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SUMMARY

The misuse of dihydrotestosterone (DHT) (17β-hydroxy-5α-androstan-3-one) as an anabolic steroid by athletes has been investigated by different authors to set criteria for differentiation between exogenous application of DHT and its endogenous excretion. In this study deuterated DHT ([16,16,17,2H$_3$]-17β-hydroxy-5α-androstan-3-one) was synthesized with introduction of three deuterium at C-16,16 and C-17. The metabolism of DHT was reinvestigated by administration of 10 mg of trideuterated DHT to a male volunteer. Urinary excretion of deuterated DHT and its deuterated metabolites 3α-hydroxy-5α-androstan-17-one (And), 5α-androstane-3α,17β-diol (3α-diol) and 5α-androstane-3β,17β-diol (3β-diol), all excreted as conjugates, was confirmed.

INTRODUCTION

The use of synthetic anabolic steroids and the endogenous steroid testosterone by athletes is banned by the International Olympic Committee (IOC) and National and International sports Federations.

The misuse of these steroids is controlled by gas chromatography/mass spectrometry (GC/MS) analysis of urine samples delivered from athletes after competition or out-of competition. The identification of synthetic anabolic steroids is based on comparison of the EI-mass spectrum and GC retention time of the isolated steroid and/or its metabolite with the EI-mass spectrum and GC retention time of an authentic reference substance [1]. For identification of an exogenous application of testosterone the ratio of urinary excreted testosterone (T) glucuronide to epitestosterone (E) glucuronide is determined by GC/MS. If the T/E ratio is greater than six the athlete is further investigated to estimate his individual T/E quotient. The doping regulation not only prohibits the use of testosterone, all treatments (e.g. use of HCG or 4-androstene-
Fig. 1

Androstenedione

HSD = HydroxySteroid Dehydrogenase

Metabolic pathway of testosterone to dihydrotestosterone and further 5α-metabolites.

5α-Androstene-3β,17β-diol
5α-Androstane-3α,17α-diol

5α-Reduction

5β-Androstene-3α,17β-diol (Eiocholanolone, 5α-Metabolites)
Testosterone
3,17-dione) which result in an increase of the T/E ratio > 6 are banned. The 5α reduced metabolite of testosterone, in cells such as the prostate, is a the more potent androgen than testosterone itself [2]. Dihydrotestosterone is a direct metabolite of testosterone and is produced by enzymatic reduction of the C-4,5 double bond by 5α-reductase (Fig.1). This reduction is not reversible, which means that testosterone cannot be produced via C-4,5 dehydrogenation of DHT.

Because of the anabolic effects of it is more likely that athletes will try to misuse DHT. The application of DHT does not influence the T/E ratio. Possible indices of an exogenous application of DHT are an increased urinary excretion of DHT and/or one of its main metabolites (androsterone, 3α-diol, 3β-diol). This has been reported by Southan et al. [3] and by investigations of Donike et al. [4] and Schindler [5].

In this study the identification of urinary metabolites originating from dihydrotestosterone was elucidated after oral application of deuterated dihydrotestosterone.

EXPERIMENTAL

Steroids and Chemicals

Dihydrotestosterone, androsterone, etiocholanolone, 5α-androstane-3α,17β-diol, 5β-androstane-3α,17β-diol, 5α-androstane-3β,17β-diol, testosterone, epitestosterone and methyltestosterone were purchased from SIGMA, D-82039 Deisenhofen, Germany. β-Glucuronidase from Escherichia coli K12 was obtained from (Boehringer, Mannheim, Germany), β-glucuronidase/arilsulphatase from Helix pomatia was obtained from SERVA (Heidelberg, Germany). MSTFA [6], [2,2,4,4-2H₄]-etiocholanolone [7], and [16,16,17-2H₃]-testosterone [7] were synthesized in our laboratory. All other reagents and solvents were of analytical grade.

Metabolism studies

10 mg of [16,16,17-2H₃]-DHT was applied to a male person (75kg, 43 years). Urine samples following administration were collected, stabilized with sodium azide and stored at 4°C.

Isolation of steroids

Enzymatic hydrolysis: 2 ml of urine were adsorbed on Amberlite XAD-2 polystyrene resin. The XAD-2 column (pasteur pipette, closed with a glass pearl, bed height: 2 cm) was washed with 2 ml of bidistilled water, and conjugated and unconjugated steroids were eluted with 2 ml of methanol. The methanolic eluate was evaporated to dryness,
and the residue was enzymatically hydrolyzed with (A) 50 μl of β-glucuronidase from E. coli in 1 ml 0.2 M phosphate buffer, pH 7.0 for 1 hour at 50°C, or (B) 50 μl of β-glucuronidase/arylsulphatase from Helix pomatia in 1 ml of 0.2 M sodium acetate buffer, pH 5.0, for 3 hours at 50°C. After hydrolysis, the buffer solution was alkalized with 250 μl of 5% potassium carbonate solution, and the steroids were extracted with 5 ml tert.butylmethyl ether. After centrifugation, the ether layer was transferred and evaporated to dryness under vacuum.

Solvolyis: For solvolysis the aqueous residue of the enzymatic hydrolysis with the E.coli enzyme is diluted with 1 ml of bidistilled water and applied to a new XAD-2 column. The column is washed with 2 ml of water, eluted with 2 ml of methanol and the methanolic layer is dried under reduced pressure. The dried residue is dissolved with 1 ml of a mixture of ethyl acetate/methanol/conc. sulfuric acid, 50:5:0.05 (v:v:v) and heated for 1 hour at 60°C. After the solvolysis 1 ml of 5% potassium carbonate solution is added and steroids are extracted after addition of 4 ml of tert.butylmethyl ether. The organic layer is concentrated, dried and derivatized.

Derivatization for GC/MS

The dry residue was derivatized with 100 μl of MSTFA/NH₄I/ethanethiol TMS, 1000:2:6 (v:w:v), which is equivalent to a MSTFA/trimethylsilyl isocyanate solution 1000:2 (v:v) [8], and heated for 15 min at 60°C.

GC/MS determination

EI mass spectra were registered with a double focusing mass spectrometer [Finnigan MAT 95] coupled with a Hewlett Packard 5890 gas chromatograph (Hewlett Packard Ultra 1 capillary column, I.D. 0.2 mm, film thickness 0.11 μm, helium carrier gas 1 ml/min, split 1:20). The electron energy was 70eV and the ion source temperature was 200°C.

Quantification of excreted steroids were performed with a GC/MS system [GC/MSD Hewlett-Packard (GC 5890/MS 5970)], with the electron impact set at 70 eV. Column: Hewlett-Packard, HP1, fused silica capillary column cross-linked methyl silicone (OV 1), 17 m, ID 0.20 mm, film thickness 0.11 μm. The carrier gas was helium (1 ml/min, split 1:10), and the temperature program was as follows: initial temperature 180°C, program rate 5°C/min, final temperature 300°C.
Selected ion monitoring (SIM) was used with the following ions recorded

m/z 436  
[16,16-2H₂]-DHT bis-TMS,

m/z 437  
[16,16,17-2H³]-DHT bis-TMS,

m/z 434  
androsterone bis-TMS,

eucholestanolone bis-TMS,

m/z 435  
[16-2H₁]-androsterone bis-TMS,

m/z 436  
isotope of m/z 435: [16-2H₁]-androsterone bis-TMS,

m/z 436  
5α-androstan-3α,17β-diol bis-TMS (3α-diol),

m/z 436  
5β-androstan-3α,17β-diol,bis-TMS,

m/z 438  
5α-androstan-3β,17β-diol bis-TMS (3β-diol),

m/z 438  
[16,16-2H₂]-5α-androstan-3α,17β-diol bis-TMS,

m/z 439  
[16,16,17-2H³]-5α-androstan-3α,17β-diol bis-TMS,

m/z 439  
[16,16,17-2H³]-5α-androstan-3α,17β-diol bis-TMS,

m/z 432  
testosterone bis-TMS, and

epitestosterone bis-TMS.

Urine concentrations were calculated by the peak areas of the recorded signals relative to the internal standard [2,2,4,4,2H₄]-eucholestanolone bis-TMS with m/z 438.

Synthesis of [16,16,17-2H³]-DHT

1. [16,16,17-2H³]-DHT

300 mg of [16,16,17-2H³]-testosterone were reduced with hydrogen in 14 ml of 96% acetic acid using 20 mg of palladium on charcoal (Aldrich, Steinheim, Germany) as a catalyst. The reaction mixture was diluted with 600 ml of diethyl ether, washed three times with 100 ml of 2% aqueous water and two times with 100 ml of water. The organic layer was concentrated to dryness and the reaction products separated by preparative HPLC (1×25 cm RP-18, 7μm, Macherey-Nagel, D-52355 Düren, Germany), yielding 170 mg of [16,16,17-2H³]-DHT (yield 57.0). The EI-spectrum of the bis-TMS product is shown in Fig.3.

RESULTS

Synthesis of [16,16,17-2H³]-DHT

The synthesis of [16,16,17-2H³]-DHT follows the catalytic reduction of [16,16,17-2H³]-testosterone (synthesis [7]) with hydrogen in acetic acid. The reduction yielded a mixture of 5α- and 5β-isomer, with a ratio of approximately of 2:1 (Fig.2).
Deuterated Testosterone (300 mg)

Hydrogen, 10% Palladium on charcoal in acetic acid

Deuterated Dihydrotestosterone

Isolated by HPLC (170 mg)

Fig.2 Synthesis [16,16,17-2H3]-Dihydrotestosterone

The EI mass spectra of the per-TMS derivatives of DHT and [16,16,17-2H3]-DHT are presented in Fig.3. The dominant ion fragments m/z 127, 142 and 143 originate from rearrangement processes of the A-ring. The fragment ions m/z 405 (408 deuterated) and 377 (380 deuterated) are produced by a loss of 29 and 57 respectively, and are assumed to be generated by A-ring fragmentation as C-16 and C-17 are not involved in the cleaved fragments. A comparison of the mass spectra over the mass range from m/z 370 to 450 is shown in Fig.4. The molecular ion m/z 437 of the deuterated bis-TMS derivative indicates that three deuteriums were introduced into the molecule. The deuteration yield was higher than 98%. 

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Fig. 3  EI mass spectra of (A) dihydrostosterone bis-TMS $M^+ 434$ (main TMS isomer) and (B) $[16,16,17-^2\text{H}_3]$-dihydrostosterone bis-TMS $M^+ 437$ (main TMS isomer).
Derivatization and GC/MS analysis

Derivatization of DHT with MSTFA/TMIS yields two bis-TMS isomers: TMS 3,5-dienol ether 17-TMS ether (5-6%) and TMS 2,4-dienol ether 17-TMS ether (94-95%) as the main isomer (Fig. 5).

Fig. 4  Partial EI mass spectra from m/z 370 to 450 from DHT and [16,16,17-\textsuperscript{2}H\textsubscript{3}]-DHT (main TMS isomers).

Fig. 5  Derivatization of dihydrotestosterone with MSTFA/TMIS.
Metabolism of [16,16,17-\textsuperscript{2}H\textsubscript{3}]-DHT

After oral administration of 10 mg of [16,16,17-\textsuperscript{2}H\textsubscript{3}]-DHT urinary excreted conjugates of deuterated DHT and deuterated metabolites were identified (Table 1).

Table I Excretion of conjugated steroids following oral administration of 10 mg of [16,16,17-\textsuperscript{2}H\textsubscript{3}]-dihydrotosterone. Comparison of enzymatic hydrolysis of conjugates with \(\beta\)-glucuronidase/arylsulfatase from Helix pomatia, \(\beta\)-glucuronidase from E. coli, and solvolysis.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Helix pomatia</th>
<th>E. coli</th>
<th>Solvolysis\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\mu g)</td>
<td>%</td>
<td>(\mu g)</td>
</tr>
<tr>
<td>[16,16-\textsuperscript{2}H\textsubscript{2}]-And</td>
<td>3692</td>
<td>36.9</td>
<td>3322</td>
</tr>
<tr>
<td>[16,16-\textsuperscript{2}H\textsubscript{2}]-3(\alpha)-Diol</td>
<td>76</td>
<td>0.8</td>
<td>72</td>
</tr>
<tr>
<td>[16,16,17-\textsuperscript{2}H\textsubscript{3}]-3(\alpha)-Diol</td>
<td>198</td>
<td>2.0</td>
<td>184</td>
</tr>
<tr>
<td>[16,16-\textsuperscript{2}H\textsubscript{2}]-3(\beta)-Diol</td>
<td>18</td>
<td>0.2</td>
<td>4</td>
</tr>
<tr>
<td>[16,16,17-\textsuperscript{2}H\textsubscript{3}]-3(\beta)-Diol</td>
<td>139</td>
<td>1.4</td>
<td>85</td>
</tr>
<tr>
<td>[16,16-\textsuperscript{2}H\textsubscript{2}]-DHT</td>
<td>52</td>
<td>0.5</td>
<td>48</td>
</tr>
<tr>
<td>[16,16,17-\textsuperscript{2}H\textsubscript{3}]-DHT</td>
<td>610</td>
<td>6.1</td>
<td>674</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>4785</td>
<td>47.9</td>
<td>4389</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Solvolysis was performed after separation of steroids which were first hydrolyzed with \(\beta\)-glucuronidase from E. coli.

And
Androsterone (3\(\alpha\)-hydroxy-5\(\alpha\)-androstan-17-one)
3\(\alpha\)-Diol 5\(\alpha\)-Androstan-3\(\alpha\),17\(\beta\)-diol
3\(\beta\)-Diol 5\(\alpha\)-Androstan-3\(\beta\),17\(\beta\)-diol
DHT Dihydrotosterone (17\(\beta\)-hydroxy-5\(\alpha\)-androstan-3-one)

Hydrolysis of these steroids with \(\beta\)-glucuronidase from E. coli supports the assumption that these steroids are excreted as \(\beta\)-glucuronic acid conjugates. Approximately 44% of the applied DHT was recovered. Hydrolysis with Helix pomatia enzyme results in an increased amount of the 3\(\beta\)-hydroxy sulphated conjugate metabolites and a small percentage of androsterone, which is excreted as a sulphate conjugate. The major metabolites were deuterated androsterone 36.9\%, deuterated DHT (6.6\%), deuterated 5\(\alpha\)-diol (2.8\%) and 5\(\beta\)-diol (1.6\%) all hydrolyzed with the Helix pomatia enzyme. Deuterated androsterone and deuterated 3\(\alpha\)- and 3\(\beta\)-diol were detected 24 h after application (Table 2).
Table 2  Excretion rates of deuterated DHT and its metabolites following oral administration of 10 mg of [16,16,17-2H3]-DHT.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>D2-Androsterone (ng/min)</th>
<th>D2-3α-Diol (ng/min)</th>
<th>D3-3α-Diol (ng/min)</th>
<th>D2-3β-Diol (ng/min)</th>
<th>D3-3β-Diol (ng/min)</th>
<th>D2-DHT (ng/min)</th>
<th>D3-DHT (ng/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-1</td>
<td>13133</td>
<td>153</td>
<td>432</td>
<td>37</td>
<td>385</td>
<td>147</td>
<td>3164</td>
</tr>
<tr>
<td>1-2</td>
<td>18315</td>
<td>199</td>
<td>542</td>
<td>40</td>
<td>493</td>
<td>195</td>
<td>2888</td>
</tr>
<tr>
<td>2.0-4.2</td>
<td>7341</td>
<td>127</td>
<td>349</td>
<td>22</td>
<td>226</td>
<td>78</td>
<td>874</td>
</tr>
<tr>
<td>4.2-6.1</td>
<td>2547</td>
<td>73</td>
<td>204</td>
<td>14</td>
<td>127</td>
<td>34</td>
<td>348</td>
</tr>
<tr>
<td>6.1-8.0</td>
<td>1700</td>
<td>69</td>
<td>194</td>
<td>19</td>
<td>122</td>
<td>29</td>
<td>220</td>
</tr>
<tr>
<td>8.0-10.5</td>
<td>691</td>
<td>41</td>
<td>99</td>
<td>10</td>
<td>28</td>
<td>12</td>
<td>69</td>
</tr>
<tr>
<td>10.5-14.0</td>
<td>340</td>
<td>33</td>
<td>67</td>
<td>23</td>
<td>61</td>
<td>4</td>
<td>49</td>
</tr>
<tr>
<td>14.0-19.2</td>
<td>157</td>
<td>19</td>
<td>35</td>
<td>20</td>
<td>55</td>
<td>-</td>
<td>22</td>
</tr>
<tr>
<td>19.2-24.0</td>
<td>137</td>
<td>12</td>
<td>15</td>
<td>-</td>
<td>35</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

D2  $[16,16\cdot{\text{2H2}}]$-  
D3  $[16,16,17\cdot{\text{2H3}}]$-AndAndrosterone (3α-hydroxy-5α-androstan-17-one)  
3α-Diol  5α-Androstane-3α,17β-diol  
3β-Diol  5α-Androstane-3β,17β-diol  
DHT  Dihydrotestosterone (17β-hydroxy-5α-androstan-3-one)

Approximately 7% of excreted deuterated DHT contains only 2 deuterium atoms, indicating a loss of deuterium at C-17. This loss can be explained via the reduction of the 17β-hydroxy group to the 17-keto steroid (5α-androstane-3,17-dione). This reaction is reversible and the enzyme 17β-hydroxysteroid dehydrogenase can convert the 17-oxidized product back to dihydrotestosterone (Fig.6). For the 3β-diol the loss of one deuterium was about 5% and for the 3α-diol 28% (following E. coli hydrolysis). The high extent of exchanged deuterium at position C-17 in the 3α-diol can be accounted via its formation from androsterone.
Fig. 6  Metabolism of [16,16,17-2H₃]-dihydrotestosterone

Of interest is the finding that a significant amount of the 3β-diol metabolite was detected in the glucuronide fraction. This is surprising as 3β-hydroxy steroids in general are conjugated with sulphate. The result can be accounted for DHT-glucuronide is metabolized to the 3β-hydroxy steroid prior to excretion into the urine. In this case the 3β-diol is glucuronidated at the 17β-hydroxy group (Fig. 7) and not at the 3β-hydroxy position. This metabolic pathway seems possible as in the first hours after application a high amount of DHT enters to the system and is quickly metabolized yielding DHT-glucuronide to a high extent. This hypothesis should be proved by further experiments and exact confirmation of the proposed excreted conjugate.
Fig. 7  Proposed metabolism for generation and excretion of 5α-androstane-3β,17β-diol (3β-diol) detected in the glucuronide fraction following enzymatic hydrolysis with E. coli.
REFERENCES


