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GC-MS(/MS) investigations on long-term metabolites of 17-methyl steroids

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Abstract

17β-Hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one was identified as long-term metabolite of metandienone in human urine resulting in an increased number of adverse analytical findings in doping control for this anabolic steroid within the last few years. As the metabolic fate of the D-ring of other 17-methylated steroids was found to be similar up to now, the excretion of analogous 17-hydroxymethyl-17-methyl-18-norandrost-13-ene derivatives ("night watch", NW) in human urines after the application of further 17-methyl steroids was studied. Therefore, following single oral doses of the respective steroid, post-administration urines were analysed. After dehydrochloromethyltestosterone (DHCMT), Methyl-1-testosterone and Methyltestosterone administration, the respective derivatives were detected in the glucuronide fraction. From their detection times in the urine, 4-chloro-17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one (after DHCMT) and 17β-hydroxymethyl-17α-methyl-18-nor-5α-androsta-1,13-dien-3-one (after methyl-1-testosterone) may serve for long-term detection in doping control while 17β-hydroxymethyl-17α-methyl-18-norandrosta-4,13-dien-3-one appeared to be excreted only shortly after administration of methyltestosterone. Thus, its detection may only be suitable as supplement in tracing methyltestosterone administration. Also following oxandrolone administration, traces of 17β-hydroxymethyl-17α-methyl-18-nor-2-oxa-5α-androsta-13-en-3-one could be detected in the glucuronide fraction. Following the administration of bolasterone, fluoxymesterone, methandriol, 17-methyl-19-nortestosterone, mibolerone, oxymesterone, and stanozolol no analogous metabolites were so far detected in the urines analysed. The 17-hydroxymethyl-17-methyl-18-norandrost-13-enes are characterised by mass spectrometric techniques.

Introduction

Investigations concerning a new long-term metabolite of metandienone have been performed since October 2005. It was identified by mass spectrometric experiments (precursor ion scans, product ion scans, full scans) as 17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one [1]. Final structure confirmation was recently achieved by comparison with biotechnologically produced reference material [2]. Its generation in metabolism is proposed as illustrated in Figure 1. The implementation in routine screening procedures largely increased the detection time of a misuse of metandienone in sports and thereby the number of adverse analytical findings [3].

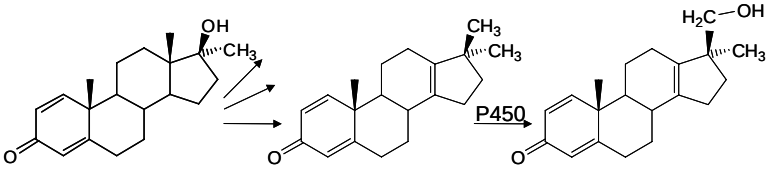

Figure 1: Proposal of 17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one generation from metandienone by cytochrome P450 catalysed hydroxylation in metabolism

An analogue metabolite was tentatively identified by GC-MS after the administration of methyl-1-testosterone [4]. As the metabolic fate of the D-ring of other 17-methylated steroids was reported to be similar, those steroids may also yield analogue metabolites. Thus, in the reported study we investigated the detectability in post administration urines of bolasterone, dehydrochloromethyltestosterone (DHCMT), fluoxymesterone, methandriol, methyl-1-testosterone, methyltestosterone, 17-methyl-19-nortestosterone, mibolerone, oxandrolone, oxymesterone, and stanozolol.

Materials and Methods

Instrumentation

The analyses were performed on a Trace GC Ultra gas chromatograph (Thermo Fisher, Bremen, Germany) coupled to a TSQ Quantum GC triple quadrupole mass spectrometer (Thermo Fisher, Bremen, Germany) applying the following parameters: column: Agilent Ultra-1 (17 m; 0.20 mm i.d.; 0.1 µm film thickness), carrier gas: helium, constant pressure: 1.14 Bar, oven temperature program: 0 min 183°C, +3°C/min, 0 min 232°C, +40°C/min, 2 min 310°C, injection volume: 1.5 µL, split 1:16, injection temperature: 300°C, ionization: 70 eV, EI, collision gas: argon. Prior to injection the samples were derivatized with TMIS reagent (MSTFA/ NH_4I/ ethanethiol, 1000:2:3, v:w:v) by heating for 20 min at 60°C.

Wagner-Meerwein rearrangement

17,17-Dimethyl-18-norandrost-13-en-3-one analogues of several 17-methyl steroids were obtained by Wagner-Meerwein rearrangement of the respective compounds: About 10 mg of the respective 17-methyl steroids (DHCMT, fluoxymesterone, methyltestosterone, methyl-1-testosterone, 17-methyl-19-nortestosterone, mibolerone, oxandrolone, and oxymesterone) were refluxed overnight in a mixture of 0.5 mL of methanol and 0.5 mL of aqueous hydrochloric acid (1 mol/L) as described by Segaloff and Gabbard [5]. The resulting 17,17-dimethyl-18-norandrost-13-ene derivatives were extracted with 5 mL of n-pentane and the organic layer was evaporated to dryness. Following derivatisation the products were analysed by GC-MS(/MS).

Biotransformation studies

Incubation of solutions of the 17,17-dimethyl-18-norandrost-13-en-3-ones (1 mM in cultivation medium) for 24 h at 30ºC under mild agitation was performed with *S. pombe* strains expressing the human CYP3A4 or CYP21 enzymes [6]. After twofold extraction with ethyl acetate aliquots were derivatised and analysed by GC-MS(/MS).

Administration studies

Administration studies approved by the local ethics committee of the German Sport University Cologne with orally ingested DHCMT (15 mg, 22 d), methyl-1-testosterone (10 mg, 2 d and 3 more morning urines), methyltestosterone (5 mg, 13 d), oxymesterone (16 mg, 26 d), mibolerone (10 mg, 3 d), 17-methyl-19-nortestosterone (4 mg, 2 d) and spot urines after bolasterone, fluoxymesterone, methandriol, oxandrolone and stanozolol administration were analysed.

The samples were prepared according to the routine steroid screening procedure [7]. In brief, after addition of the internal standard, 2 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, derivatised and analysed by GC-MS/MS.

Results

The Wagner-Meerwein rearrangement of DHCMT, fluoxymesterone, methyltestosterone, methyl-1-testosterone, 17-methyl-19-nortestosterone, mibolerone, oxandrolone, and oxymesterone resulted in the corresponding 17,17-dimethyl-18-norandrost-13-ene derivatives. The EI mass spectra of their TMS derivatives are displayed in Figure 2. In post administration urines 17,17-dimethyl-18-norandrosta-4,13-dien-3-one (from methyltestosterone), 4-hydroxy-17,17-dimethyl-18-norandrosta-4,13-dien-3-one (from oxymesterone) and 17,17-dimethyl-2-oxa-5α-androst-13-en-3-one (from oxandrolone) were detectable in considerable amounts while the other compounds did not appear in the urines analysed.

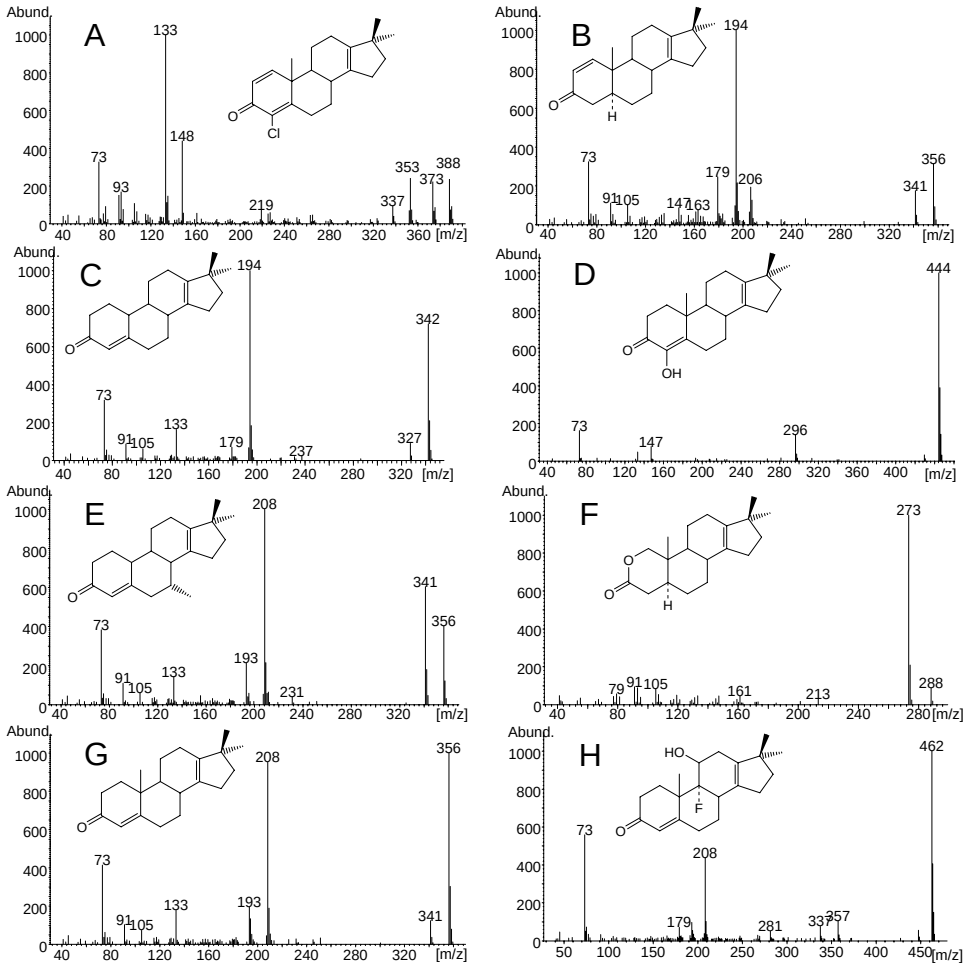

Figure 2: Mass spectra (GC-MS, EI, scan) of Wagner-Meerwein product of DHCMT (A), mono-TMS, M^+=388, of methyl-1-testosterone (B), mono-TMS, M^+=356, of 19-normethyltestosterone (C), mono-TMS, M^+=342 of oxymesterone (D), bis-TMS, M^+=444, of mibolerone (E), mono-TMS, M^+=356, of oxandrolone (F), M^+=288, of methyltestosterone (G), mono-TMS, M^+= 356, of fluoxymesterone (H), bis-TMS, M^+= 462

Biotransformation studies

The TMS derivatives of the different 17ξ-hydroxymethyl-17ξ-methyl-18-norandrost-13-enes obtained in the biotransformation studies were recorded in full scan mode. Both corresponding 17-epimers showed similar mass spectra (spectra of 17β-hydroxymethyl-17α-methyl epimer depicted in Figure 3) with analogous fragments compared to the metandienone long-term metabolites 17ξ-hydroxymethyl-17ξ-methyl-18-norandrosta-1,4,13-trien-3-one. Some proposals of fragment generation are indicated in the mass spectra. All spectra show dominant losses of 103 u corresponding to $^{\bullet}CH_2$-O-TMS. M/z 133 is generated from the C/D-ring (C-8,9,11-18,20) after a loss of 103 u as already reported in case of the metandienone metabolite [1]. Interestingly regarding of the fluoxymesterone product (9α-fluoro-11β-hydroxy-17β-hydroxymethyl-17α-methyl-18-norandrosta-4,13-en-3-one) no fragment with m/z 133 appears in the spectrum. This spectrum is dominated by subsequent losses of HF (20 Da) and TMS-OH (90 Da) from M^+-103 (m/z 447), generating the fragments m/z 427, 357, and 337.

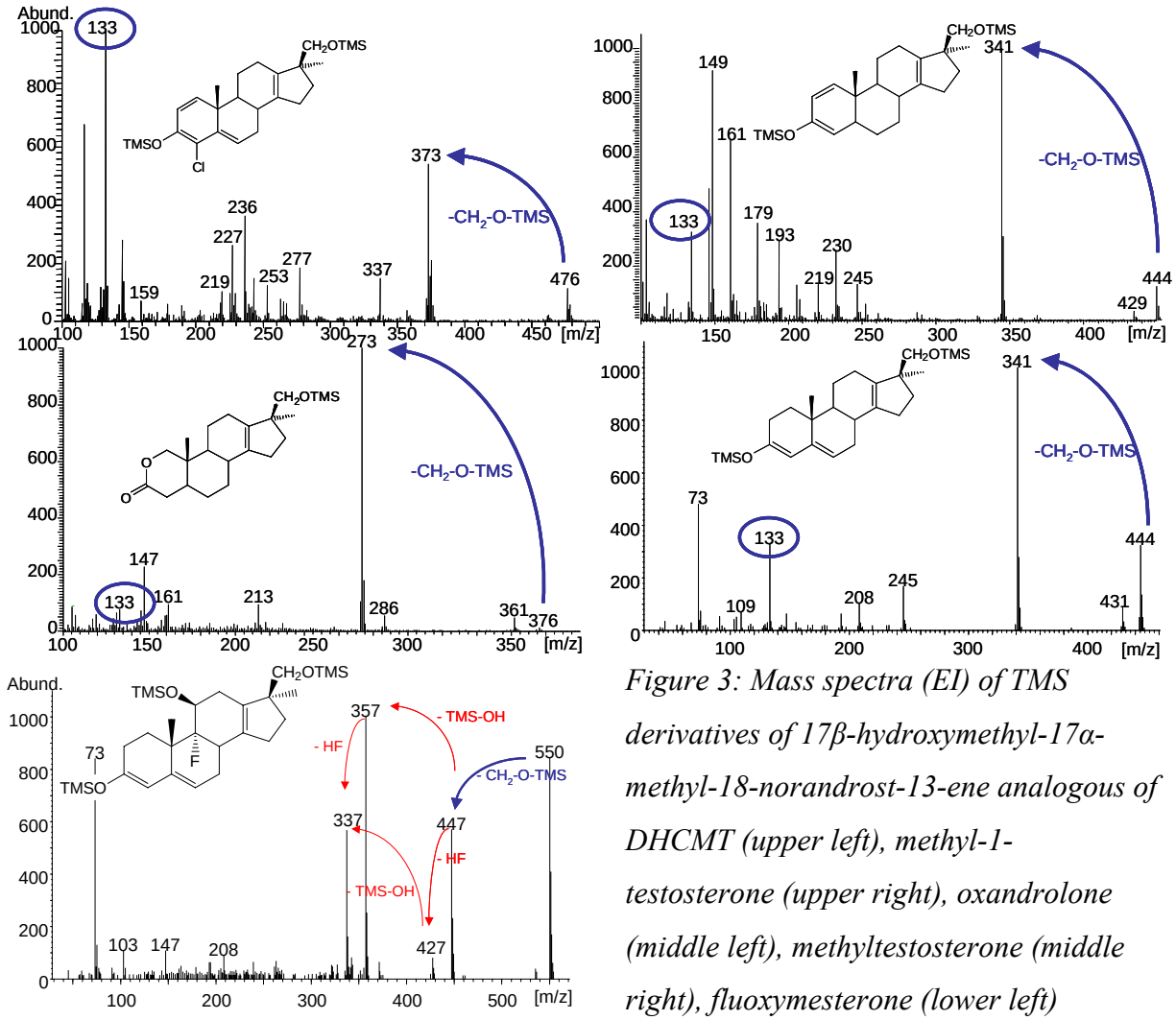

Figure 3: Mass spectra (EI) of TMS derivatives of 17β-hydroxymethyl-17α-methyl-18-norandrost-13-ene analogous of DHCMT (upper left), methyl-1-testosterone (upper right), oxandrolone (middle left), methyltestosterone (middle right), fluoxymesterone (lower left)

Excretion studies

Monitoring the characteristic ion transitions, the excretion studies were analysed for the presence of the respective 17β-hydroxymethyl-17α-methyl-18-norandrost-13-enes. Their detectability was compared to the metabolites traditionally used in doping control analyses. Following DHCMT administration, the urinary excretion was surveyed monitoring the characteristic ion transitions of m/z 236 → 133 representing the loss of $^{\bullet}CH_2OTMS$ from the C/D-ring fragment [1]. Two metabolites (RT_{NW} = 16.62 min and RT_{epiNW} = 16.24 min) were detected. As confirmed for the metandienone metabolites, the 17β-hydroxymethyl epimer most likely elutes later, identifying NW as 4-chloro-17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one while epiNW is assigned to 4-chloro-17α-hydroxymethyl-17β-methyl-18-norandrosta-1,4,13-trien-3-one.

Comparison of the detection periods for the possible identifiers of a DHCMT administration revealed similar detection periods (22 days) for the NW, epiNW and the actual long-term metabolite 4ξ-chloro-3α,6β,17β-trihydroxy-17α-methyl-5β-androst-1-en-16-one (M2) in GC-MS/MS (MRM) analysis while DHCMT, 17-epi-DHCMT and 6β-hydroxy-DHCMT (M2) were only detectable for 12 or 8 days respectively (Figure 4).

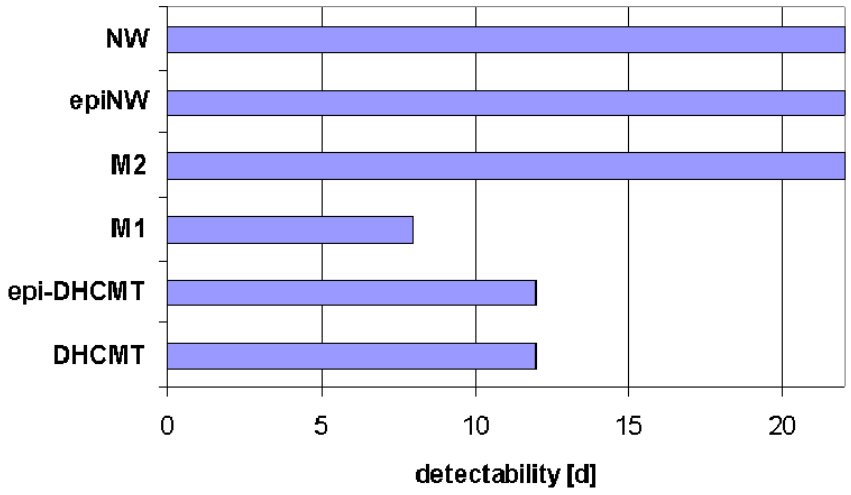

Figure 4: GC-MS/MS detection times (MRM) in post-administration urines after DHCMT (5 mg, p.o.), NW: 4-chloro-17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one, epiNW: 4-chloro-17α-hydroxymethyl-17β-methyl-18-norandrosta-1,4,13-trien-3-one, M1: 6β-hydroxy-DHCMT, M2: 4ξ-chloro-3α,6β,17β-trihydroxy-17α-methyl-5β-androst-1-en-16-one

In the excretion study with oral methyltestosterone, the appearance of 17ξ-hydroxymethyl-17ξ-methyl-18-norandrosta-4,13-dien-3-one was surveyed by monitoring the characteristic ion transitions of m/z 444 → 341 and m/z 444 → 133.

Comparison of the detection periods for the possible identifiers in MRM mode (Figure 5) revealed that 17β-hydroxymethyl-17α-methyl-18-norandrosta-4,13-dien-3-one (NW, RT_{NW} = 13.17 min) was detectable only for 2 days after the administration of 5 mg of methyltestosterone. Its 17-epimer was not detectable in the urine samples at all. Yet, the "classical metabolites" 3α5α-THMT and 3α5β-THMT could be detected up to 7 days. Additionally epi-3α5β-THMT was detected up to 4 days and 17α-hydroxy-17β-methylandrosta-4,6-dien-3-one (epi-6-ene) for 3 days in this study. As epi-6-ene was identified as long-term metabolite in LC-MS/MS analysis by Pozo et al [8], it would be interesting to compare the detection periods also in LC-MS/MS analysis.

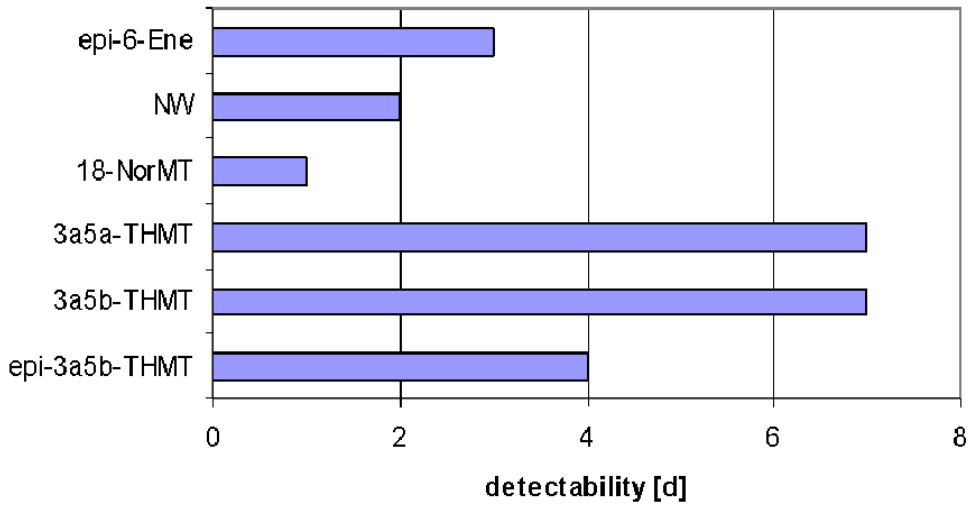

Figure 5: GC-MS/MS detection times (MRM) in post-administration urines after methyltestosterone (5 mg, p.o.), epi-6-ene: 17α-hydroxy-17β-methylandrosta-4,6-dien-3-one, NW: 17β-hydroxymethyl-17α-methyl-18-norandrosta-4,13-dien-3-one, 18-NorMT: 17,17-dimethyl-18-nor-androsta-4,13-dien-3-one , 3α5α-THMT: 17α-methyl-5α-androstane-3α,17β-diol, 3α5β-THMT: 17α-methyl-5β-androstane-3α,17β-diol, epi-3α5β-THMT: 17β-methyl-5β-androstane-3α,17α-diol

In case of methyl-1-testosterone all samples (collection period 4 days) were found to contain 17β-hydroxymethyl-17α-methyl-18-nor-5α-androsta-1,13-dien-3-one as well as its 17-epimer (ion transitions m/z 341 → 161 and m/z 341 → 147, RT_{NW} = 12.19 min and RT_{epiNW} = 11.81 min). The classical metabolites 17α-methyl-5α-androstane-3α,17β-diol (3α5α-THMT), 17β-methyl-5α-androstane-3α,17α-diol (epi-3α5α-THMT), 17α-methyl-5α-androst-1-ene-3α,17β-diol (3OH-M1T), 17β-methyl-5α-androst-1-ene-3α,17α-diol (epi-3OH-M1T), 17α-hydroxy-17β-methyl-5α-androst-1-en-3-one (epi-M1T), and the parent compound were also detected in all urines available. Thus, the assignment of long-term metabolites remained unclear.

In contrast, no 17ξ-hydroxymethyl-17ξ-methyl-18-norandrost-13-enes were detected following the administration of 17α-methyl-19-nortestosterone, mibolerone and oxymesterone. However, in the early oxymesterone urines 4-hydroxy-17,17-dimethyl-18-norandrosta-4,13-dien-3-one was detectable up to 10 hours post administration as additional metabolite.

Spot urines

In case of oxandrolone, 17β-hydroxymethyl-17α-methyl-2-oxa-18-nor-5α-androst-13-en-3-one was detected in MRM mode. None of the spot urines collected after bolasterone, fluoxymesterone, methandriol, and stanozolol administration was found to contain the respective 17ξ-hydroxymethyl-17ξ-methyl-18-norandrost-13-enes.

Discussion

The extent of 17ξ-hydroxymethyl-17ξ-methyl-18-norandrost-13-ene derivatives formed in the metabolism of 17α-methyl steroids appears to be highly dependent on the structure of the parent compound. In general, compounds that are more stable to other routes of metabolism and thus show longer half-lives, tend to yield the investigated 17-hydroxymethyl metabolites. For their creation in the body two ways are possible:

a) formation of 17,17-dimethyl-18-norandrost-13-ene derivative via sulfate as described by Schänzer et al. [9] and subsequent hydroxylation of 17-CH_3 as illustrated in Figure 1, or
b) hydroxylation of the parent compound on C18 and subsequent Wagner-Meerwein rearrangement.

The yeast experiments suggest pathway a), where the 17-hydroxymethyl metabolites can be obtained from 17,17-dimethyl-18-norandrost-13-ene by hydroxylations involving CYP3A4 and CYP21. However, further investigations are needed to elucidate the metabolic pathway.

Summary

17β-Hydroxymethyl-17α-methyl-18-nor-androst-13-ene metabolites were detected after oral administration of metandienone, DHCMT, methyltestosterone, methyl-1-testosterone, and oxandrolone. On Agilent Ultra-1 columns, they showed retention times almost identical to the 17-epimer of the parent compound. Characteristic fragments were m/z 133 and M^+-103, characteristic ion transitions $M^{+\bullet} \rightarrow [M - 103]^+$, $M^{+\bullet} \rightarrow 133$, and $[M-103]^+ \rightarrow 133$. Prolonged detectability was shown only for metandienone administration. However, long-term kinetics of methyl-1-testosterone and oxandrolone still have to be investigated. The DHCMT-analogue offers a potential perspective for confirmations after biotechnological production of reference material as the other long-term metabolite M2 is not available as reference.

Up to now no 17ξ-hydroxymethyl-17ξ-methyl-18-nor-androst-13-ene metabolites have been detected after administration of bolasterone, fluoxymesterone, methandriol, methyl-19-nortestosterone, mibolerone, oxymesterone, and stanozolol. However, 4-hydroxy-17,17-dimethyl-18-norandrosta-4,13-dien-3-one was found as additional metabolite with a detection time of ~10 h post administration of oxymesterone.

Acknowledgements

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