Masato Okano¹, Tomoharu Osada², Mitsuhiko Sato¹, Ayako Uchiyama², Shinji Kageyama¹

Mass spectrometric identification of epitiostanol metabolite: In vitro metabolism using human embryonic stem cell (hESC)-derived hepatocytes and in vivo excretion study in human

Anti-Doping Laboratory, Mitsubishi Chemical Medience Corporation, Tokyo, Japan¹; Advanced Medical Science Research Center, Mitsubishi Chemical Medience Corporation, Tokyo, Japan²

Abstract

Mepitiostane (2α,3α-epithio-17β-(1-methoxycyclopentylxylo)-5α-androstane), which is a prodrug of epitiostanol (2α,3α-epitio-5α-androstan-17β-ol), is an epitiosteroid having anti-estrogenic activity. The aim of this study was to identify a specific metabolite for the detection of mepitiostane doping using LC/ESI-MS/MS. In vitro studies with human cryopreserved hepatocytes and human embryonic stem cell (hESC)-derived hepatocytes were performed. An in vivo administration study of mepitiostane was also conducted to evaluate the usefulness of the in vitro metabolism studies. In the TOFMS experiment, the observed protonated ions in human hepatocytes and hESC-derived hepatocytes were m/z 323.2052 and 323.2045, respectively. Product ions generated from ion m/z 323 were observed at m/z 305 (MH⁻ – H₂O) and 255 (MH⁻ – H₂O – H₂O – S). The peak of interest was suggested to be a protonated epitiostanol sulfoxide (C₁₉H₃₁O₂S, theoretical m/z 323.2039). Chemical synthesis was performed and synthetic epitiostanol sulfoxide was identified by NMR analysis. Epitiostanol sulfoxide as its gluco-conjugate was identified in human urine after oral administration of mepitiostane. Epitiostanol sulfoxide, a specific metabolite with a sulfur atom was identified by in vitro and in vivo studies. Monitoring epitiostanol sulfoxide by LC/ESI-MS/MS in positive mode is suitable for the doping control of epitiostanol and mepitiostane.

Introduction

Mepitiostane (2α,3α-epithio-17β-(1-methoxycyclopentylxylo)-5α-androstane), which is a prodrug of epitiostanol (2α,3α-epitio-5α-androstan-17β-ol), is an epitiosteroid having anti-estrogenic activity (Figure 1). The aim of this study was to identify a specific metabolite for the detection of mepitiostane doping using LC/MS. The use of primary human hepatocytes is frequently established for the investigation of drug metabolism; however, the availability of the same lots is limited. Therefore, human hepatocytes prepared by differentiating human stem cells may be one of the ideal resources available [1]. In vitro studies with human cryopreserved hepatocytes and human embryonic stem cell (hESC)-derived hepatocytes were performed. An in vivo administration study of mepitiostane was also conducted to evaluate the usefulness of the in vitro metabolism studies.

Experimental

Materials

Epitiostanol, 2,(5α)-androsten-17β-ol and Thioderon® (5 mg mepitiostane) were purchased from the Society of Japanese Pharmacopoeia (Tokyo, Japan), Steraloids (Newport, RI, USA), and Shionogi (Osaka, Japan), respectively. Human cryopreserved hepatocytes (HU8072) and William’s E medium were from Life Technologies. (Tokyo, Japan). hESC-derived hepatocytes SA181(46XY) were from Cellartis AB (Gothenburg, Sweden). Epitiostanol sulfoxide was synthesized from epitiostanol using hydrogen peroxide oxidation of episulfide (30% H₂O₂/glacial acetic acid, 40 min) [2], and its chemical structure was confirmed by NMR spectroscopy.
In vitro study
Hepatocytes were attached to 24-well plates with collagen coating prior to substrate incubations in William's E Medium. Reactions (37°C, 5% CO₂) with substrate epitiostanol (100 μM) were performed during 24 h.

In vivo study
Thioderon® (10 mg mepitiostane) was administered orally to healthy male volunteers.

Sample preparation
In brief, 3 mL human urine or 200 μL of culture supernatant with 2.8 mL of distilled water was incubated with β-glucuronidase from E.coli (pH7.0, 50°C, 60 min). After target steroids were extracted (TBME, pH 9.6), extracts were dissolved in 50% CH₃OH for LC/MS.

LC/MS
The LC/MS system was an Acquity UPLC®/Synapt G2 HDMS Q/TOFMS from Waters (Milford, MA, USA). The analytical column was an Acquity UPLC® BEH C8 (2.1 mm x 50 mm, 1.7 μm), and the mobile phases were 0.1% CH₃COOH (A) and CH₃OH (B). A gradient elution was as follows: 0–1 min (A 50%), 1–5 min (A 46%), 5–5.5 min (A 30%), 5.5–7.9 min (A 24%), 7.9–9 min (A 10%), and 9.1 min (A 50%). The column temperature was 40 °C. The flow rate was 0.5 mL/min. Ionization was accomplished using ESI in positive mode. The TOF mass range varied from m/z 70 to 400 (resolution: 40000, lock mass: leucine- enkephalin). The precursor ion was m/z 323.2 (MH⁺, CV: 10 V, CE: 22 eV).

Results and Discussion
The most abundant peak (tᵢ = 2.0 min) on UPLC/TOFMS analysis was observed in the in vitro studies. The peak of interest was suggested to be a protonated epitiostanol sulfoxide (C₁₉H₃₁O₂S, theoretical m/z 323.2039). In the UPLC/MS/MS experiment, product ions generated from ion m/z 323 (MH⁺) were observed at m/z 305 (MH⁺ – H₂O) and 255 (MH⁺ – H₂O –H₂O– S) (Figure 2). As shown in Table 1, accurate masses and elemental compositions were determined. Furthermore, the peak of interest was confirmed to be epitiostanol sulfoxide by comparing it against a synthetic compound. Synthetic epitiostanol sulfoxide was identified by Bruker Avance III 400 NMR system (Rheinstetten, Germany). H-NMR(δ(C²HCl₃)) ppm at 400 MHz: 2.45 (1H, dd, CH₂-1), 1.28 (1H, m, CH₂-1'), 0.40 (1H, dt, CH-5). ¹³C-NMR(δ(C²HCl₃)) ppm at 100 MHz: 35.48 (C-1), 51.14 (C-2), 53.51 (C-3), 25.53 (C-4), and 40.48 (C-5). Differences in ¹³C chemical shifts between epitiostanol and the corresponding sulfoxide (ΔδC in ppm) were −4.80 (C-1), 16.06 (C-2), 15.45 (C-3), −5.04 (C-4), and 5.37 (C-5), respectively.
The phase II metabolite gluco-conjugate of epitiostanol sulfoxide could be detected in culture supernatant by \textit{in vitro} examination using human hepatocytes; however, the preliminary result from the hESC-derived hepatocyte study showed insufficient glucuronidation (data not shown). The \textit{in vitro} study using hESC-derived hepatocytes should be further optimized. Although the proton affinity of epitiostanol and 2,\( (5\alpha) \)-androsten-17\( \beta \)-ol is too low for ionization, the sensitivity of epitiostanol sulfoxide was much higher (Figure 3). As shown in Figure 3 and Table 1, epitiostanol sulfoxide was identified in human urine collected 8 h after oral administration of mepitiostane and it could be detected up to an investigated period of 48 h after administration.

<table>
<thead>
<tr>
<th>Precursor ion ([M+H]^+)</th>
<th>Elemental composition</th>
<th>Reference standard (m/z) (m/z) (m/z)</th>
<th>error (ppm)</th>
<th>error (ppm)</th>
<th>error (ppm)</th>
<th>error (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(m/z)</td>
<td>(m/z) (found)</td>
<td>(m/z) (found)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>323.2</td>
<td>C(<em>{19})H(</em>{26})O(_{4})S</td>
<td>323.2039</td>
<td>323.2054</td>
<td>323.2043</td>
<td>323.2055</td>
<td>323.2028</td>
</tr>
<tr>
<td></td>
<td>C(<em>{17})H(</em>{24})OS</td>
<td>305.1934</td>
<td>305.1941</td>
<td>305.1951</td>
<td>305.1938</td>
<td>305.1955</td>
</tr>
<tr>
<td></td>
<td>C(<em>{17})H(</em>{27})S</td>
<td>287.1828</td>
<td>287.1842</td>
<td>287.1832</td>
<td>287.1839</td>
<td>287.1824</td>
</tr>
<tr>
<td></td>
<td>C(<em>{19})H(</em>{25})O</td>
<td>273.2213</td>
<td>273.2212</td>
<td>273.2221</td>
<td>273.2231</td>
<td>273.2228</td>
</tr>
<tr>
<td></td>
<td>C(<em>{19})H(</em>{27})</td>
<td>255.2107</td>
<td>255.2112</td>
<td>255.2119</td>
<td>255.2113</td>
<td>255.2113</td>
</tr>
<tr>
<td></td>
<td>C(<em>{19})H(</em>{21})</td>
<td>201.1638</td>
<td>201.1645</td>
<td>201.1649</td>
<td>201.1642</td>
<td>201.1648</td>
</tr>
<tr>
<td></td>
<td>C(<em>{17})H(</em>{19})</td>
<td>199.1481</td>
<td>199.1485</td>
<td>199.1489</td>
<td>199.1492</td>
<td>199.1498</td>
</tr>
<tr>
<td></td>
<td>C(<em>{17})H(</em>{15})</td>
<td>159.1168</td>
<td>159.1172</td>
<td>159.1171</td>
<td>159.1171</td>
<td>159.1178</td>
</tr>
<tr>
<td></td>
<td>C(<em>{17})H(</em>{12})</td>
<td>145.1012</td>
<td>145.1013</td>
<td>145.1022</td>
<td>145.1018</td>
<td>145.1025</td>
</tr>
</tbody>
</table>

Table 1. Characteristic product ions of epitiostanol sulfoxide using UPLC/ESI-Q/TOF MS/MS.

![Product ion mass spectrum](image)
Conclusions

Epitiostanol sulfoxide, a specific metabolite with a sulfur atom was identified by in vitro and in vivo studies. As reported earlier [3,4], we confirmed that epitiostanol is metabolized to olefin steroid (5α-androst-2-en-17β-ol) by oxygenation and dethionylation. The common steroid screening procedure by GC/MS is suitable for the detection of mepitiostane abuse and the target metabolite should be 2,(5α)-androsten-17β-ol as a TMS derivative [4]. However, the metabolite 2,(5α)-androsten-17β-ol is an isomer of endogenous steroid 16,(5α)-androsten-3β-ol and is not so specific due to loss of a sulfur atom from epitiostanol. Monitoring epitiostanol sulfoxide by LC/MS/MS in ESI mode is suitable for the doping control of epitiostanol and mepitiostane.

References


**Acknowledgements**

The work was funded by grants from the Ministry of Education, Culture, Sports, Science and Technology (MEXT, Japan) and Japan Anti-Doping Agency (JADA).