NEW BOLDENONE HYDROXYL METABOLITES IN CATTLE URINE

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Section of Chemical risk in the chain of production and control quality
General Outline

- Introduction
- Boldenone hydroxyl metabolites
- *In vitro* LC-MS/MS studies
- *In vivo* LC-MS/MS studies
- Preliminary results
- Conclusions
Introduction

- **$17\beta$-Boldenone** ($17\beta$-Bol) is an androgenic steroid of synthetic origin endowed of potent anabolic properties, and forbidden for meat production.

- It is illegally used for treatment of athletes and racehorses to improve sport performance and in cattle.
Introduction

- As for other androgenic steroids, $17\beta$-Bol is classified by the IARC as a probable human carcinogen

- Role of $17\alpha$-Bol in the development of human prostate carcinomas

- *In vitro* and *in vivo* metabolism studies (phase I and phase II) can be of help in efficiently comparing urinary excretion profiles of “control” and “treated” animals
Structures

ADD (1,4-androstadiene-3,17-dione)

17β-Bol
(1,4-androstadiene-17β-ol-3-one)

17α-Bol
(1,4-androstadiene-17α-ol-3-one)
Investigated hydroxyl metabolites of Boldenone:

- \( 16\beta,17\beta\text{-OH-Bol} \)
- \( 16\alpha,17\alpha\text{-OH-Bol} \)
- \( 6\beta,17\beta\text{-OH-Bol} \)
- \( 6\beta,17\alpha\text{-OH-Bol} \)
LC-MS/MS system

GRADIENT ELUTION

(A) CH$_3$COOH 1%
(B) ACN

Flow 200 $\mu$L/min

- AB API 3000 mass spectrometer - triple quadrupole
- TIS (Turbo Ion Spray source, PI mode)
- $T = 300 \, ^\circ$C
LC-MS/MS studies

- Q1 scan
- Product Ion scan (mass fragments)
- Q1-Q3 (MRM, Multiple Reaction Monitoring)
LC-MS/MS studies: \((6\beta,17\alpha-\text{OH-Bol})\)

Product Ions of 303

Collision Energy = 30

Selected fragments
## LC-MS/MS Analysis

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Precursor m/z</th>
<th>Product m/z</th>
<th>t_R (min)</th>
<th>CE (eV)</th>
<th>CC_{β} (ng mL^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>6β,17β-OH-Bol</td>
<td>303 (M + H)^+</td>
<td>121, 171, 147</td>
<td>6.0</td>
<td>30</td>
<td>0.6</td>
</tr>
<tr>
<td>6β,17α-OH-Bol</td>
<td>303 (M + H)^+</td>
<td>121, 171, 147</td>
<td>7.0</td>
<td>30</td>
<td>0.3</td>
</tr>
<tr>
<td>16β,17β-OH-Bol</td>
<td>303 (M + H)^+</td>
<td>121, 147, 171</td>
<td>7.8</td>
<td>30</td>
<td>0.6</td>
</tr>
<tr>
<td>16α,17α-OH-Bol</td>
<td>303 (M + H)^+</td>
<td>121, 147, 171</td>
<td>9.1</td>
<td>30</td>
<td>0.6</td>
</tr>
<tr>
<td>β-Bol</td>
<td>287 (M + H)^+</td>
<td>121, 135, 173</td>
<td>11.7</td>
<td>21</td>
<td>0.4</td>
</tr>
<tr>
<td>α-Bol</td>
<td>287 (M + H)^+</td>
<td>121, 135, 173</td>
<td>13.3</td>
<td>21</td>
<td>0.4</td>
</tr>
<tr>
<td>ADD</td>
<td>285 (M + H)^+</td>
<td>121, 147, 151</td>
<td>13.1</td>
<td>21</td>
<td>0.5</td>
</tr>
<tr>
<td>17β-T-d_{2}</td>
<td>291 (M + H)^+</td>
<td>99.1</td>
<td>13.4</td>
<td>27</td>
<td>-</td>
</tr>
</tbody>
</table>

- Product ions are the same, but ion ratios are different
LC-MS/MS

16β,17β → 6β,17α → 16α,17α

6β,17β → 17β-Bol

17α-Bol → ADD

Standard solution
While in literature m/z 121 and 147 are well documented, we hypothesized the structure of m/z 171.

Dehydration of 6-OH
Hypothesis of fragmentation

Incubation with $\beta$-Bol

6$\beta$, 17$\beta$-OH, or ?? 16$\alpha$, 17$\beta$-OH

16$\beta$, 17$\alpha$-OH
Hypothesis of fragmentation

Incubation with ADD

Product Ions of 301
Collision Energy = 30
Putative 6-OH ADD

Dehydration of 6-OH
Sample Extraction

Urine (4mL)

Spiking IS, on blank matrix

2 mL of ABS 0.9 M

pH 5 with acetic acid 2M

50 µL enzyme β-glucuronidase/arylsulphatase

IS (17β-T-d₂)
Sample Extraction

vortex for 15”

Overnight incubation under stirring at 37 °C

1 mL NaOH 1M

(2 x 15 mL) DCM
Sample Extraction

Evaporation under reduced pressure

Dissolution in 6 mL ABS

C18 SPE

LC-MS/MS analysis
LC-MS/MS: in vivo

Spiked blank matrix – I cal. level
$6\beta,17\alpha-\text{OH BOL}$ is present in the treated, but not in the control sample.
Conclusions

- $17\beta$-Bol is confirmed as a marker of illegal use of anabolic agents.

- The presence after treatment of the hydroxyl metabolite $6\beta,17\alpha$-OH-Bol, even though additional studies on other metabolites are currently in due course, candidate this as another possible marker of anabolic abuse.
Conclusions

RESEARCH is ongoing.........
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