

## Determination of Adrenosterone and its Metabolites in Human Urine by LC/APCI/MS and GC/MS

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This study was done for the determination and excretion profile of adrenosterone and its metabolites in human urine using both liquid chromatography with atmospheric pressure chemical ionization mass spectrometry and gas chromatography with mass spectrometry. Adrenosterone and its two metabolites were detected in human urine after administration a healthy volunteer with 75 mg of adrenosterone. We found that adrenosterone-M1 (C<sub>19</sub>H<sub>26</sub>O<sub>5</sub>) was a reduction and adrenosterone-M2 (C<sub>19</sub>H<sub>26</sub>O<sub>4</sub>) was a hydroxylation at C-ring, which did not know the exact position of the C-ring. The adrenosterone parent was detected by GC/TOF-MS, but not detected by LC/APCI/MS because of low intensity. Adrenosterone and its two metabolites were excreted as their glucuronidated fractions. The recovery of this method ranged from 100.7 to 118.4% and the reproducibility and accuracy test were 85.5 to 112.0% and 1.1 to 8.4%, respectively. The excretion studies showed that adrenosterone and its metabolites were detectable in human urine during a 48 h period after oral administration, with maximum level of excretion at 4.1 h. The glucuro-/sulfa-conjugated ratio of adrenosterone, M1 and M2 was 0.73 ± 0.03, 0.96 ± 0.06 and 0.89 ± 0.03 (n = 6), respectively. The amounts of adrenosterone excreted in urine were 14.75 ng for 48 h. Also, the maximum level of androsterone and 11β-hydroxy androsterone, which were endogenous steroids, were reached 4.1 h after the oral administration of adrenosterone.

**Key Words:** Adrenosterone, Metabolites, LC/APCI/MS, GC/MS

### Introduction

Anabolic-androgenic steroids (AAS) have been widely used as therapeutic agents for restoring muscle strength and increasing muscle size.<sup>1,2</sup> However, abuse of AAS causes many adverse effects in several organs systems, particularly the liver and heart.<sup>3,4</sup>

Over the last decade, a number of certain type of anabolic steroids, so called prohormones, have become available in the United States as nutritional supplements.<sup>5</sup> The term "prohormone" is used because initially these substances were precursors of testosterone (T) or nortestosterone. These products Supplement Health and Education Act, although the recently are sold as over-the-counter products under the 1994 Dietary signed "Anabolic steroid Control Act of 2004" is intended to put a ban on these substances. Global distribution via the internet has resulted in a huge international commercial success for products including androst-4-ene-3,17-dione (AD), 19-norandrostenedione and dehydroepiandrosterone. The use of these substances constitutes a doping offence according to the regulations<sup>6</sup> of the World Anti-Doping Agency (WADA).

In general, adrenosterone (androst-4-ene-3,11,17-trione, Fig. 1), a tri-ketosteroid, was isolated from the cortical extracts by Reichstein.<sup>7</sup> Adrenosterone exhibits a variety of biological properties such as androgenic properties and activities.<sup>8,9</sup> It also possesses aromatase (estrogen synthetase) inhibitory activity which plays an important role managing the estrogen-mediated events especially in ovulation and the growth of estrogen-dependent tumors.<sup>10</sup>

Generally, metabolites in urine are polar, so it is not easy to use gas chromatography/mass spectrometry (GC/MS) without

derivatization for their analysis.<sup>11-14</sup> More recently, liquid chromatography/mass spectrometry (LC/MS) has been used for trace bioanalysis.<sup>15-18</sup> This technique allows highly sensitive determinations without derivatization as required for GC/MS. It is also possible to devise methods in specificity for metabolites and structurally similar compounds.<sup>19</sup>

The present work reports a method to detect the metabolites of adrenosterone in human urine after oral administration, using both LC/MS and GC/MS as methods for screening and confirmation. The determination of the adrenosterone parent and its metabolites in human urine was also studied. In addition, we studied the excretion profile of adrenosterone and its metabolites after oral administration.

### Experimental

**Chemicals.** Adrenosterone (androst-4-ene-3,11,17-trione) and 17-methyltestosterone (used as an internal standard) were purchased from Sigma Chemical Co. (MO, USA). Adrenosterone tablets were purchased from Ergopharm (Champaign, IL, USA). Sordolite<sup>®</sup> PAD-1 resin (particle size 0.1-0.2 mm) was supplied by Serva Co. (Heidelberg, Germany) and was washed with acetone, methanol and distilled water before use. KH<sub>2</sub>PO<sub>4</sub> and K<sub>3</sub>PO<sub>4</sub> were obtained from KANTO Chemical Co (Tokyo, Japan). β-Glucuronidase was obtained from Roche Diagnostics (Mannheim, Germany). Diethyl ether and acetonitrile were purchased from J. T. Baker (Phillipsburg, MT, USA). Ammonium acetate was purchased from YAKURY Chemical Co (Tokyo, Japan). Acetonitrile and distilled water were used after filtering through Millipore filters (0.45 μm, MA, USA) and sonication for 20 min. *N*-Methyl-*N*-trimethyl-

**Table 1.** Operating conditions for LC/APCI/MS

LC conditions					
Column :	Capcell Pak, Type UG120, C18 (150 mm × 2.0 mm i.d., particle size 5 μm)				
Flow rate :	0.2 mL/min				
Mobile phase					
solvent A	10 mM acetic acid in D.W				
solvent B	10 mM acetic acid in Acetonitrile				
Gradient time table					
Time (min)	0	3	7	9	10
A solvent (%)	75	50	50	20	75
B solvent (%)	25	50	50	80	25
Run time :	13 min				
Injection volume :	10 μL				
Column temperature :	45 °C				

**MS conditions**

Ionization method: APCI  
 Ionization mode: positive ion mode  
 Corona current: 5 uA  
 Desolvation gas flow: 1000 cc/min (N<sub>2</sub>)  
 Nebulizer pressure: 410 kPa  
 Desolvation gas temperature: 400 °C  
 Scan range: *m/z* 100 ~ 400  
 Scan rate: 6.25 spectra/sec

silyltrifluoroacetamide (MSTFA), ammonium iodide (NH<sub>4</sub>I) and dithioerythritol (DTE) were obtained from Sigma.

**Instruments and equipment.** The LC/TOF-MS system consisted of an HP 1100 series LC with an atmospheric pressure chemical ionization (APCI) source from Agilent Technologies (Palo Alto, CA, USA) and TOF-MS from LECO (St. Joseph, MI, USA), was used for the separation and estimation of the molecular weights of metabolites. Liquid chromatographic separation was done on a Capcell Pak C<sub>18</sub> column (150 mm × 2.0 mm i.d., particle size 5 μm) from Shiseido (Chuoku, Japan). The 6890N GC from Agilent with TOF-MS from LECO was used to detect suspected metabolites. 6890N GC-5975 MSD from Agilent was used for the excretion studies for adrenosterone and its urinary metabolites. The column was an Ultra-2 cross-linked 5% phenylmethylsiloxane capillary column (25 m × 0.2 mm i.d., 0.11 μm film thickness). Operating conditions for LC/APCI/MS and GC/MS are shown in Tables 1 and 2, respectively.

HS501D shaker from IKA (Staufen, Germany) for liquid extraction, Varifuge-F from Heraeus (Hanau, Germany) for centrifugation, vortex mixer from Scientific Industry (Bohemia, USA), Turbovap<sup>®</sup> LV evaporator from Zymark (Hopkinton, MA, USA) and WB 2000 evaporator from Hiedolph (Schwabach, Germany) for sample concentration, and ecoline RE112 Freezer from LAUDA (Lauda-Königshofen, Germany) to take organic layer from extraction procedure were used.

**Volunteer sample.** A healthy male volunteer (age 30, weight 75 kg) was orally dosed with one tablet of 11-oxo (trade name) which included adrenosterone 75 mg. Urine samples were collected to 49 h (1, 1.5, 2.2, 4.1, 5, 11, 16.5, 18, 31, 49 h) and were kept at 2 ~ 3 °C.

**Sample preparation.** The extraction was based on a method described for anabolic steroids in doping control test procedure.<sup>20,21</sup> A Pasteur pipette was filled with Aqueous Serdolite<sup>®</sup>

**Table 2.** Operating conditions for GC/MS

GC conditions			
Column :	Ultra-2 (cross-linked 5% phenylmethylsiloxane, 0.2 mm i.d. × 25 mm length × 0.11 μm film thickness)		
Carrier gas :	He at 1.0 mL/min		
Oven temperature program :			
Rate (°C/min)	Temp. (°C)	Hold(min)	
	180	1	
	20	260	0
	10	325	2
Run time :	14.50 min		
Injection volume :	2 μL		
Injection port temperature :	280 °C		
Transfer line temperature :	280 °C		

**TOF-MS conditions**

Source temperature: 230 °C  
 mass range: *m/z* 100 ~ 650  
 Scan rate: 10 spectra/sec

**MSD conditions**

Source temperature: 230 °C  
 mass range: *m/z* 100 ~ 650  
 Acquisition mode: SIM (Selected Ion Monitoring) mode  
 Selected ion (*m/z*): 301, 411, 413, 446, 501, 503, 516, 518, 589, 604

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 3 mL of a urine sample spiked with 17-methyltestosterone (50 μL, 10 μg mL<sup>-1</sup>) 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 6.8).

β-Glucuronidase (50 μL) was added and the solution was heated at 55 °C for 1 h in order to hydrolyze the glucuronide conjugate. After hydrolysis and cooling to room temperature, 5 mL of ether was added, and then the mixture was shaken for 10 min and centrifuged at 2500 rpm for 5 min. The organic layer was transferred to another test tube and then concentrated at 40 °C under a gentle stream of nitrogen. The residue was dissolved in 100 μL of methanol, and 10 μL was injected into the LC/APCI/MS.

The ratio of free, glucuro- and sulfa-conjugated forms of adrenosterone and its metabolites were studied to the excreted forms.

**Trimethylsilylation for GC/MS.** For characterization of adrenosterone and its urinary metabolites proposed by GC/MS, the residue was dried in a vacuum desiccator for 30 min. The dried residue was dissolved in 50 μL of MSTFA/NH<sub>4</sub>I/DTE (500:4:2, w/w/w) and the solutions was heated for 30 min at 70 °C. Two μL was injected into the GC/MS.<sup>22,23</sup>

**Method validation.** For a calibration curve of adrenosterone, the proper amount of standard and internal standard to 1, 2, 5, 10 and 20 ng mL<sup>-1</sup> were fortified into the 3 mL blank urine and used for GC/MSD after trimethylsilylation.

The accuracy, recovery and reproducibility of this method were tested at three levels (1, 20, 50 ng mL<sup>-1</sup>) for adrenosterone.

The 3 mL blank urines ( $n = 6$ ) were spiked standard and internal standard at three levels (1, 20, 50  $\text{ng mL}^{-1}$ ) and used for GC/MSD after trimethylsilylation.

## Results and Discussion

**Determination of adrenosterone and its metabolites by LC/APCI/MS.** The molecular mass of adrenosterone is 300 Da and its structure is shown in Figure 1. The urine sample extracts were analyzed by LC/APCI/MS. Figure 2 shows total ion chromatograms (TICs) of standard, blank urine, spiked

urine and dosed urine, which is the urine of 4.10 h after oral administration, by LC/APCI/MS. Two unknown peaks design-

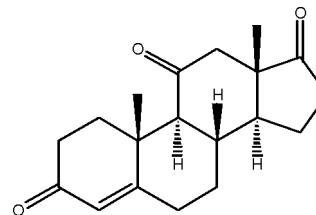
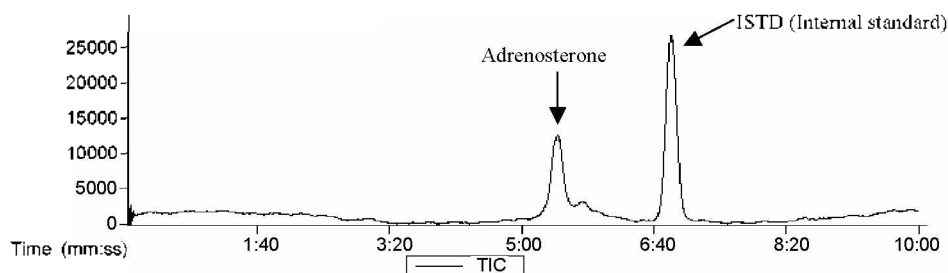
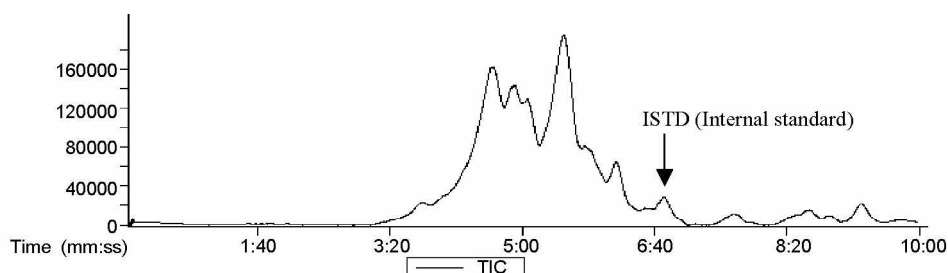


Figure 1. Structure of adrenosterone.

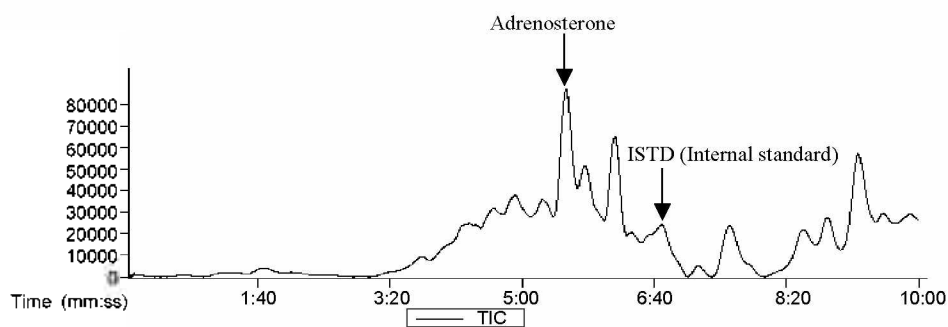
(a) Standard



(b) Blank urine



(c) Spiked urine



(d) Dosed urine

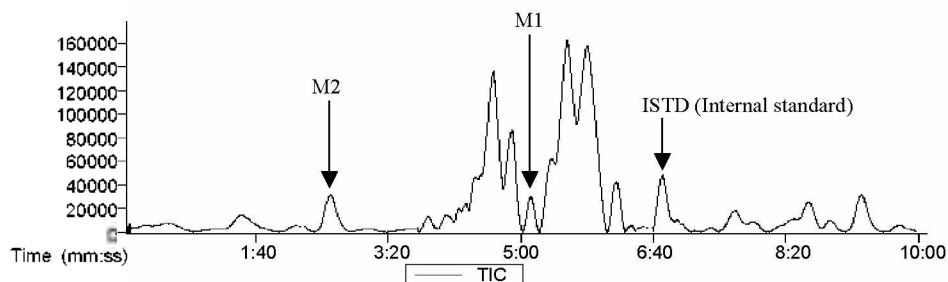
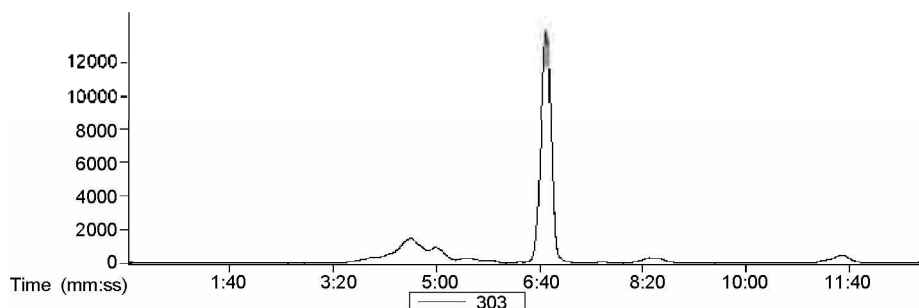
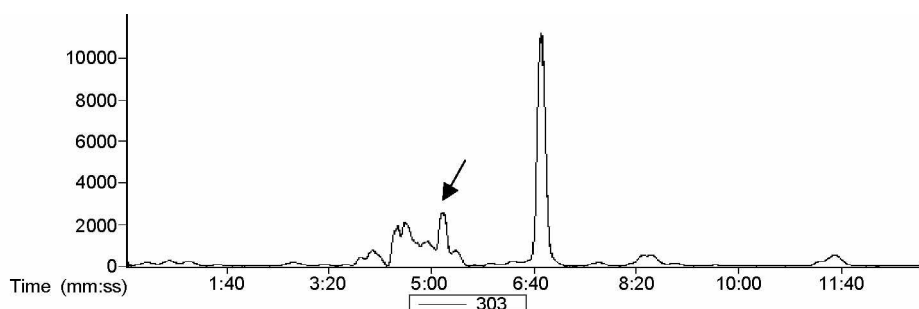
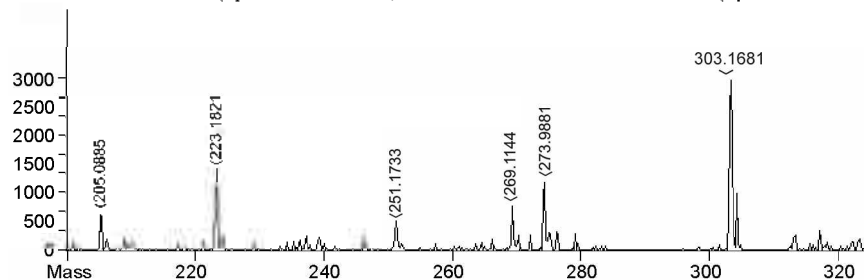


Figure 2. Total ion chromatograms of (a) standard (10  $\mu\text{g/mL}$ ), (b) blank urine, (c) spiked urine at 10  $\mu\text{g/mL}$ , and (d) dosed urine after the oral administration of adrenosterone showing adrenosterone-M1 and M2 peaks by LC/APCI/MS.

(a) Blank urine ( $m/z$  303)(b) Dosed urine ( $m/z$  303)

(c) Mass spectrum

Caliper - sample "dosed2 re:080807\*", 5:11.96 min:sec to 5:11.96 min:sec (Spec #1931 to 1931) - (5:07.48 min:sec to 5:07.48 min:sec (Spec #1903 to 1903) + 5:34.04 min:sec to 5:34.04 min:sec (Spec #2069 to 069))

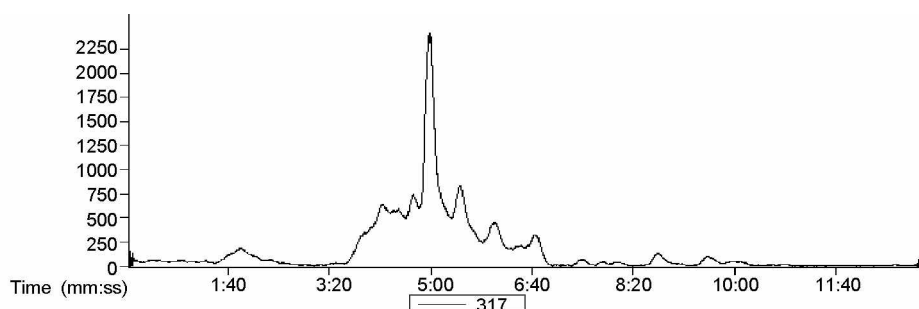
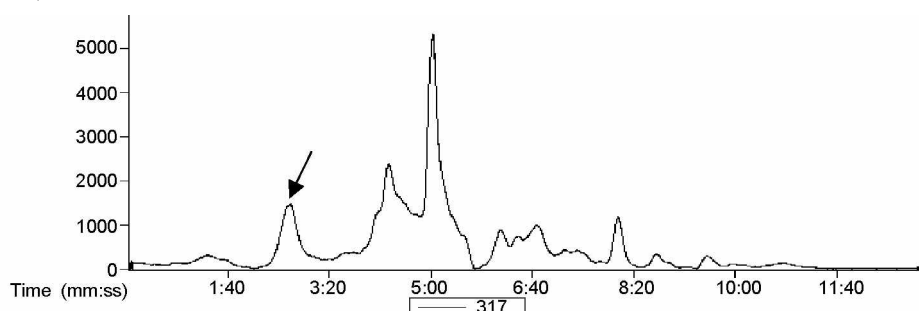


**Figure 3.** Extracted ion chromatograms ( $m/z$  303) of (a) blank urine, (b) dosed urine showing adrenosterone-M1 peak (c) mass spectrum corresponding to adrenosterone-M1.

nated adrenosterone-M1 (5.11 min) and adrenosterone-M2 (2.40 min) detected before adrenosterone elution (5.26 min). Figures 3 and 4 show the extracted ion chromatograms (EICs) of  $m/z$  303 and 317 for blank urine and dosed urine and the mass spectra of adrenosterone-M1 and adrenosterone-M2, respectively. From these results, we suggest that adrenosterone-M1 and M2 have  $m/z$  302 ( $C_{19}H_{26}O_3$ ) and 316 ( $C_{19}H_{26}O_4$ ) as molecular ions, respectively. We checked that the adrenosterone parent was not detected by LC/APCI/MS.

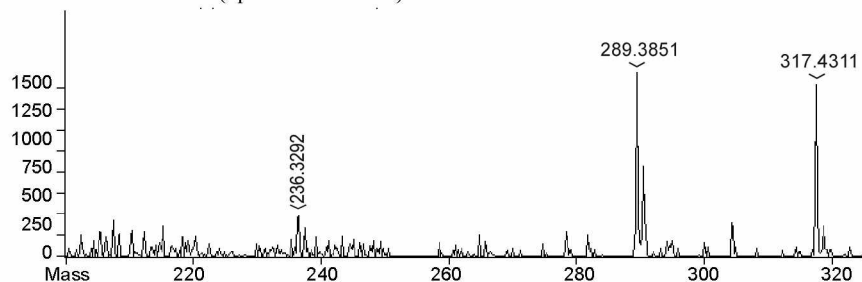
**Characterization of adrenosterone and its metabolites by GC/MS.** The adrenosterone and its metabolites were also analyzed by GC/TOF-MS after trimethylsilylation. Figure 5 showed the mass spectra of 3TMS-adrenosterone, 3TMS-adrenosterone-M1 and 4TMS-adrenosterone-M2. The mass spectrum of 3TMS-adrenosterone (Fig. 5a) showed the molecular ion at  $m/z$  516 and fragment ions at  $m/z$  129, 169, 411 ( $[M-CH_3-OTMSH]^+$ ) and 501 ( $[M-CH_3]^+$ ) ions. The  $m/z$  169 ion, already well known in 17-keto steroid fragmentation,

derived from the C/D-ring cleavage. The  $m/z$  129 ion could be associated with the D-ring fragmentation hypothesis (confirmed by the absence of  $m/z$  169).<sup>20,22</sup> The mass spectrum of 3TMS-adrenosterone-M1 showed the figure 5b. The main fragment ions of 3TMS-adrenosterone-M1 were observed at  $m/z$  503 ( $[M-CH_3]^+$ ) and 413 ( $[M-CH_3-OTMSH]^+$ ). The mass spectrum of 3TMS-adrenosterone-M1 contained  $m/z$  129 and 169 ions. We suggest that adrenosterone-M1 was a reduction with  $m/z$  303 ( $[M+H]^+$ ) from LC/MS result, which did not know exact position. The mass spectrum of 4TMS-adrenosterone-M2 (Fig. 5c) is characterized by the molecular ion at  $m/z$  604 and fragment ions at  $m/z$  169, 209 (cleavage of C 7-8 and 9-10), 437 ( $[M-C/D \text{ ring}]^+$ ), 589 ( $[M-CH_3]^+$ ) ions.<sup>22</sup> We could deduce that adrenosterone-M2 was substituted by one hydroxyl group on the C-ring with  $m/z$  317 ( $[M+H]^+$ ) from LC/MS result, which did not know exact configuration of the C-ring. Table 4 summarized the retention time and fragment ions for compared adrenosterone and its metabolites obtained

(a) Blank urine ( $m/z$  317)(b) Dosed urine ( $m/z$  317)

(c) Mass spectrum

Caliper - sample "dosed:081001:2\*": 2:38.84 min:sec to 2:38.84 min:sec (Spec #974 to 974) - (4:08.76 min:sec to 4:08.76 min:sec (Spec #1536 to 1536)



**Figure 4.** Extracted ion chromatograms ( $m/z$  317) of (a) blank urine, (b) dosed urine showing adrenosterone-M2 peak and (c) mass spectrum of adrenosterone-M2.

LC/APCI/MS and GC/TOF-MS. The adrenosterone parent was detected by GC/TOF-MS, but not detected by LC/APCI/MS because of low intensity.

**Method validation.** The regression line was  $y = 0.0111x + 0.0077$ , and correlation coefficient ( $r^2$ ) was 0.9995. The results for recovery, reproducibility and accuracy are summarized in Table 3. As shown in Table 3, the recovery ranged from 100.7 to 117.7%. The reproducibility of this method was represented by percent relative standard deviation (RSD) at each fortification level and these values were within 10%. The accuracy ranged from 85.6 to 112.0%. The limit of detection (LOD) and the limit of detection quantification (LOQ) were  $0.35 \text{ ng mL}^{-1}$  and  $0.7 \text{ ng mL}^{-1}$ , respectively.

**Phase II metabolite study.** For the determination of ratio of free, glucuro- and sulfa-conjugated forms, hydrolysis of glucuro-conjugates was experimented with  $\beta$ -glucuronidase from *E. coli*, whereas sulfa-conjugates was efficiently hydrolyzed by  $\beta$ -glucuronidase/arylsulfatase from *H. pomatia*. To confirm

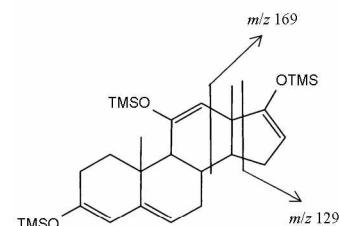
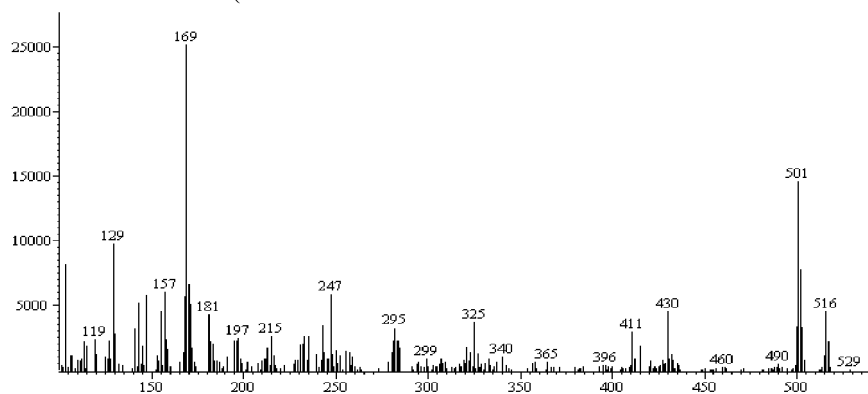
the free form, we experimented without hydrolysis step. Because adrenosterone and its metabolites did not detect without hydrolysis procedure, we could suggest that adrenosterone and its metabolites were mainly excreted as conjugated form.

From these results, we could suggest that the sulfa-conjugated forms and glucuro-forms were excreted in human urine. When we compared with the excreted forms of sulfa-conjugation and glucuro-conjugation, the level of sulfa-conjugated forms were higher than that of glucuro-conjugated forms in urine. The glucuro-/sulfa-conjugated ratio of adrenosterone, M1 and M2 was  $0.73 \pm 0.03$ ,  $0.96 \pm 0.06$  and  $0.89 \pm 0.03$  ( $n = 6$ ), respectively.

**The excretion profile of adrenosterone and its metabolites.** The urinary excretion of adrenosterone and its metabolites in human urine were followed for 48 h after the oral administration of a 75 mg dose of adrenosterone. The excretion profiles of adrenosterone and its metabolites from dosed urine, detected by GC/MSD, are shown in Figure 6. Adrenosterone, adreno-

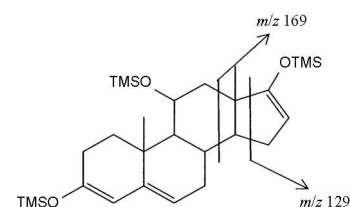
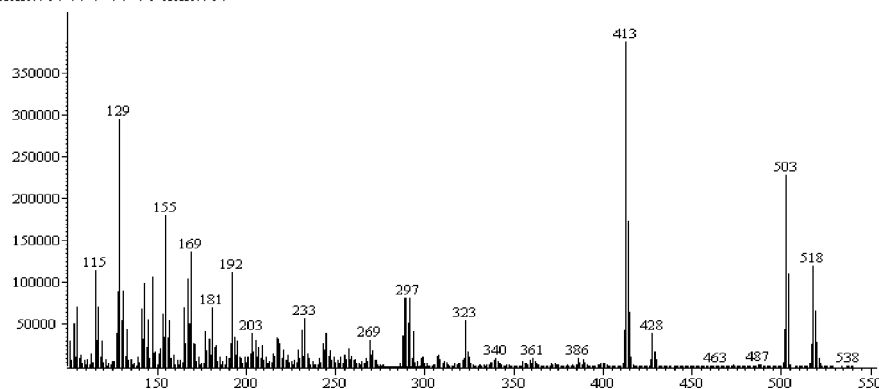
## (a) Mass spectrum of 3TMS-adrenosterone

Caliper - sample "dosed5 (hydrolysis):1", 8:19.98 min:sec to 8:19.98 min:sec (Spec #1999 to 1999) - 8:19.28 min:sec to 8:19.28 min:sec (S)



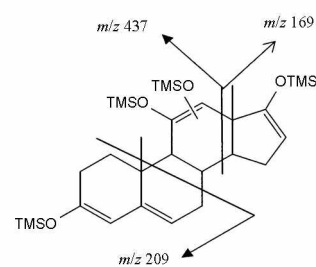
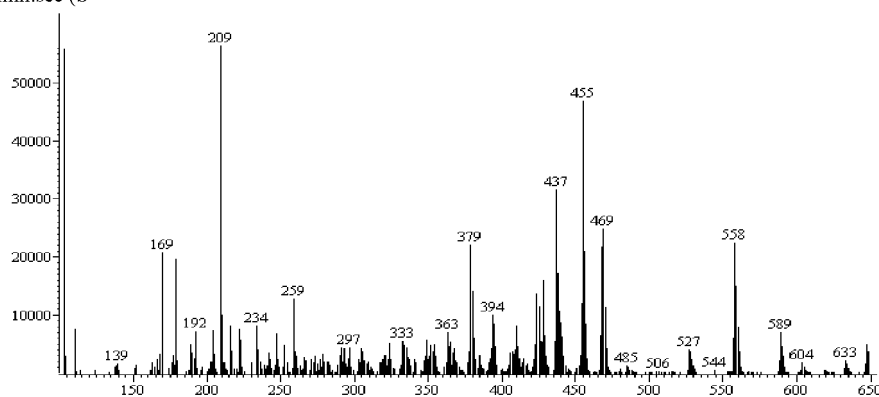
## (b) Mass spectrum of 3TMS-adrenosterone-M1

Caliper - sample "dosed5 (hydrolysis):1", 7:20.98 min:sec to 7:20.98 min:sec (Spec #1409 to 1409) - (5:00.08 min:sec to 5:00.08 min:sec)



## (c) Mass spectrum of 4TMS-adrenosterone-M2

Caliper - sample "dosed5 (hydrolysis):1", 9:30.18 min:sec to 9:30.18 min:sec (Spec #2701 to 2701) - (9:27.28 min:sec to 9:27.28 min:sec (S)

**Figure 5.** Mass spectra and main fragmentations of (a) 3TMS-adrenosterone, (b) 3TMS-adrenosterone-M1 and (c) 4TMS-adrenosterone-M2 by GC/TOF-MS.**Table 3.** The results for recovery, reproducibility and accuracy

n = 6

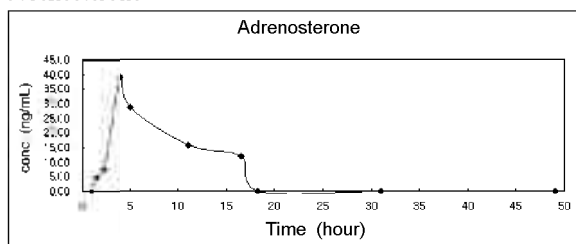
Conc. (ng/mL)	recovery			reproducibility			accuracy		
	1	20	50	1	20	50	1	20	50
adrenosterone	117.7	108.8	100.7	2.5	8.4	3.6	88.4	85.6	112.0

**Table 4.** LC/APCI/MS and GC/TOF-MS data of adrenosterone and its metabolites

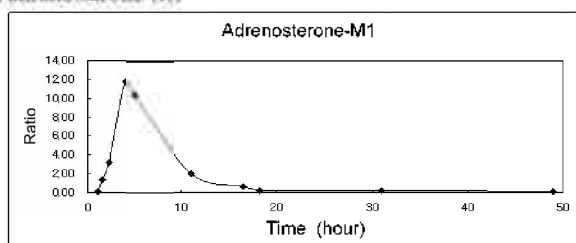
Compounds	LC/APCI/MS		GC/TOF-MS			
	[M+H] <sup>+</sup>	RT (min)	[M] <sup>+</sup>	RT (min)	[M-15] <sup>-</sup>	Characteristic ions ( <i>m/z</i> )
adrenosterone	301	5.26	516	8.18	501	169, 411
adrenosterone-M1	303	5.11	518	7.20	503	169, 413
adrenosterone-M2	316	2.40	604	9.27	689	169, 209, 437

\*RT: retention time

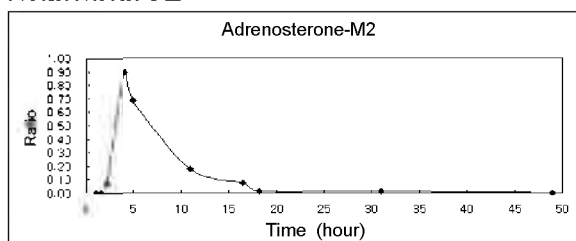
(a) adrenosterone



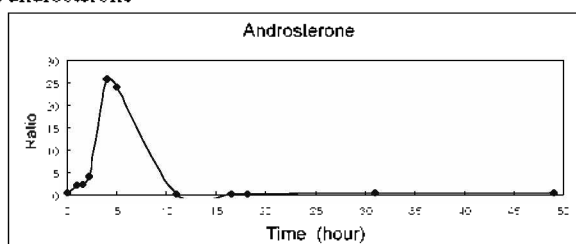
(b) adrenosterone-M1



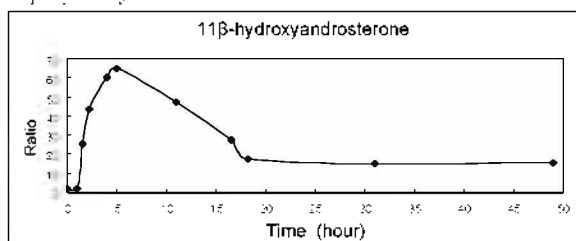
(c) adrenosterone-M2



(d) androsterone



(e) 11β-hydroxy androsterone

**Figure 6.** Excretion profiles of (a) adrenosterone, (b) adrenosterone-M1, (c) adrenosterone-M2, (d) androsterone and (e) 11β-hydroxy androsterone after the administration of adrenosterone 75 mg.

sterone-M1 and adrenosterone-M2 had a maximum level of excretion at 4.1 h. Adrenosterone and its metabolites were no longer detectable 16 h after administration, because they were rapidly excreted from the body. In case of the nandrolone, it had a maximum level of excretion at 2 h after administration.<sup>24</sup> Also, androsto-4-ene-3,17-dione and boldenone reached maximum levels at 4 h and 5 h after administration, respectively.<sup>25,26</sup> The excreted amounts from adrenosterone were 14.75 ng for 48 h. The half-life of adrenosterone was 5 h after oral administration. In cases of the M1 and M2 metabolites, for which no standards were available, the abundances plotted the peak area ratios of the metabolites to the internal standard.

**The excretion profile of endogenous steroids after oral administration.** To confirm the androgenic effects of adrenosterone, levels of six endogenous steroids (testosterone, epitestosterone, androsterone, dehydroepiandrosterone, 11β-hydroxy androsterone and 11β-hydroxyetiocholanolone) in human urine were simultaneously analyzed by GC/MSD after oral administration. Two endogenous steroids (androsterone and 11β-hydroxy androsterone) of them were increased the level in urine flow. Figure 7 showed the excretion profiles of androsterone and 11β-hydroxy androsterone from dosed urine after oral administration. The urinary excretions of androsterone and 11β-hydroxy androsterone were increased, and the maximum urinary excretion was reached 4.1 h, the same time as that of adrenosterone and its metabolites. As described by S. H. Lee *et al.*, endogenous steroids levels, especially androsterone, were increased after oral administration of clostebol, mesterolone and nandrolone.<sup>27</sup> In the other hand, H. R. Norli *et al.* and M. Donike *et al.* showed that it was decreased after administration of bolasterone, norethandrolone and boldenone.<sup>28,29</sup>

## Conclusions

We have analyzed adrenosterone and its two metabolites in human urine using both LC/APCI/MS and GC/MS. As the chromatograms of the blank urine and dosed urine were compared, we found adrenosterone-M1 and M2 by LC/APCI/MS. After then, we applied the GC/TOF-MS for confirmation of adrenosterone-M1 and M2 with trimethylsilylation. We suggested that adrenosterone-M1 was a reduction, and adrenosterone-M2 was substituted one hydroxyl group on the C-ring. The adrenosterone was detected by GC/TOF-MS, but was not detected by LC/APCI/MS because of low intensity. Adrenosterone and its metabolites were excreted as conjugated forms. Sulfa-conjugated forms were shown higher levels in urine than glucuro-conjugated forms. In the excretion profile

studies, adrenosterone and its metabolites could be detected in urine for 48 h after the oral administration (75 mg), and the maximum level of adrenosterone and its metabolites were observed at 4.1 h. The amounts of adrenosterone excreted were 14.75 ng after a 48 h. Furthermore, the endogenous steroids, androsterone and 11 $\beta$ -hydroxy androsterone, reached maximum level at 4.1 h.

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