Masato Okano[1], Tomoharu Osada[2], Mitsuhiko Sato[1], Ayako Uchiyama[2], Shinji Kageyama[1]

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, Japan[1]; Advanced Medical Science Research Center, Mitsubishi Chemical Medience Corporation, Tokyo, Japan[2]

Abstract

Mepitiostane (2α,3α-epithio-17β-(1-methoxycyclopentyloxy)-5α-androstane), which is a prodrug of epitiostanol (2α,3α-epitio-5α-androstane-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 ($MH^+ - H_2O$) and 255 ($MH^+ - H_2O - H_2O - S$). The peak of interest was suggested to be a protonated epitiostanol sulfoxide ($C_{19}H_{31}O_2S$, 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-methoxycyclopentyloxy)-5α-androstane), which is a prodrug of epitiostanol (2α,3α-epitio-5α-androstane-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% H_2O_2/glacial acetic acid, 40 min) [2], and its chemical structure was confirmed by NMR spectroscopy.

 ISBN 978-3-86884-038-4

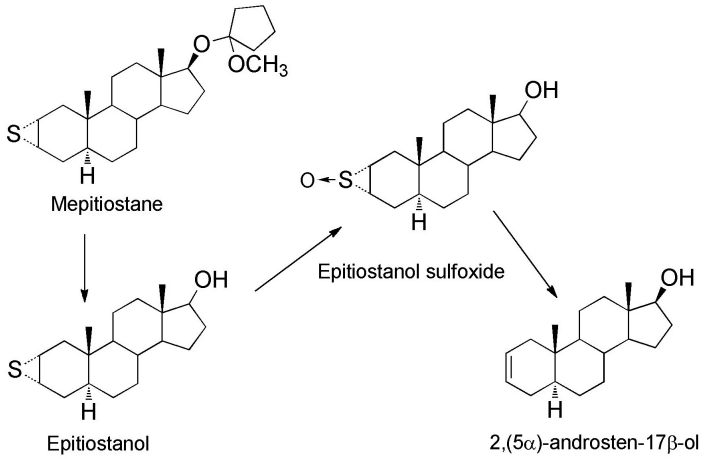

Figure 1. Metabolism of mepitiostane.

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% CO_2) 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% CH_3OH 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% CH_3COOH (A) and CH_3OH (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 (t_R = 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 ($C_{19}H_{31}O_2S$, 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 ($MH^+ - H_2O$) and 255 ($MH^+ - H_2O - H_2O - S$) (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). ^{1}H-NMR[$\delta_H(C^2HCl_3)$ ppm at 400 MHz]: 2.45 (1H, dd, CH_2-1), 1.28 (1H, m, CH_2-1'), 0.40 (1H, dt, CH-5). ^{13}C-NMR[$\delta_C(C^2HCl_3)$ 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 ^{13}C chemical shifts between epitiostanol and the corresponding sulfoxide ($\Delta\delta_C$ 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.

 ISBN 978-3-86884-038-4

The phase II metabolite gluco-conjugate of epitiostanol sulfoxide could be detected in culture supernatant by *in vitro* examination using human hepatocytes; however, the preliminary result from the hESC-derived hepatocyte study showed insufficient glucuronidation (data not shown). The *in vitro* study using hESC-derived hepatocytes should be further optimized. Although the proton affinity of epitiostanol and 2,(5α)-androsten-17β-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.

Precursor ion $[M+H]^+$	Elemental composition	Product ion								
			Reference standard		*in vitro* hESC-derived hepatocytes		*in vitro* human hepatocytes		*in vivo* post 8h	
m/z		*m/z* (theor.)	*m/z* (found)	error (ppm)	*m/z* (found)	error (ppm)	*m/z* (found)	error (ppm)	*m/z* (found)	error (ppm)
323.2	$C_{19}H_{31}O_2S$	323.2039	323.2054	4.6	323.2043	1.2	323.2055	5.0	323.2028	-3.4
	$C_{19}H_{29}OS$	305.1934	305.1941	2.3	305.1951	5.6	305.1938	1.3	305.1955	6.9
	$C_{19}H_{27}S$	287.1828	287.1842	4.9	287.1832	1.4	287.1839	3.8	287.1824	-1.4
	$C_{19}H_{29}O$	273.2213	273.2212	-0.4	273.2221	2.9	273.2231	6.6	273.2228	5.5
	$C_{19}H_{27}$	255.2107	255.2112	2.0	255.2119	4.7	255.2113	2.4	255.2113	2.4
	$C_{15}H_{21}$	201.1638	201.1645	3.5	201.1649	5.5	201.1642	2.0	201.1648	5.0
	$C_{15}H_{19}$	199.1481	199.1485	2.0	199.1489	4.0	199.1492	5.5	199.1498	8.5
	$C_{12}H_{15}$	159.1168	159.1172	2.5	159.1171	1.9	159.1171	1.9	159.1178	6.3
	$C_{11}H_{13}$	145.1012	145.1013	0.7	145.1022	6.9	145.1018	4.1	145.1025	9.0

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

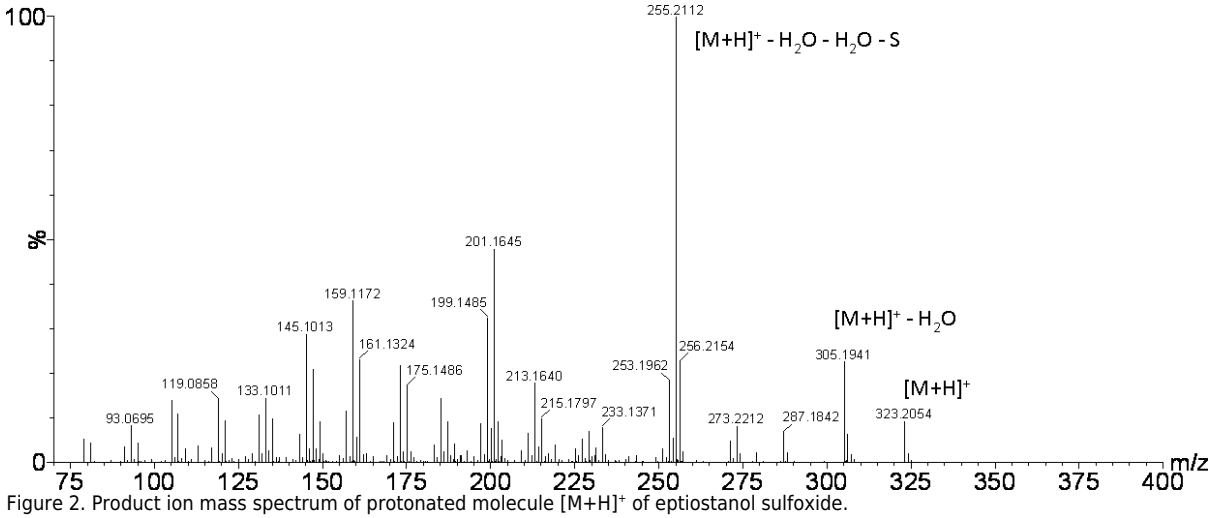

Figure 2. Product ion mass spectrum of protonated molecule $[M+H]^+$ of eptiostanol sulfoxide.

ISBN 978-3-86884-038-4

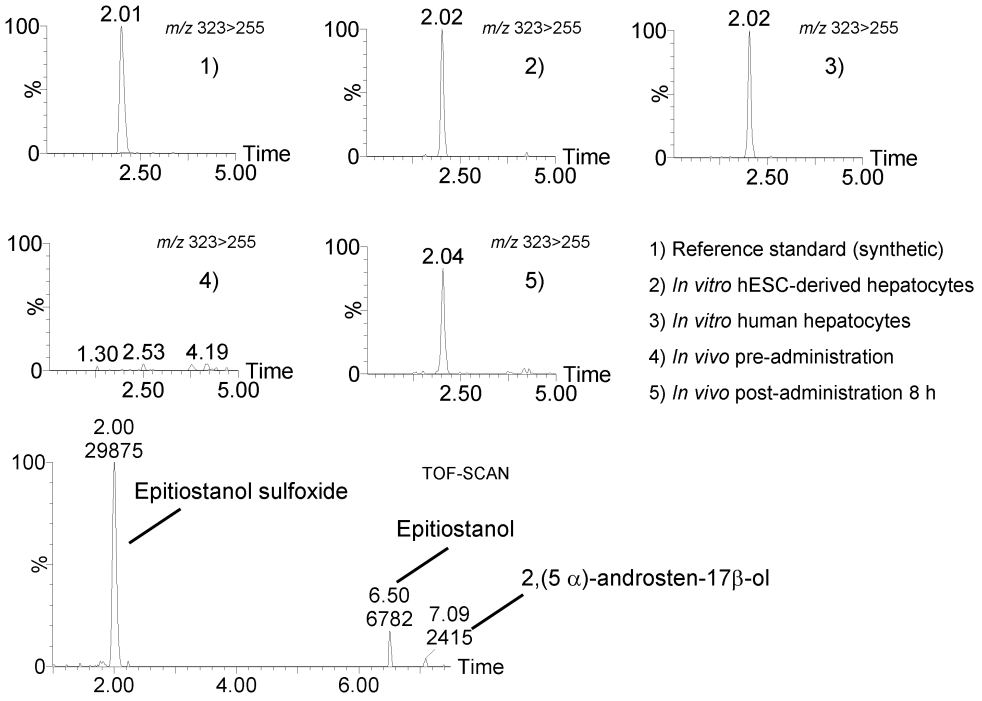

Figure 3. Product ion mass chromatogram of epitiostanol sulfoxide (upper and middle). Comparison of ESI TOFMS sensitivity (lower, each 6 ng injection).

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

[1] Sartipy P, Björquist P, Strehl R, Hyllner J. (2007) The application of human embryonic stem cell technologies to drug discovery. *Drug Discov. Today* **12**, 688-699.
[2] Golchoubian H, Hosseinpoor F. (2007) Effective oxidation of sulfides to sulfoxides with hydrogen peroxide under transition-metal-free conditions. *Molecules* **12**, 304-311.

[3] Ichihashi T, Kinoshita H, Shimamura K, Yamada H. (1991) Absorption and disposition of in rats (1): Route of administration and plasma levels of epitiostanol. *Xenobiotica* **21**, 865-872.
[4] Okano M, Ikekita A, Sato M, Kageyama S. (2007) Detection of mepitiostane in doping analysis. In: Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) *Recent Advances in Doping Analysis (15)*, Köln, pp 123-132.

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).