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Detection of Mepitiostane in Doping Analysis

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Introduction

Hematopoetics, anabolic androgenic steroids used in doping purposes are prohibited by WADA. Table-1 shows most of the clinically used hematopoietics in Japan except for iron preparations [1], our laboratory already has readied analytical methods for EPO and almost anabolic steroids, however, analytical methods have not been conducted for mepitiostane and epitiostanol.

Table-1 Hematopoietics used clinically in Japan [1]

Commercial name	Active compound
Epogin, Exprex	Erythropoetin alfa
Espo, Recomon	Erythropoetin beta
Aranesp	Darbepoetin alfa
Hemataide	Synthetic peptide
Mircera	Erythropoietin receptor activator
Duran	Nandrolone
Duramin	Nandrolone cyclohexylpropionate
Deca Durabolin, Deca Duramin	Nandrolone decanoate
Durabolin	Nandrolone phenylpropionate
Mesanolon	Mestanolone
Primobolan	Metenolone
Thiodol	Epitiostanol
Thioderon	Mepitiostane

(except for sideropenia anemia medicine)

Mepitiostane (Thioderon®, 2α, 3α-epithio-17β-(1-methoxycyclopentyloxy)-5α-androstane) -17β-ol), the prodrug of epitiostanol (2α, 3α-epithio-5α-androstane-17β-ol) is an epithiosteroid having anti-estrogenic activity, a concomitant weak androgenic and myotropic activity, and have been used in the treatment of mammary cancer. Mepitiostane and epitiostanol increase hemoglobin in the circulation by stimulating the maturation of CFU-E in

the bone marrow. Thioderon® was developed and produced by Shionogi Pharmaceutical Co. Ltd. (Osaka, Japan) [2]. Production of Thiodol® was discontinued in 2001, however, Thiodol® has been therapeutically used for the same purposes as Thioderon®. After oral administration of mepitiostane, it is metabolized to the active hematopoetic compound epitiostanol via detachment of methoxycyclopentyloxy from the 17th position and epitiostanol is metabolized to 2,(5α)-androsten-17β-ol (M-1) and 2, (5α)-androsten-17-one (M-2) mainly by oxygenation (Fig.1) [3, 4].

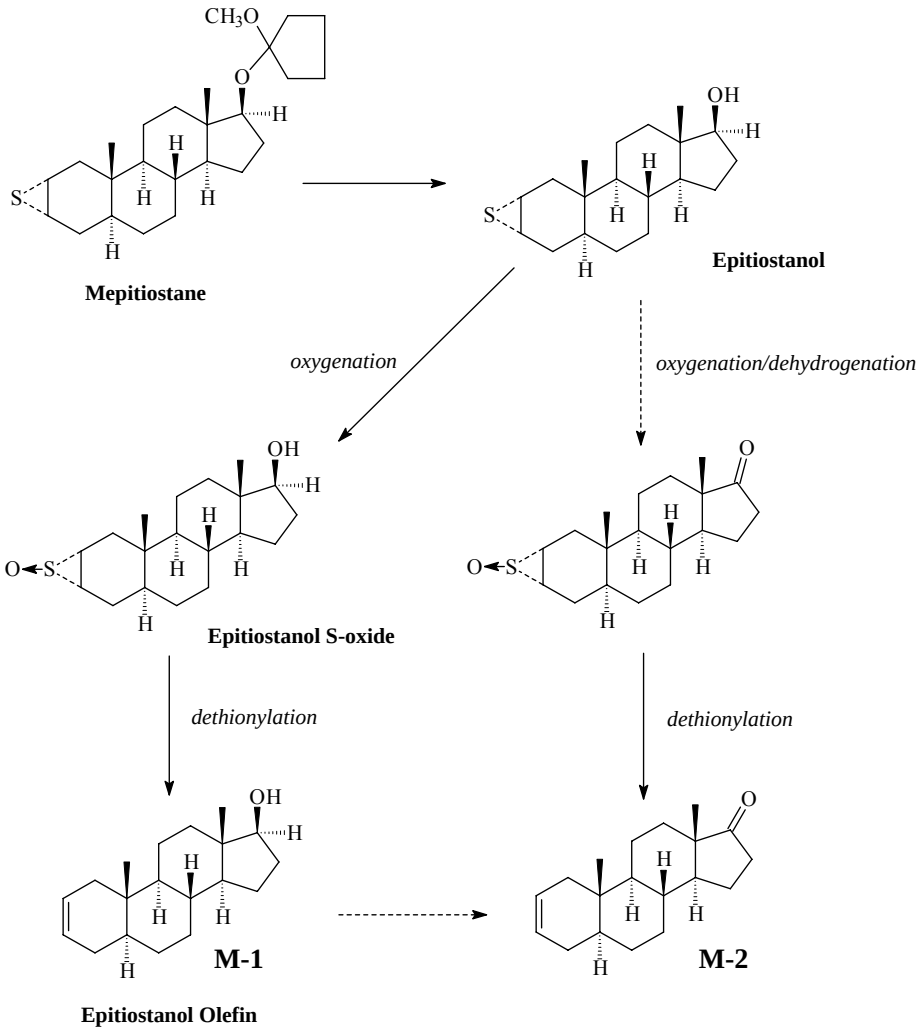

Fig.1 The metabolic pathway of mepitiostane[3, 4]

Mepitiostane and epitiostanol are not mentioned in the WADA prohibited List in 2007[5]. There were no reports concerning the abuse of this substance by athletes, however, it is possible that some athletes use them for doping purposes. Therefore the aim of this study was to establish adequate GC/MS and LC/MS parameters for the screening of mepitiostane and also to start the screening in routine doping tests.

Chemicals

All reagents were analytical grade, acetic acid, hydrochloride, diethyl ether, ethanol, potassium carbonate anhydrous, formic acid, sulphuric acid, ethyl acetate, sodium acetate trihydrate, sodium sulfate, sodium sulfate anhydrous and HPLC grade methanol were purchased from Kokusan Chemical Works (Tokyo, Japan). Dithioerytritol was purchased from Nakalai Tesuque (Osaka, Japan), iodotrimethylsilane (TMSI) was purchased from Aldrich (Steinheum, Germany) and N-methyl-N-trimethylsilylfluoroacetamide (MSTFA) was purchased from Marchery Nagel (Düren, Germany). XAD-2 and Bond Elut C_{18} 3 cc/500 mg were products of Sigma-Aldrich Japan (Tokyo, Japan) and Varian (Walnut Creek, CA, USA). Glufatase type A-2(β-glucuronidase; arylsulfatase = 42,000 Fishman U: 21,000 Roy U per ml each) from Ampullaria was purchased from Nippon Bio-Test (Tokyo, Japan), β-glucuronidase from E.coli (80 unit/ml) was purchased from Boehringer Manheim (Düsseldorf, Germany). Thioderon® and mepitiostane were purchased from Shionogi Pharmaceutical Co. Ltd. (Osaka, Japan). Epitiostanol was from Society of Japanese Pharmacopoeia (Tokyo, Japan).
2, (5α)-androsten-17β-ol and 2, (5α)-androsten-17-one were purchased from Steraloids Inc. (Newport, RI, USA).

Sample preparations

2 mL of human urine sample was applied to a solid-phase an Amberlite XAD-2. And the solid-phase was washed with 2 mL of distilled water. Elution was performed with 2.5 mL of methanol. The elute was dried under nitrogen stream at 40 °C. The dried residue was dissolved in 1 mL of 0.2 M sodium acetate buffer (pH 5.2) followed by addition of 50 μL of internal standard solution (20 μg/ml 17α-methyltestosterone). Hydrolysis was performed with 30 μL of Glufatase type A-2 at 60 °C for 1 hr. After addition of 0.25 mL of 7 % K_2CO_3, the steroids were extracted with 5 mL of diethyl ether. The organic layer was evaporated under nitrogen stream at 40 °C. The dried residue was dissolved in 50 μL of MSTFA/TMSI/DTE, 1000/2/2(v/v/w) and heated at 60 °C for 15 min for GC-MS analyses and was dissolved in 1 % CH_3COOH/CH_3CN: 65/35(V/V) for LC/MS analyses.

Instrumentation and GC-MS and LC-MS parameters

The GC-MS system was an Agilent 6890/5973 inert mass equipped with an Ultra-1 capillary column, length 17 m, 0.25 mm internal diameter, 0.11 μm film thickness (Agilent Technologies, Palo Alto, CA, USA). The LC-MS experiments were performed using an

Agilent HPLC 1100 Series (Agilent Technologies, Palo Alto, CA, USA), which was interfaced to a QSTAR XL MS/MS (Applied Biosystems, Foster City, CA, USA). The analytical column was a Supelco Discovery C_{18} column (id = 4.0 mm; length = 50 mm). Table-2 shows the detailed analytical parameters of GC-MS and LC-MS system.

Excretion study

Two capsules of Thioderon® containing mepitiostane 10mg were administered to a 37-year-old healthy male volunteer. Blank sample was collected before an oral administration. Urine samples obtained were collected for the first 49 hours after the administration to detect target metabolites of mepitiostane.

Table-2 GC-MS and LC-MS parameters

GC/MS system		
Injection parameters:	Volume: 2 µL, Temp.: 280 °C Split mode (11:1)	
Carrier gas:	Helium at constant flow rate of 1mL/min.	
Oven temp. program:	Initial 180 °C hold 1 min, 3 °C/min to 229 °C and 40 °C/min to 300 °C, hold 2 min	
Ionisation:	70 eV, electron impact (EI)	
Data acquisition:	Scan range 70 to 500, SIM mode	
Monitored ions and dwell time:	129, 256 and 346 m/z; 10, 20, 25 mesec.respectively	
LC/MS system		
Mobile phase:	A: 1 % CH_3COOH, B: CH_3CN, Flow rate: 250 µL/min Temp: 25 °C	
Gradient:	0 min - 10 min: A 65 % to 10 %, 10.1 min: A 65 %	
Run time:	11 min	
Ionization condition(Polarity):	ESI (Positive mode)	APPI (Positive mode)
Ion spray Temp.:	450 °C	400 °C
Ion spray Voltage:	5,500 V	1,300 V
Neblizer Gas:	2.85 L/min(Zero Air)	3.94 L/min(N_2)
Dopant:		Toluene 20 µL/mim
Aux. Gas:	4.80 L/min(Zero Air)	2.00 L/min(N_2)
TOF MS range:	m/z 140 to 600	m/z 140 to 600

Results and Discussion

The total ion chromatograms and mass spectrum of TMS-derivatives of epitiostanol, M-1 and M-2 are shown in Fig.2. The relative retention time of eptitiostanol, M-1 and M-2 were 0.93, 0.43 and 0.41 respectively. The typical fragment ions of epitiostanol-O-TMS were at m/z 378(M^+), 346(M^+ -S) and 129 (D-ring fragment) , those of M-1 as TMS derivative were at m/z 346(M^+), 256(M^+ -TMSOH) and 129 (D-ring fragment) and those of M-2 as TMS derivative

were at m/z 344(M^+), 329(M^+ -CH_3) and 169 (D-ring fragment).

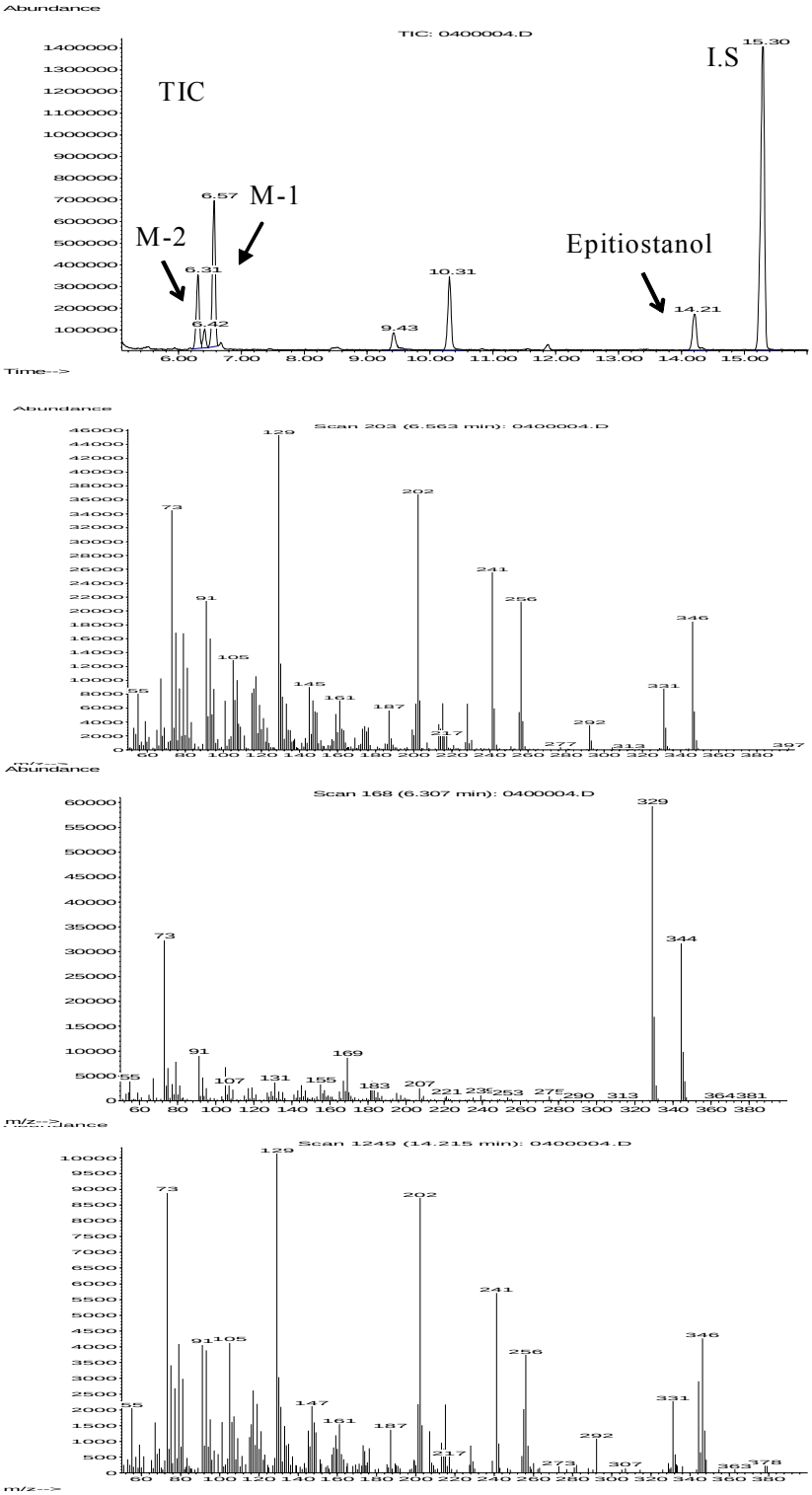

Fig.2 The total ion chromatograms and mass spectrum of standard mixture (TMS-derivatives of epitiostanol, M-1 and M-2)

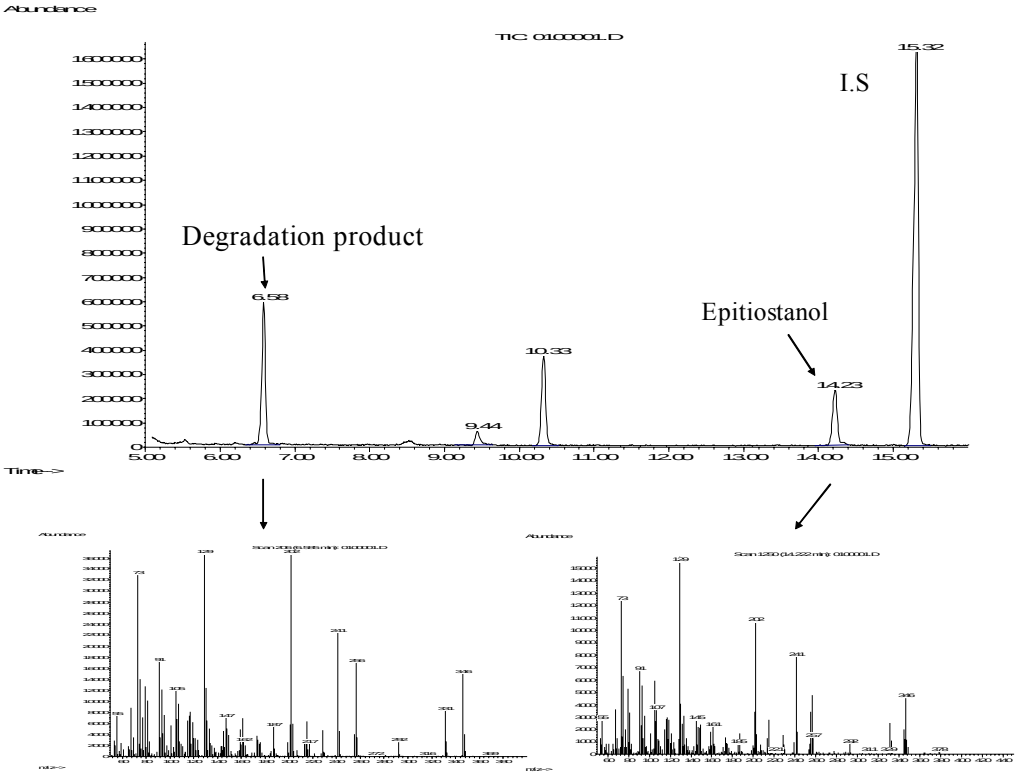

Fig.3 TIC and the mass spectrums obtained after delivatization of epitiostanol

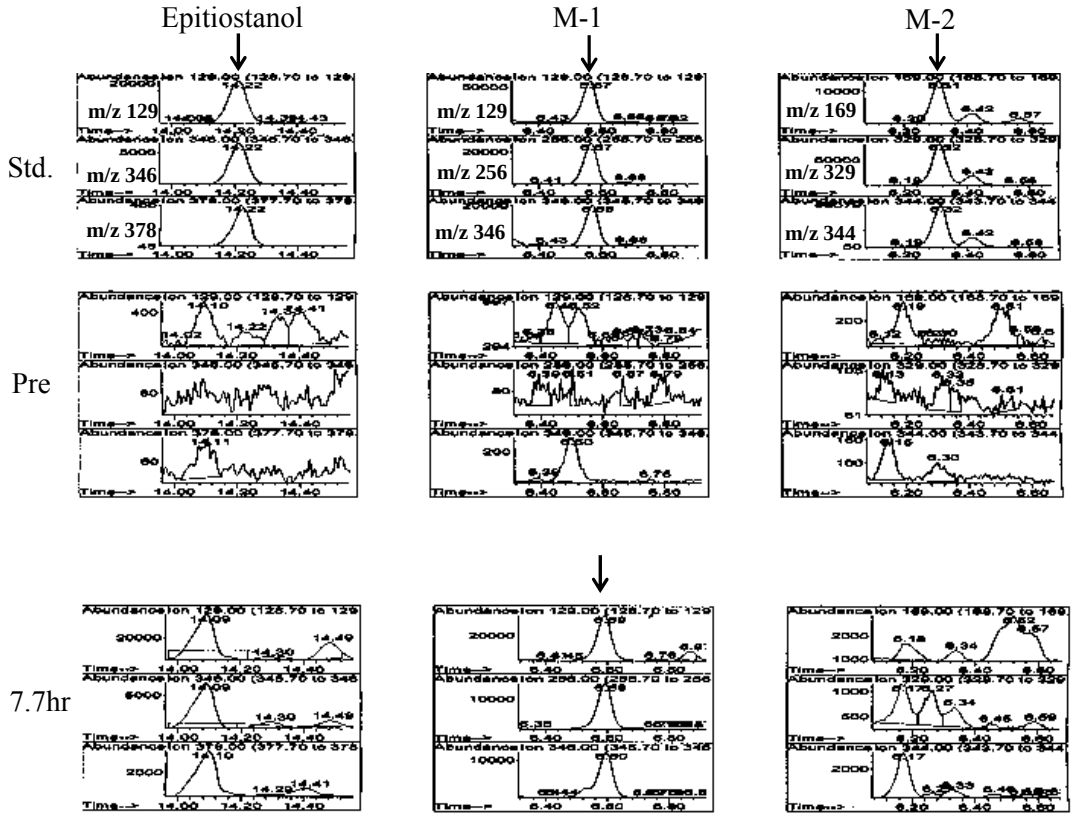

Fig. 4 Detection of mepitiostane metabolites by GC/MS

The peak at the relative retention time of 0.43 was also observed on the total ion chromatogram of epitiostanol, and its mass spectrum agreed with that of M-1. Therefore, our interpretation is that epitiostanol was pyrolized to M-1 during the derivatization or GC/MS analysis (Fig.3).

Metabolite M-1 could be detected in the urine sample collected 7.7 hours after a 10 mg oral administration of mepitiostane (Fig.4), however, epitiostanol and M-2 could not be detected.

Table-3 Metabolites of mepitiostane and their proposed TMS derivatives

	Chemical name	Derivatives	MW
Epitiostanol	2α,3α-epitio-5α-androstan-17β-ol	mono-TMS	378
M-1	2,(5α)-androsten-17β-ol	mono-TMS	346
M-2	2,(5α)-androsten-17-one	mono-TMS	344
M-3	2α-hydroxy-epi-androsterone	tris-TMS	522
M-4	2β,16β-dihydroxy-androsterone	tris-TMS	538
M-5	2-keto-epi-androsterone	tris-TMS	520
M-6	5α-androstan-2β,3α,17β-triol	tris-TMS	524
M-7	2β-OH-epiandrosterone	tris-TMS	522
M-8	2β-OH-androsterone	tris-TMS	522
M-9	5α-androstan-2β,3α,17β-triol-16-one	tetra-TMS	610

The other metabolites of mepitiostane are shown in Table-3[3, 4], but the reference standards are not available in our laboratory. The ions m/z 522 and 520 are extracted from full scans, and four peaks could be observed in the administrated urine sample (Fig.5). These peaks were not observed in the urine before the administration. It may be suggested that these were metabolites of mepitiostane listed in Table-3.

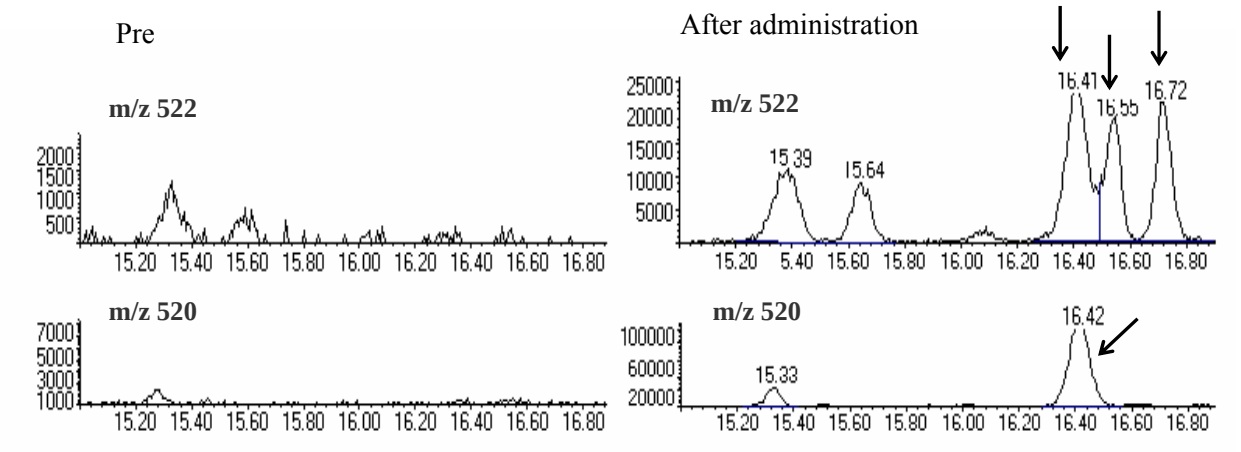

Fig. 5 Other proposed metabolites of mepitiostane

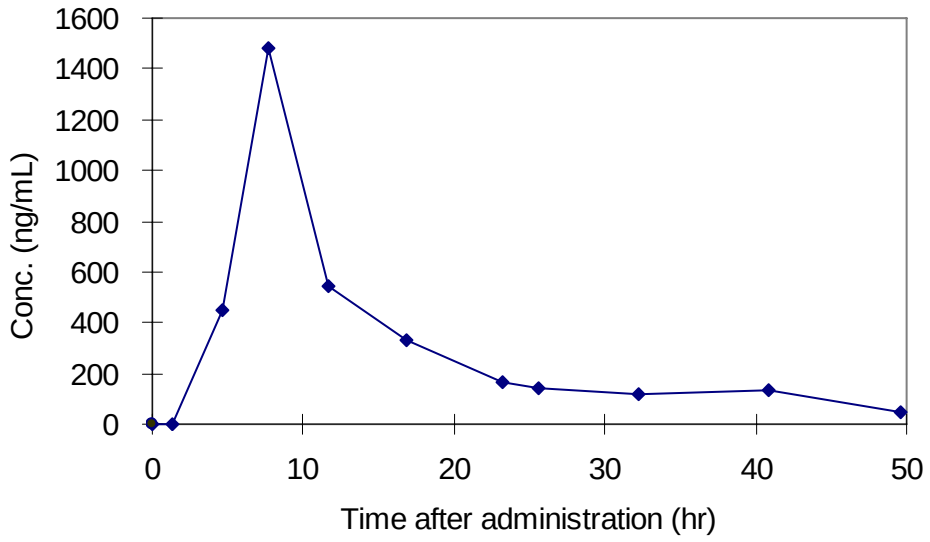

Fig.6 Urinary concentration of 2, (5α)-androsten-17β -ol (M-1) after administration of mepitiostane

M-1 was detected up to 49 hours after a 10 mg administration of mepitiostane (Fig.6). Epitiostanol and M-2 were not detected over a time period of 49 hours.
Furthermore, the metabolic profile of mepitiostane was investigated according to Axelson's ion-exchange method [6]. The result showed that M-1 was excreted as its glucuronide.

Table-4 Concentrations of endogenous steroids after administration

Time course	0'00"	1'20"	4'40"	7'40"	11'40"	16'50"	23'10"	25'40"	32'15"	40'50"	49'35"
Androsterone	508	1149	1531	2158	1247	997	1151	1240	1743	1556	1200
Etiocholanolone	756	1527	1884	2595	1400	1410	1610	1802	2131	1965	1655
Testosterone	6.0	5.6	3.2	3.8	3.8	2.9	3.7	4.0	4.2	5.3	4.8
Epitestosterone	8.4	6.9	10.9	13.7	8.6	6.2	11.3	7.8	12.3	11.0	12.9
5α-Dihydrotestosterone	0.0	0.4	0.7	1.5	1.0	1.3	0.7	1.3	1.2	1.8	1.6
5β-androstan-3α,17βdiol	12.4	29.1	37.9	45.5	38.4	32.6	29.0	30.4	36.3	56.4	33.8
5α-androstan-3α,17βdiol	10.0	20.0	33.2	32.0	28.0	38.3	22.8	22.7	35.3	49.4	39.0
Dehydroepiandrosterone	70.8	136.0	107.4	192.4	154.5	126.1	137.3	190.5	193.8	222.5	203.7
6-OH-Androstenedione	3.2	3.6	1.6	1.7	1.8	1.7	2.0	2.8	2.0	2.8	2.5

Unit: ng/mL

Steroid profiles after administration are shown in Table-4 in order to investigate the effects of anti-estrogen substances on steroid parameters. It seems that concentrations of androsterone and etiocholanolone were increased by the administration, however, we are not able to judge

whether it was significantly influenced by the administration. Further excretion studies (more volunteers and multiple dosages) must be performed.

In addition, it was necessary to perform LC/MS identification in order to verify the existence of epitiostanol in the urine after administration. Epitiostanol, M-1 and M-2 could not be detected by our screening procedure for heat labile compounds (THG and gestrinone) by LC/MS analysis in positive ESI mode, because the proton affinity of the metabolites is too low for ionization. Atmospheric photo spray ionization (APPI) is known to be more effective for non-polar compounds than ESI. Hence, LC/MS analysis in APPI mode was performed (Fig.7). The monitoring ions of epitiostanol, M-1 and M-2 were m/z 289 $[M+H-H_2O]^+$, 257 $[M+H-H_2O]^+$, and 255$[M+H-H_2O]^+$ respectively. Mepitiostan was detected successfully as epitiostanol over a time period of 4.7 hours after the administration. M-1 could be detected 7.7 hours after the administration.

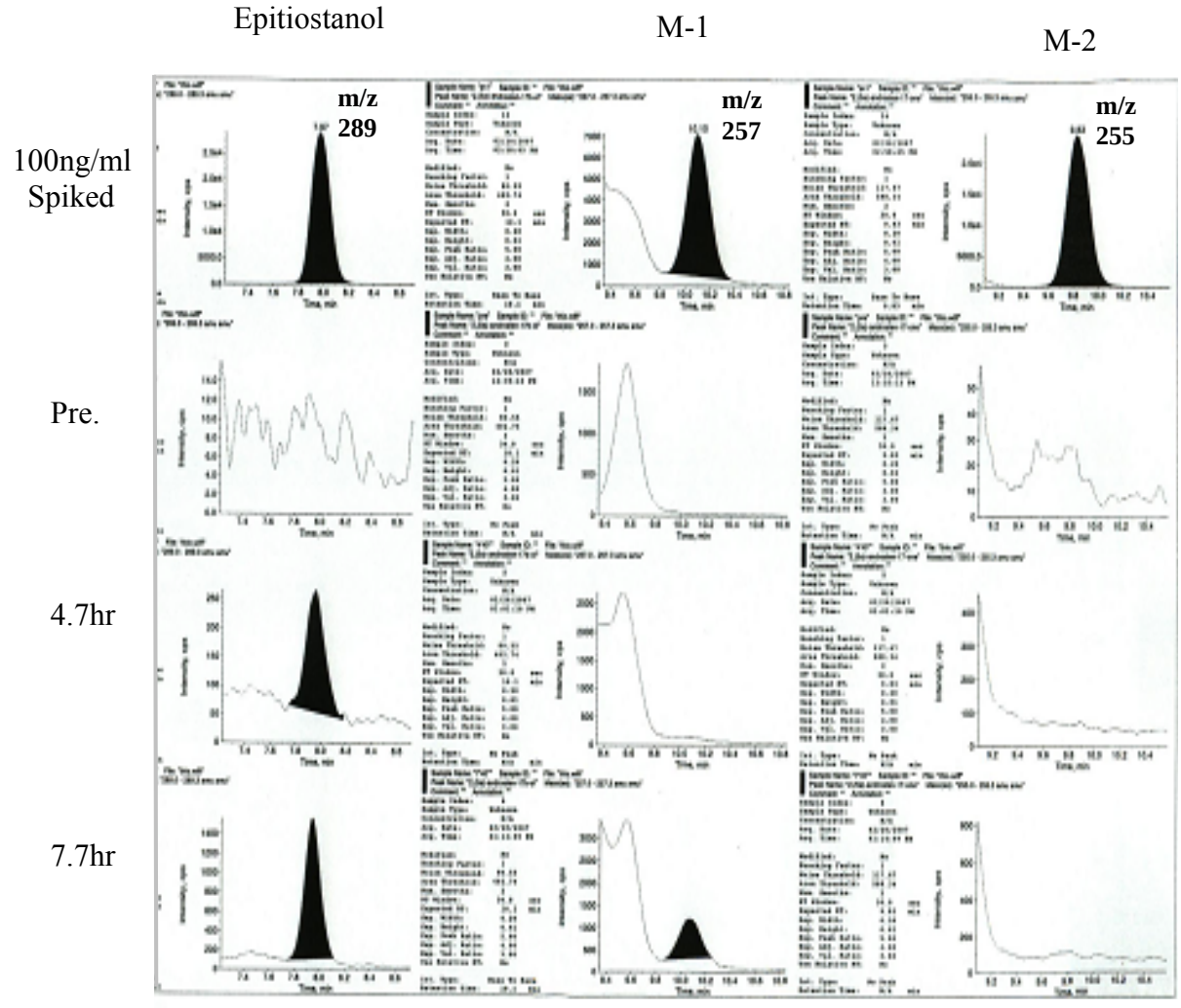

Fig.7 Detection of metabolites of mepitiostane by LC/APPI (+)-TOFMS

Conclusion

The common steroid screening procedure by GC/MS is suitable for the detection of mepitiostan abuse, and then the target substance should be 2, (5α)-androsten-17β-ol as TMS derivative. The detection of specific metabolite epitiostanol by LC/MS in APPI-mode could be proposed for the confirmation analysis. The mentioned analytical methods for the hydroxy-metabolites are still in investigation.

References

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