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Recent steroid findings in “Designer Supplements”

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Abstract

Several supplements have been purchased from two different internet-based suppliers and were analysed for their steroid content. Besides the findings of DHEA (and DHEA esters) products were found to contain 6-bromoandrostenedione, androstanoisoxazoles, and estra-4,9-diene-3,17-dione.

Additionally Δ 6-methyltestosterone was detected in the product “Jungle Warfare” from ALR Industries, androst-4-ene-3,11,17-trione in the product “11Oxo” from Ergopharm, and finally the product “Oxyguno” distributed by Spectra Force Research was found to contain 4-chloro-11-oxomethyltestosterone. These three compounds have been reported in the 1960s to show weak androgenic (about 1/2 of testosterone) and from weak to strong anabolic properties.

The urinary metabolism was studied following the administration of one capsule to one test person each.

The administration of Δ 6-methyltestosterone was detectable for more than one week by monitoring the metabolites 17 α -hydroxy-17 β -methylandrosta-4,6-dien-3-one, 17 α -methyl-5 β -androstane-3 α ,17 β -diol, and 17 β -methyl-5 β -androstane-3 α ,17 α -diol. Oral administration of androst-4-ene-3,11,17-trione resulted in several reduced metabolites with 11 β -hydroxyandrosterone as main product. Isotope ratio mass spectrometry confirmed the exogenous origin of 11 β -hydroxyandrosterone and 11 β -hydroxyetiocholanolone as well as their 11-oxo precursors for up to 24 hours.

4-Chloro-11 β -hydroxymethyltestosterone (4-chloro-11 β ,17 β -dihydroxy-17 α -methylandrost-4-en-3-one) was identified as main urinary metabolite following the ingestion of 4-chloro-11-oxomethyltestosterone.

Introduction

Since a few years more and more products appear on the market for dietary supplements containing steroids that had never been marketed as approved drugs, mostly without proper labelling of the contents [1-12]. Syntheses and few data on pharmacological effects are available dated back mainly to the 1950s or 1960s. Only little knowledge exists about effects and side effects of these steroids in humans. Also only little information is available on their metabolism. They are only produced for the “supplement market” and are advertised as anabolic steroids and/or aromatase inhibitors. The legal status of these supplements is not clear in several countries. Most likely they are designed and marketed to evade existing laws (legal and sports), by modifying the molecular structures to produce effects similar to controlled drugs.

According to the doping regulations of the World Anti-Doping Agency (WADA), anabolic androgenic steroids as well as aromatase inhibitors are prohibited for use in sports. Most of the steroids found in “designer supplements” are not explicitly mentioned in the list of prohibited substances, but are covered by the wording “and other substances with a similar chemical structure or similar biological effect(s)”.

To include the new substances into routine steroid screening the steroids available have to be identified and the urinary metabolism has to be investigated. For the accessibility of reference material possibilities for the synthesis of the parent compounds as well as their metabolites have been studied.

Materials and Methods

Supplements, chemicals and reagents

Reference material of 11 β -hydroxyandrostenedione (11 β -hydroxyandrost-4-ene-3,17-dione, 11 β -OHAdione), 11 β -hydroxytestosterone (11 β ,17 β -dihydroxyandrost-4-en-3-one, 11 β -OHT), 11-oxotestosterone (17 β -hydroxyandrost-4-ene-3,11-dione, 11-oxoT), 11 β -hydroxy-etiocholanolone (3 α ,11 β -dihydroxy-5 β -androstane-17-one, 11 β -OHEt), 11-oxoetiocholanolone (3 α -hydroxy-5 β -androstane-11,17-dione, 11-oxoEt), and 3 α ,17 β -dihydroxy-5 β -androstane-11-one (11-oxo $\beta\alpha\beta$) were obtained from Steraloids (Newport, USA).

Estra-4,9-diene-3,17-dione was obtained from Thinker Chemical (Hangzhou, China), 17 α -methyl-5 α -androstane-3 α ,17 β -diol (3 α ,5 α -THMT), 17 α -methyl-5 β -androstane-3 α ,17 β -diol (3 α ,5 β -THMT), 17 β -methyl-5 α -androstane-3 α ,17 α -diol (3 α ,5 α -EpiTHMT) and 17 β -methyl-

5 β -androstane-3 α ,17 α -diol (3 α ,5 β -EpiTHMT) were synthesised in our laboratory as described by Schänzer *et al.* [13,14].

17 β -Hydroxy-17 α -methylandrosterone (methyltestosterone), 11 α ,17 β -dihydroxy-17 α -methylandrosterone (11 α -hydroxymethyltestosterone), 3 β -hydroxyandrost-5-en-17-one (DHEA), androst-4-ene-3,11,17-trione, 11 β -hydroxyandrosterone (3 α ,11 β -dihydroxy-5 α -androst-17-one, 11 β -OHA), 11-oxoandrosterone (3 α -hydroxy-5 α -androst-11,17-dione, 11-oxoA), androsterone (3 α -hydroxy-5 α -androst-17-one, A), etiocholanolone (3 α -hydroxy-5 β -androst-17-one, Et), 5 α -androst-3 β -ol, tetrachloro-1,4-benzoquinone (Chloranil, TCQ), hydrogen peroxide solution (H₂O₂, 30% in H₂O), chromium(VI) oxide (CrO₃), manganese(IV) oxide (MnO₂), sodium borohydride (NaBH₄) and LS-Selectride (lithium tris-*i*-amylborohydride, 1M in tetrahydrofuran) were obtained from Sigma-Aldrich GmbH (Steinheim, Germany). β -Glucuronidase from *E.coli* was obtained from Roche Diagnostics (Mannheim, Germany), N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) from Chem. Fabrik Karl Bucher (Waldstetten, Germany). All other reagents and solvents were of analytical grade and obtained from Merck (Darmstadt, Germany).

The following products were purchased from web-based stores for sport supplements:

Product name	Company	Labelled steroid ingredients
Anavar	Hi-Tech Pharmaceutical	DHEA, DHEA-Esters
Mayhem	BCS Labs	17 α -Methyl-1, 4-Androstadiene-3, 17 Diol
Propadrol	EST Nutrition LLC	12-Ethyl-3-Methoxy-gona-dien 17 6-17dihydroxy-etiocholone-3-ol
Halo T-400	Anabolics Formulation	4-chloro-17 α -methyl-androst-1,4-diene-3-17 β -diol Estra-4, 9-diene-3, 17-dione
Trenadrol	Kilo Sports	17 β -methoxy-trienbolone
Super Tren-MG	Black China Labs	Estra-4, 9-diene-3, 17-dione
Regenesen	Neogenix	
Furaguno	Spectra Force Research	
Oxodrol	IDS	2 α ,3 α -epithio-17 α -methyletioallocholanol
11-Oxo	Ergopharm Performance Nutrition	11-OXO™(Adrenosterone)
Oxyguno	Spectra Force Research	4-Chloro-17 α -Methyl-Etioallochol-4-Ene-17 β -Ol-3, 11-Dione
Jungle Warfare	ALR	17 α -Methyl-5 α - Dehydro-Etiochol-4,6-Dien-3-One- 17-Ol

Supplement analysis

The homogenised content of one capsule was suspended in 5 mL of methanol. After shaking for 5 min and centrifugation for 5 min at 800 g, the methanolic layer was separated. Aliquots were analysed by GC-MS (underivatized and as TMS derivatives).

Synthesis of reference material

4-Chloro-17 β -hydroxy-17 α -methylandrosta-4-ene-3,11-dione (1)

11 α -Hydroxymethyltestosterone was epoxidised with H₂O₂/NaOH_{aq} followed by epoxide opening in aqueous hydrochloric acid. The resulting 4-chloro-11 α ,17 β -dihydroxy-17 α -methylandrosta-4-en-3-one was oxidised by cautious addition of a solution of CrO₃ in acetic acid. After addition of 6 ml of 7 N KOH the products were extracted three times with 5 ml of t-butyl methyl ether (TBME), each. Crystallisation from n-hexane/TBME (5:9, v:v) yielded 4-chloro-17 β -hydroxy-17 α -methylandrosta-4-ene-3,11-dione (**1**).

Reduction of 4-chloro-17 β -hydroxy-17 α -methylandrosta-4-ene-3,11-dione

The reduction of 4-chloro-17 β -hydroxy-17 α -methylandrosta-4-ene-3,11-dione (**1**) with LS-Selectride in THF (1.5 fold molar excess) yielded the two isomeric 4-chloro-3 ξ ,17 β -dihydroxy-17 α -methylandrosta-4-en-11-ones (**3a** and **3b**). Using an excess of NaBH₄ two isomeric 4-chloro-17 α -methylandrosta-4-en-3 ξ ,11 β -17 β -triols (**4**) were obtained. By oxidation of the 3-hydroxy group utilising MnO₂ 4-chloro-11 β ,17 β -dihydroxy-17 α -methylandrosta-4-en-3-one (**5**) was obtained.

Dehydrogenation of (epi-)methyltestosterone

Methyltestosterone or epimethyltestosterone were dehydrogenated with an excess of chloranil in refluxing t-butanol within 4 h following the principle described by Fiesers and Fieser (Fieser and Fieser, 1967). The products (17 β -hydroxy-17 α -methylandrosta-4,6-dien-3-one (**11**) and 17 α -hydroxy-17 β -methylandrosta-4,6-dien-3-one (**12**), respectively) were extracted with TBME from an aqueous KOH solution and the extract was washed with water. Following evaporation under reduced pressure crystallization from n-hexane/dichloromethane yielded crystals of the corresponding 6-ene-products.

Administration study

The following p.o. administration studies were performed in one healthy male volunteer, each: three capsules of 11-Oxo (urine collection 24 h pre- and 50 h post-administration), one capsule of Oxyguno (urine collection 27 h post-administration) and one capsule of Jungle Warfare (urine collection for 24 hours, afterwards morning urines for 11 days). Ethical approval was obtained from the Human Research Ethics Committee of the Ministry of Sports, Tourism and Youth Policy of the Russian Federation.

Sample pre-treatment

The samples were prepared according to the routine steroid screening procedure[15]. In brief, after addition of the internal standard methyltestosterone 2-5 mL of urine were incubated at pH 7 with β -glucuronidase from *E.coli* at 50°C for 1 h. The steroids were extracted with 5 mL of TBME at pH 9.6, the organic layer was evaporated to dryness. For GC-MS analyses the residues were derivatised with TMIS reagent (MSTFA/ NH₄I/ ethanethiol, 1000:2:3, v:w:v) by heating for 20 min at 60°C and injected into the GC-MS.

For IRMS analysis the urine samples of the 11-Oxo administration study were additionally prepared as described by Flenker *et al.* [16].

Instrumentation

GC-MS analyses

For the measurement of the TMS derivatives the analyses were carried out on an Agilent 6890 GC coupled to an Agilent 5973 mass selective detector (MSD) using the following parameters: injection volume: 3 μ L, split 1:16, injection temperature: 300°C, column: Agilent Ultra-1 column (17 m; 0.20 mm inner diameter (i.d.); 0.11 μ m film thickness), carrier gas: helium, head pressure 1 bar, oven temperature program: 0 min 183°C, +3°C/min, 0 min 232°C, +40°C/min, 2 min 310°C, ionisation: 70 eV, electron ionisation (EI), data acquisition: full scan mode, 40-800 Da.

For GC-MS/MS analysis a Trace GC Ultra (Thermo Fisher, Bremen, Germany) coupled to a TSQ quantum triple quadrupol mass spectrometer was utilized with the same parameters as described for GC-MS analysis of the TMS derivatives. Divergently to the above mentioned 2 μ l were injected.

Results and Discussion

Supplement analysis

The analysis of the products revealed that the products Anavar™, Mayhem™, Propadrol™, and Halo T-400™ only contained DHEA, even if labelled differently.

Comparison of the mass spectra with reference material and data available from the literature [8,12,17,18] identified estra-4,9-diene-3,17-dione in Trenadrol™ and Super Tren-MG™, 6-bromoandrost-4-ene-3,17-dione in Regenesen™, 2 α ,3 α -epithio-17 α -methyl-5 α -androstan-17 β -ol in Oxodrol, androst-4-ene-3,11,17-trione in 11-Oxo™ and 17 β -hydroxy-5 α -androstan-2-one in Furaguno™. These findings confirmed the structures that were deduced from the labelling.

The analysis of the TMS derivatized extract derived from Oxyguno revealed an ingredient with a previously unreported mass spectrum (Fig. 1). The two proposals for the structure are displayed in Fig. 2. 4-Chloro-11 β ,17 β -dihydroxy-17 α -methylandrosta-1,4-dien-3-one (11 β -hydroxy-DHCMT, **2**) was already reported to be misused in the former GDR under the name "Substanz XII".

However, the successful synthesis of (**1**) allowed the confirmation of the steroid as 4-chloro-17 β -hydroxy-17 α -methylandrosta-4-ene-3,11-dione (4-chloro-11-oxomethyltestosterone) by GC-MS comparison.

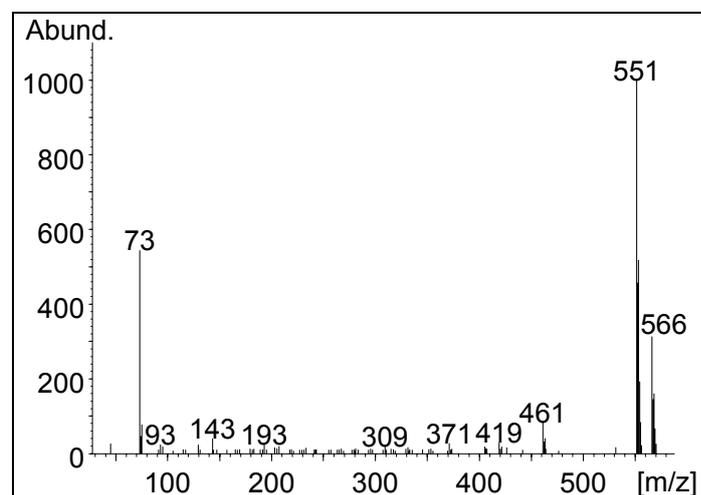


Fig. 1: Mass spectrum of the steroid ingredient present in Oxyguno™, identified as 4-chloro-17 β -hydroxy-17 α -methylandrosta-4-ene-3,11-dione (**1**), tris-TMS, $M^+ = 566$

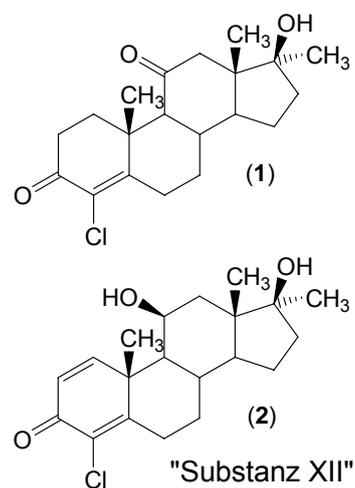


Fig. 2: Structure proposals for Oxyguno: (**1**): 4-chloro-17 β -hydroxy-17 α -methylandrosta-4-ene-3,11-dione, (**2**): 4-chloro-11 β ,17 β -dihydroxy-17 α -methylandrosta-1,4-dien-3-one

Following successful 6,7-dehydrogenation of 17 α -methyltestosterone the steroid present in the product Jungle Warfare™ was identified as 17 β -hydroxy-17 α -methylandrosta-4,6-diene-3-one (Δ 6-methyltestosterone) by GC-MS comparison of the bis-TMS derivatives (Fig. 3).

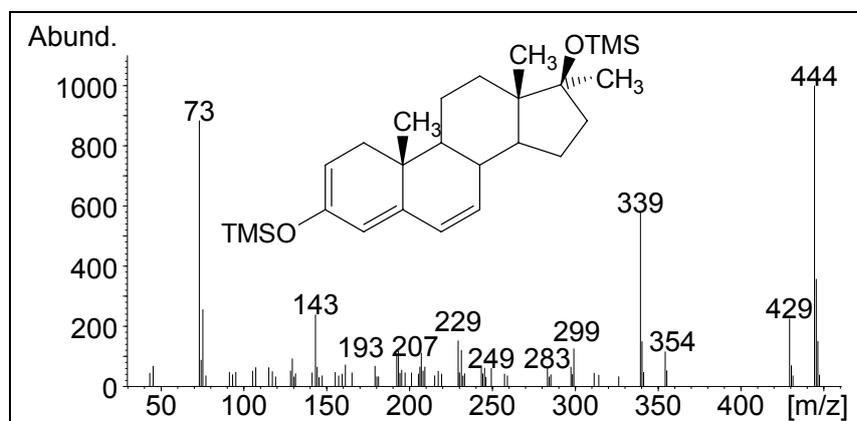


Fig. 3: Mass spectrum of 17 β -hydroxy-17 α -methylandrosta-4,6-diene-3-one, bis-TMS, $M^+ = 444$

Administration urines

Oxyguno

The administration of 4-chloro-11-oxo-17 α -methyltestosterone was detectable by its main urinary metabolite, that was identified as 4-chloro-11 β -hydroxy-17 α -methyltestosterone (**5**) by comparison with the synthesised references (mass spectrum in Fig. 4). Its elimination kinetics is displayed in Fig. 5.

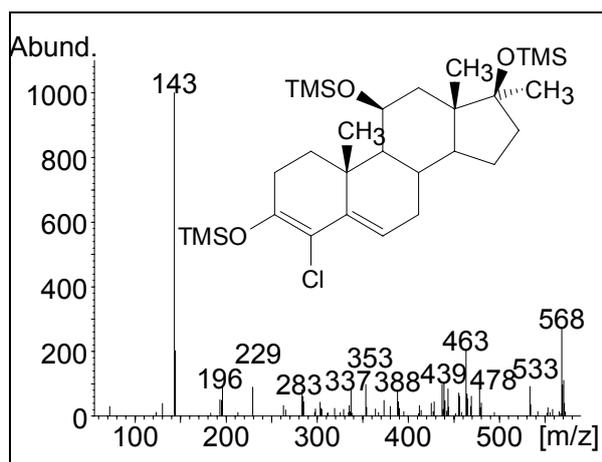


Fig. 4: Mass spectrum of 4-chloro-11 β -hydroxy-17 α -methyltestosterone (**5**), tris-TMS, $M^+ = 568$

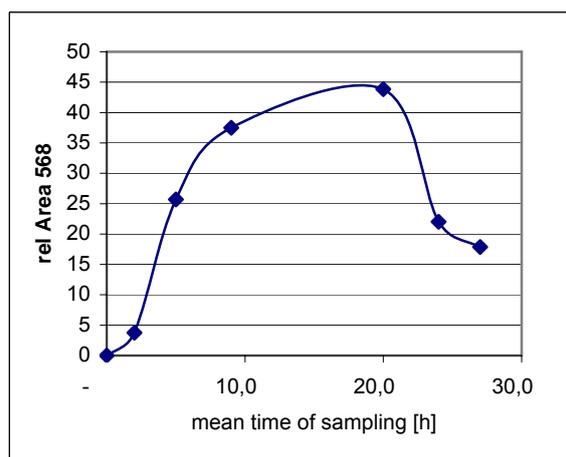


Fig. 5: Elimination kinetics of 4-chloro-11 β -hydroxy-17 α -methyltestosterone (**5**) after one capsule of Oxyguno, data shown using midpoints of collection periods

Jungle Warfare

Following the administration of Jungle Warfare the parent compound, 17 β -hydroxy-17 α -methylandrosta-4,6-diene-3-one (**11**), was detectable in all urine samples up to the 63-70 hours urine. The main urinary metabolites were identified as the 17-epimer Δ 6-epimethyltestosterone (**12**), 17 α -methyl-5 β -androstane-3 α ,17 β -diol (3 α ,5 β -THMT), and its epimer 17 β -methyl-5 β -androstane-3 α ,17 α -diol (3 α ,5 β -epiTHMT). Δ 6-Epimethyltestosterone (mass spectrum in Fig. 6) and 3 α ,5 β -THMT were still detectable in the 181-189 h urine. The time course of the elimination is displayed in Fig. 7.

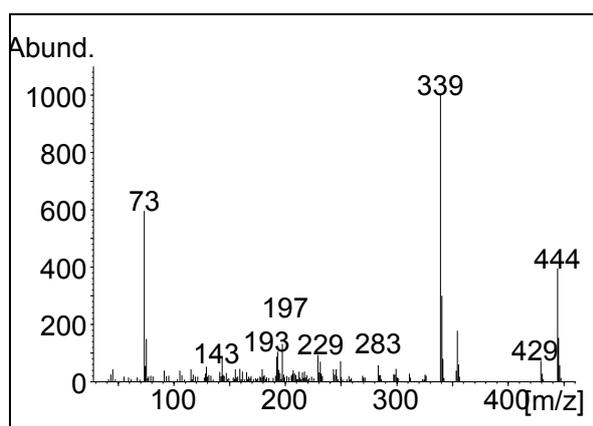


Fig. 6: Mass spectrum of Δ 6-epimethyltestosterone, bis-TMS, $M^+ = 444$

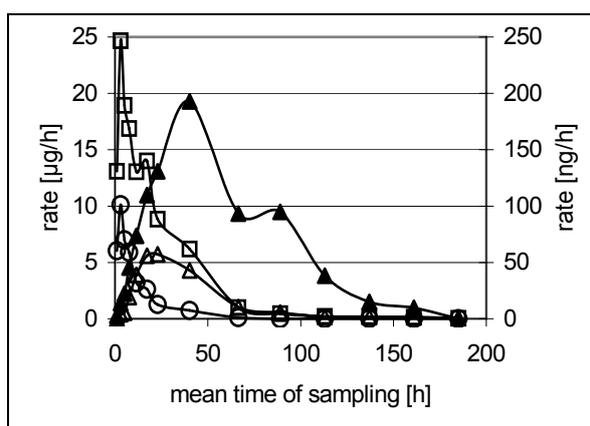


Fig. 7: Elimination kinetics of Δ 6-methyltestosterone (o), Δ 6-epimethyltestosterone (\square), 3 α ,5 β -THMT (Δ), and 3 α ,5 β -epiTHMT (\blacktriangle , right y-axis) after one capsule of Jungle Warfare

11-Oxo

The results of this investigation are published as:

Brooker L, Parr MK, Cawley A, Flenker U, Howe C, Kazlauskas R, Schänzer W, George A. Development of criteria for the detection of adrenosterone administration by gas chromatography-mass spectrometry and gas chromatography-combustion-isotope ratio mass spectrometry for doping control. Drug Testing and Analysis, in press

Properties of the steroids

The characterisation of the anabolic and androgenic properties of lots of steroids has been reviewed by JA Vida [19]. Recently, people refer to this book in chat forums as basis of information on “new” steroid ingredients. Rumours from Internet sources indicate connections to Chinese sources of raw material. The data of the steroids relevant to the reported investigation Confirming Anabolic Properties are listed in Tab. 1.

Tab. 1: Anabolic and androgenic properties of steroids taken from Vida's tables (VP= rel. weight of ventral prostate, SV=of seminal vesicle, LA=of levator anii, adapted from [19])

Name	VP	SV	LA	Reference (value = 100)
Androst-4-ene-3,11,17-trione	7	8	10	Testosterone
	48	48	70	Testosterone
4-Chloro-11-oxo-17 α -methyltestosterone	-	6	730	Metandienone
17 β -Hydroxy-17 α -methylandrosta-4,6-diene-3-one	5.1	-	7.2	17 α -Methyltestosterone
	24	-	61	17 α -Methyltestosterone
17 β -Hydroxy-estra-4,9-diene-3-one	10	10	100	17 α -Methyltestosterone
2 α ,3 α -Epithio-17 α -methyl-5 α -androstan-17 β -ol	27	27	154	Testosterone-propionate
	91	91	1100	17 α -Methyltestosterone
17 β -Hydroxy-5 α -androstan- $[2,3-d]$ isoxazole	11	-	40	Testosterone-propionate
	0	-	0	17 α -Methyltestosterone
DHEA	<10	<10	<10	Testosterone
	34	14	12	Testosterone

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