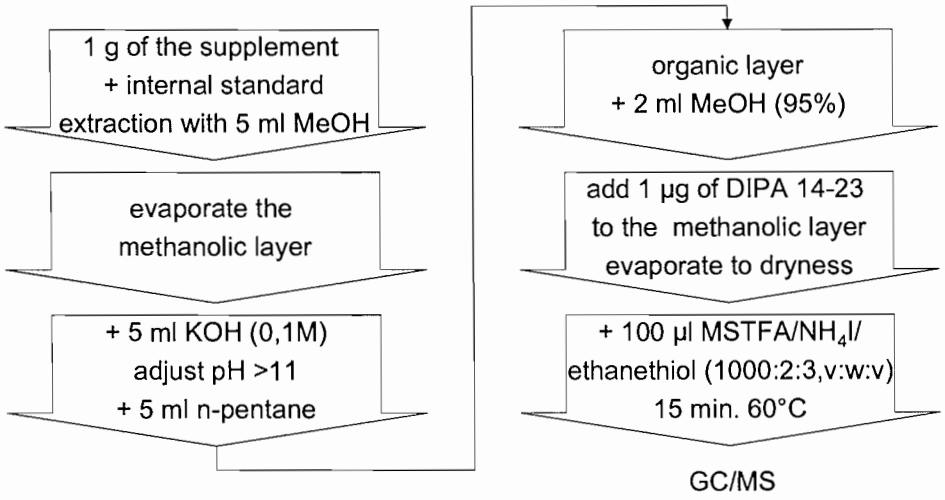

Fig. 1: Scheme of the sample preparation for supplements

Instrumentation

For the analyses the GC-MS was operated with the following parameters:

GC-MS:	GC: Hewlett Packard (HP) 6890, MSD: HP 5973
Injection param.:	Volume: 2 µl, Temp.: 300°C
Column:	HP 5 MS; 16.5 m; 0.25 mm i.d.; 0.25 µm film thickness
Carrier gas:	Helium, splitless, 1.5 ml/min, const. flow
Oven temp.:	100°C with 40°C/min to 190°C, with 5°C/min to 240°C, with 40°C/min to 320°C, 3 min hold
Ionisation:	70 eV, electron impact (EI)
Data aqu.:	SIM/SCAN

Results and Discussion

Reference standards

Isomers of Dihydrotestosterone (DHT)

The reduction of epitestosterone with hydrogen (Pd/C as catalyst, Fig. 2) yields the two isomers of dihydroepitestosterone (DHEpiT): 5α-Androstan-17α-ol-3-one and 5β-Androstan-17α-ol-3-one (ratio ~ 1:2).

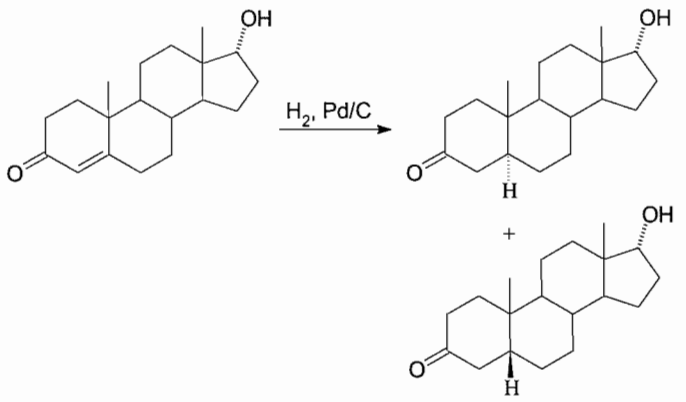

Fig. 2: Reaction schema of the hydrogenation of Epitestosterone

After derivatisation two enol-TMS ethers are obtained from each of the isomers. Both derivatives show almost the same mass spectrum but have different retention times (Tab. 1).

As for the commercially available 17β-isomers, the 2-enol-TMS derivative is the main product for 5α– (~94%) and the 3-enol-TMS for 5β– (75 %). For the 5α-Androstan-17ξ-ol-3-ones the 2-enol-TMS derivatives almost coelute with the 3-enol derivative which shows a slightly shorter retention time. However, the derivatives of the 5β-Androstan-17ξ-ol-3-ones are clearly separated from the 3-enol-TMS derivatives which are eluting first. The chromatogram of derivatised 5β-Androstan-17β-ol-3-one (5β-Dihydrotestosterone) is shown in Fig. 3.

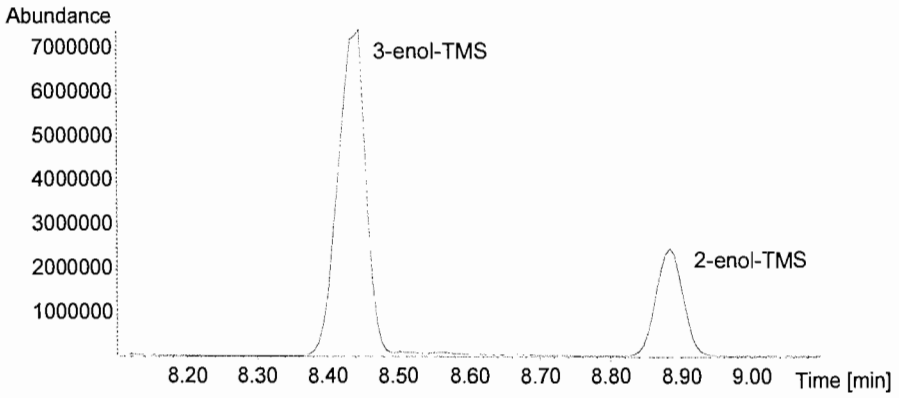

Fig. 3: Chromatogram of 5β-Dihydrotestosterone, enol-bis-TMS

Reduction of 3- and/or 17-keto groups

Another group of isomers yielding analogue mass spectra as per-TMS derivatives are the Androst-4-ene-diols.

The 4 possible isomers were synthesised by reducing the 3-keto-group of Testosterone and Epitestosterone with sodium borohydride. Both reductions yielded the 3α– and 3β-hydroxy isomers at a ratio of ~1:6. The reaction schema for Epitestosterone is shown in Fig. 4. The mass spectrometric data and the retention times of all isomeric Androst-4-ene-diols are displayed in Tab. 1.

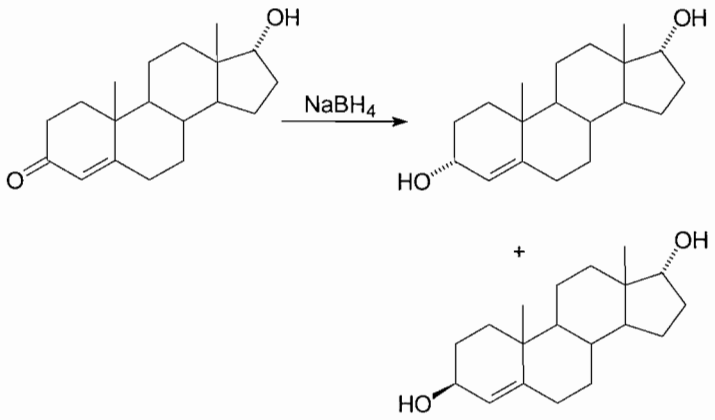

Fig. 4: Reaction schema of the reduction of Epitestosterone with $NaBH_4$

Also the isomers of Androst-1-ene-diol show analogue mass spectra. They are synthesised by reduction of 5α-Androst-1-en-17β-ol-3-one (1-Testosterone), 5α-Androst-1-ene-3,17-dione (1-Androstenedione), and 5β-Androst-1-en-17β-ol-3-one (metabolite of Boldenone) with sodium borohydride. As an example the reaction scheme for 5α-Androst-1-en-17β-ol-3-one is

shown in Fig. 5. Up to now the 17α-isomers could not be synthesised. For the other Androst-1-ene-diols the mass spectrometric data and the retention times are also included in Tab. 1.

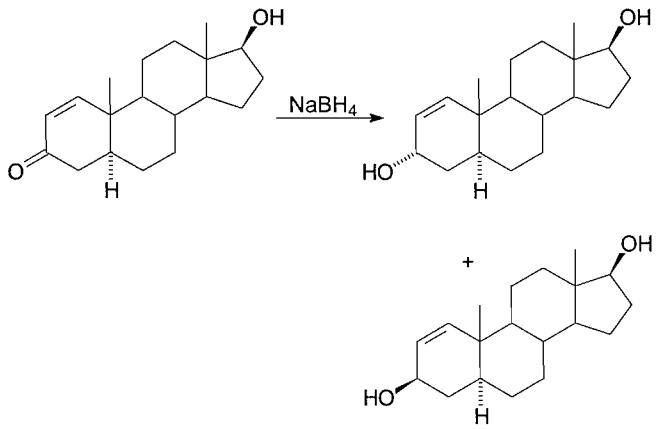

Fig. 5: Reaction schema of the reduction of 5α-Androst-1-ene-17β-ol-3-one with $NaBH_4$

Tab. 1: Retention times and relative abundances of characteristic fragment ions of the isomers of dihydrotestosterone and androstenediol

	RT [min]	m/z 143	m/z 434	m/z 142	m/z 405	m/z 202
5α-DHT, 3enol TMS	10,41	100,0%	71,0%	70,6%	25,5%	22,5%
5α-DHT, 2enol TMS	10,49	100,0%	65,2%	68,4%	21,1%	26,7%
5β-DHT, 3enol TMS	8,42	100,0%	56,6%	58,1%	10,0%	16,1%
5β-DHT, 2enol TMS	8,83	100,0%	54,8%	59,4%	9,2%	18,4%
5α-DHEpiT, 2enol TMS	9,89	100,0%	63,5%	56,7%	23,3%	23,1%
5α-DHEpiT, 3enol TMS	9,81	100,0%	49,6%	33,2%	11,8%	14,5%
5β-DHEpiT, 2enol TMS	7,56	100,0%	47,7%	42,4%	7,4%	19,1%
5β-DHEpiT, 3enol TMS	7,95	100,0%	42,8%	48,7%	0,0%	18,1%
5α-Androst-1-ene-3β,17β-diol	10,23	100,0%	51,2%	63,6%	23,6%	24,0%
5α-Androst-1-ene-3α,17β-diol	9,53	100,0%	76,4%	60,1%	27,1%	20,9%
5β-Androst-1-ene-3β,17β-diol	9,44	100,0%	42,2%	58,0%		15,7%
5β-Androst-1-ene-3α,17β-diol	9,24	100,0%	42,5%	67,9%		0,0%
Androst-4-ene-3α,17β-diol	8,40	100,0%	53,7%	51,0%	8,9%	16,2%
Androst-4-ene-3β,17β-diol	10,12	100,0%	65,1%	59,5%	16,2%	19,1%
Androst-4-ene-3α,17α-diol	8,10	100,0%	45,6%	41,7%	9,3%	17,0%
Androst-4-ene-3β,17α-diol	9,40	100,0%	40,1%	44,3%	9,0%	20,2%

Supplement contents

Prohormones

When applying the routine screening for prohormones in nutritional supplements [15] Androst-4-ene-3β,17β-diol, 1-Testosterone and Norandrostenedione were detected in Stanozolon-S. None of the steroids screened for was found in the other two supplements.

Prescription drugs

When operating the GC/MS in scan mode several prescriptive anabolic steroids were identified in those supplemets, namely:

in Parabolon – S: Metandienone

in Stanozolon – S: Testosterone, 5α-Dihydrotestosterone, Boldenone, Stanozolol

in 1-Adiol: 5α-Dihydrotestosterone

All these steroids are classified as Schedule III controlled substances in USA, all explicitly listed in Section 801 (41) A of the Controlled Substances Act [16].

Additionally Estrone was detected in the dried methanolic extract of Stanozolon – S after derivatisation. The mass spectrum is shown in Fig. 6.

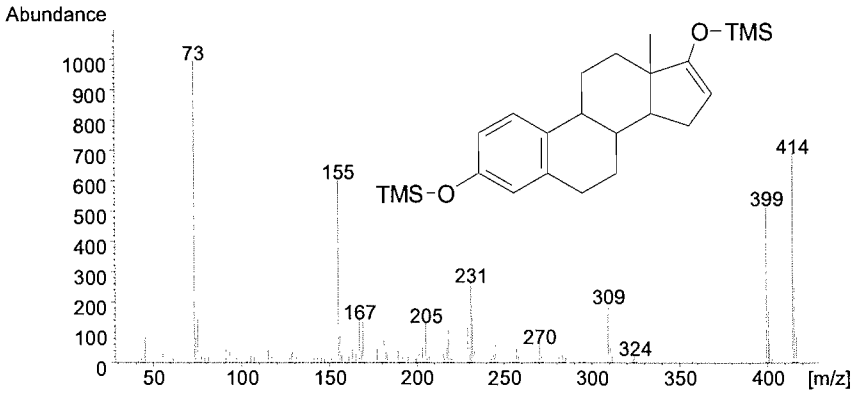

Fig. 6: Mass spectrum (EI) of Estrone, bis-TMS

Isomeric Androstanediols

Two isomeric androstanediols were detected in 1-Adiol. Both show very similar mass spectra (Fig. 7). They could be identified as the 5α-3β,17β– and the 5α-3β,17α–isomer. The GC/MS data of the bis-TMS derivatives of seven of possible isomers (5α/5β,3α/3β, 17α/17β) are listed in Tab. 2 (until now the 5β,3β,17α-isomer was not characterised).

Also for the isomers of androstanediol mass spectra with same charateristic fragment ions are obtained. The abundances of the ions vary within the isomers.

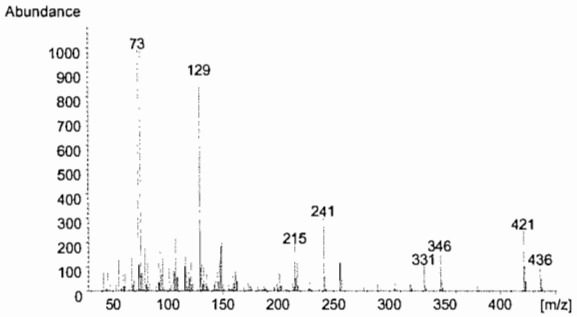

Fig. 7: Mass spectrum (EI) of 5α-Androstane-3β,17α-diol, RT 9.70 min from 1-Adiol

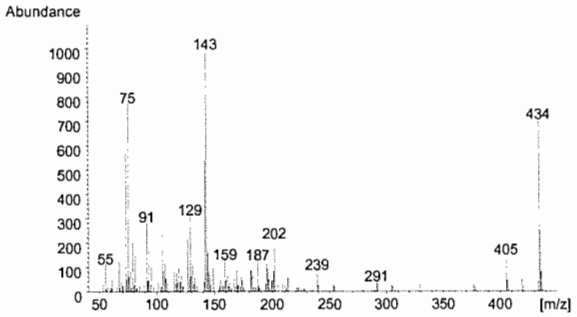

Fig. 8: Mass spectrum (EI) of two more steroids detected: same mass spectrum, different retention times (9.24 min and 9.44 min)

Tab. 2: Retention times and relative abundances of characteristic fragment ions of isomeric Androstanediols

	5β,3β,17β	5α,3α,17β	5β,3α,17β	5α,3β,17β	5β,3α,17α	5α,3α,17α	5α,3β,17α
RT [min]	9.11	9.25	9.33	10.27	7.94	8.17	9.7
m/z 436	6%	26%	4%	42%	6%	7%	36%
m/z 421	19%	21%	8%	94%	2%	9%	83%
m/z 346	46%	26%	23%	54%	32%	61%	48%
m/z 256	100%	54%	100%	39%	100%	100%	45%
m/z 241	60%	100%	64%	100%	91%	63%	100%
m/z 215	36%	57%	42%	47%	59%	54%	64%

Other steroid contents

In addition to Androst-4-ene-3β,17β-diol (RT 10.24 min), which is routinely screened for, Stanozolon - S contained two more steroids showing the same mass spectrum (Fig. 8)., but different retention times (9.24 min and 9.44 min). By comparison with the references synthesised they could be identified as 5β-Androst-1-ene-3β,17β-diol and 5β-Androst-1-ene-3α,17β-diol.

Summary

Schedule III controlled steroids occur in prohormone preparations, obviously as intentional admixtures. In the supplements analysed during this investigation Testosterone, Boldenone, 5α-Dihydrotestosterone, Stanozolol and Metandienone are detected.
For the first time Estrone is identified in a supplement.
Isomers detected for the first time on the supplement market were 5α-Androstane-3β,17α-diol, Androst-4-ene-3β,17α-diol, 5β-Androst-1-ene-3β,17β-diol and 5β-Androst-1-ene-3α,17β-diol.
For the identification isomers of Androst-4-ene-3,17-diol, Androst-1-ene-3,17-diol and Dihydrotestosterone are synthesised. They are found to have very similar mass spectra (M+=434, intense fragments at m/z 143 (B+) and 142). Their retention times and mass spectrometric data are presented.

Condensed supplement contents
The following steroids were identified in the supplements:

1-Adiol:
no 1-Androstene-3β,17β-diol
but 5α-Dihydrotestosterone, 5α-Androstane-3β,17β-diol, 5α-Androstane-3β,17α-diol

Parabolon – S:
no prohormones
but Metandienone

Stanozolon – S:
declared: 4-Androstenediol, 1-A-diol, 19-Nor-4-a-dion, 5-alpha-androsteno-(3,2-c)pyrazol-17-beta Matrix
found: Norandrost-4-ene-3,17-dione, Androst-4-ene-3β,17β-diol, Testosterone, Boldenone, 5α-Dihydrotestosterone, Stanozolol, 5β-Androst-1-ene-3β,17β-diol, 5β-Androst-1-ene-3α,17β-diol, 1-Testosterone, Estrone

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