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The Excretion Study of 1,4,6-Androstatriene-3,17-dione(ATD) and its Metabolite in Human Urine

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1,4,6-androstatriene-3,17-dione (ATD), the target compound, is known to inhibit the aromatization of androgens to estrogens by a process called aromatization.[1] *In vivo*, an injection of ATD caused inhibition of ovarian aromatase and reduced estrogens secretion in pregnant mare's serum gonadotropin-primed rats for at least 24 hr after injection.[2] ATD was used for sequential cancer drug due to the estrogen,[3] but if abused, an occurring side effect could be bisexuality[4] and sexual dysfunction.[5] Because the ATD drug can be easily purchased on the internet for muscle and endurance growth in an athlete, it can be easily misused. Therefore, it was included as a banned drug at the International Natural Bodybuilding and Fitness Federation (INBF) in 2006 year.[6] Even if not listed the World Anti-Doping agency (WADA) in 2008, classifies the administration of ATD in sports as doping due to the aromatase inhibiting properties. Even though, Parr *et al* presented the metabolite study of ATD on Manfred Donike workshop (25th Cologne workshop on Dope Analysis),[7] The time that ATD and ATD-M remained in human body were investigated through the result of investigating the excretion of ATD and its metabolite. Especially, the time that chemicals were all consumed could be assumed as the time that the metabolites were not detected.

ATD (1,4,6-androstatriene-3,17-dione) and methyltestosterone (used as an internal standard) were purchased from Sigma Chemical Co. (MO, USA). ATD tablets were purchased from Serious Nutrition Solution, LLC (Danville, VA, USA).

The LC/TOF-MS system consisted of an HP 1100 series LC with an electrospray ionization (ESI) source from Agilent (Palo Alto, CA, USA) and TOF-MS from LECO (St. Joseph, MI, USA) was used for the separation of the sample suspect molecular weight of metabolites. The LC separation was performed using a Capcell Pak C_{18} column (150 mm × 2.0 mm i.d., particle size 5 μm) (Shiseido, Chuoku, Japan) at a flow rate of 0.2 mL/min. Aqueous ammonium acetate (2 mM) and acetonitrile were selected as mobile phase solvents. A gradient program was used ; the percentage of organic solvent was linearly changed as follow: 0 min, 20%; 3 min, 40%; 20 min, 80%; 25 min, 20%. An amount of 10 μL of sample was injected into the system. The colume temperature was maintained at 40 °C. TOF-MS equipped with an electrospray source operated in the positive ion mode. The working parameters for the ESI source were the following. The desolvation gas used N2 at 6000 cc/min and the desolvation gas temperature was maintained at 300 °C. The settings for the nebulizer were 300 kPa. The mass scan rate was 3.13 spectra/min from *m*/*z* 100 to *m*/*z* 300.

6890N GC from Agilent with TOF-MS from LECO was used for detecting suspected metabolites. 6890N GC-5975 MSD from Agilent was used for the observation of excretion studies for ATD and the suspected metabolites. The column for GC was an Ultra-2 cross-linked 5% phenylmethylsiloxane capillary column (25 m × 0.2 mm i.d., 0.33 μm film thickness). The temperature of the transfer line and inlet were 280 °C. The He was used as carrier gas at a flow rate of 1.4 mL/min. An amount of 2 μL of sample was injected into the system. The oven temperature program was from 180 °C (hold for 1 min) to 245 °C at 15 °C/min , then to 260 °C at 3 °C/min, then 320 °C at 20 °C/min (hold for 2 min).

The TOF-MS from LECO condition was as follows: The mass scan rate was 10 spectra/min from *m*/*z* 50 to *m*/*z* 500. The ion source temperature was 230 °C.

The 6890N GC-5975 MSD from Agilent was as follows: The MS was in SIM (selective ion monitoring) mode. The ion source temperature was 230 °C.

A healthy male volunteer (age 28, weight 63 kg) was orally dosed with one tablet of Inhibit- E™ which was purchased from Serious Nutrition Solutions (Danville, VA, USA). There are 25.00 mg of ATD and other ingredients in which microcrystalline cellulose, dicalcium phosphate, magnesium stearate, stearic acid in Inhibit- E™. Urine samples, 60 ~ 300 mL, were collected during 48 hours, and were kept at 2 ~ 3 °C until analysis. All the donation process was accepted with volunteer.

The extraction was based on a method for anabolic steroids in doping control test procedure. A pasteur pipette was filled with aqueous Serdolite® PAD-1 resin until a bead height of 1.5 cm was achieved. The column was washed with 2 mL of distilled water and then 5 mL of a urine sample spiked with methyltestosterone (50 μL, 10 μg mL^{-1}) as the internal standard was passed through the column. The adsorbed sample was washed with 2 mL of distilled water and eluted with 4 mL of methanol. The collected solution was evaporated with a rotary evaporator, and the residue was dissolved in 1 mL of phosphate buffer (0.2 M, pH 7.0). In order to hydrolyze the glucuronide conjugate, β-glucuronidase (50 μL) was added and the solution was heated at 55 °C for 1 hour. After hydrolysis and cooling to room temperature, 5 mL of *n*-hexane was added and then shaken for 10 min and centrifuged at 450 g for 5 min. The solution was stored in a freezer (−30 °C) and the organic layer was transferred to another test tube and then dried at 40 °C under a gentle stream of nitrogen. The residue was dissolved in 200 μL of methanol, and 10 μL of this was injected into the LC/ESI-MS system.

For confirmation of the target compound by GC/MS, the residue was dried in a vacuum desiccator over P_4O_{10}/KOH for 30 min. To make the trimethylsilyl (TMS) ethers, the dried re-

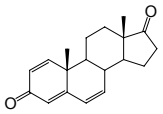

Figure 1. Structure of 1,4,6-androstatriene-3,17-dione.

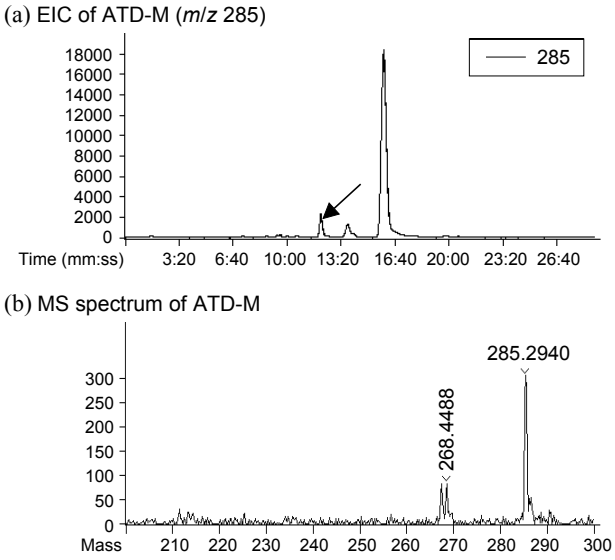

Figure 2. Extracted ion chromatograms and MS spectra of ATD-M in dosed urine by LC/ESI-MS.

sidue was dissolved in 50 μL of MSTFA/NH_4I/DTE (500:4:2, w/w/w) and heated for 30 min at 60 °C, and 2 μL of this was injected into the GC/MS system.

A calibration curve of ATD was needed for the quantitative analysis of the excretion study after administration. Standard and internal standard were added to 5 mL blank urine and determinated by GC-MSD after trimethylsilylation. The concentration was 0.1, 0.2, 0.5, 1.0, 2.0 μg mL^{-1}. Regression line was $y = 0.1996x - 0.0098$, and correlation coefficients (r^2) was 0.9973. The recovery of ATD is 98% and reproducibility of ATD is 4% at pH 6.5 with *n*-hexane for LLE solvent.

The molecular weight of ATD is 282.16 Da and its structure is shown in Figure 1. The urinary metabolism of the irreversible aromatase inhibitor ATD was investigated by Parr *et al.*,[7] and it is mainly excreted unchanged and as its 17β-hydroxy analogue (ATD-M). We tried to detect ATD and ATD-M using LC/ESI-MS, $[M+H]^+$ ion (*m*/*z* 283) of ATD was mainly produced in ESI positive mode. Extracted ion chromatograms (EIC) and mass spectra of ATD-M in dosed urine are shown in Figure 2. In Figure 2(d), [M+H]+ ion (*m*/*z* 285) and $[M+H-H_2O]+$ ion (*m*/*z* 267) of ATD-M were detected. From these results, the molecular weight of ATD-M was assumed 284 Da.

GC/TOF-MS were used to confirm the suspected metabolite of ATD after derivatization.

GC/TOF-MS was applied and the fragment ions were detected at *m*/*z* 413, 207, 323 *etc* in Figure 3.

The urinary excretion of ATD and ATD-M in human urine, 48 hr after oral administration of 25 mg of ATD drug, was observed by GC/MSD SIM mode. The excretion profiles of ATD and ATD-M from dosed urine are shown in Figure 4. ATD and ATD-M have a maximum level of excretion at 14 hr and 16 hr, respectively, after the oral dose. The total amount to be excreted of ATD was about 15.23 mg in urinary volume 2.6 L during 48 hr. We could not quantitative analysis for ATD-M in human urine because we could not get the standard.

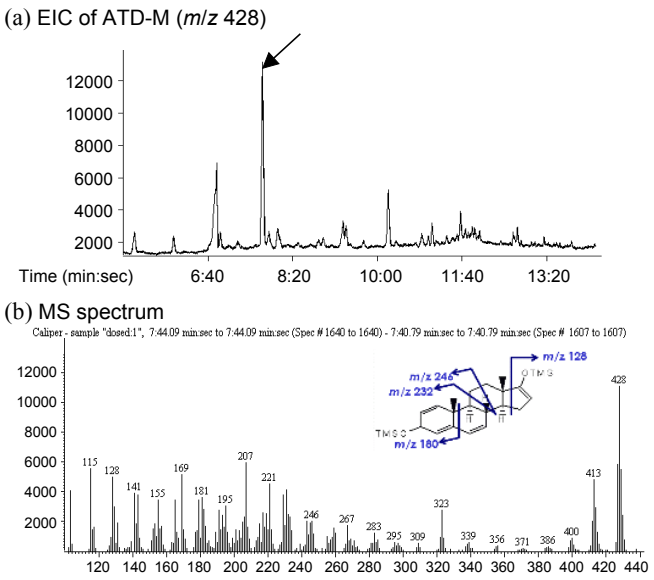

Figure 3. Extracted ion chromatograms and MS spectrum of ATD-M in dosed urine by GC/TOF-MS.

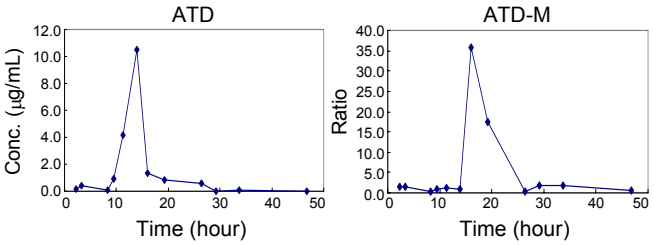

Figure 4. Excretion profiles of ATD and ATD-M.

We have studied the excretion of ATD and its metabolite after oral dose in human urine, and evaluated the characterization method for ATD and its metabolite in human urine. We could detect ATD-M by LC/TOF-MS and the molecular weight was suspected 284 Da. After then, we applied the GC/TOF-MS for confirmation of ATD-M with trimethylsilylation. In the excretion profile study, the maximum level of ATD and ATD-M was at 14 hr and 16 hr, respectively. And the total amount to be excreted of ATD from 25.00 mg does was about 15.23 mg in urinary volume 2.6 L during 48 hr.

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