Micro-Analytical Identification, by NMR, of Elevated Steroid in Urine of Cushing's Syndrome Patients

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Abstract. We previously reported that the daily urinary unidentified ketosteroid glucuronide (US-G) level in patients with Cushing's syndrome was much higher than that in the healthy subjects. Furthermore, urine samples from patients with Cushing's syndrome, including those with pituitary adenoma and adrenal adenoma, yielded almost the same high excretion levels, despite the different sites of the adenomas. We extracted US obtained by hydrolysis of US-G in urine of patients with Cushing's syndrome, purified it, and analyzed its chemical structure. Molecular weight and molecular formula were analyzed by MS spectrometry, and the chemical structure was analyzed by NMR spectrometry, utilizing small quantities of refined US. The substance has a molecular weight of 304 Da, a molecular formula of $C_{19}H_{28}O_3$, and its chemical structure is $3\alpha,11\beta$ -dihydroxyandrost-4-en-17-one.

Key Words: Cushing's syndrome, 17-Ketosteroid glucuronides, $3\alpha,11\beta$ -Dihydroxy-androst-4-en-17-one

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PREVIOUSLY, we described the daily urinary excretion levels (as free type), measured by a new method, of an unidentified ketosteroid glucuronide (US-G) in patients with Cushing's syndrome and healthy subjects. Levels in patients with Cushing's syndrome were much higher than those in the healthy subjects [1]. In this report, we describe the extraction of the US obtained by enzymatic hydrolysis of US-G in the urine of those patients, the refining process and the identification of the chemical structure by NMR spectrometry, utilizing small quantities of refined US.

Materials and Methods

Apparatus

Hitachi HPLC (655A-11) was used, equipped with a

controller (L-500 LC), UV detector (655A-21), recorder (D-2000 chromato-integrator), and column oven (655A-52). The columns for HPLC were 4.6 (i.d.) $\times$ 250 mm stainless-steel for analysis and purification, and 10 (i.d.) $\times$ 250 mm stainless-steel for separation of US, both of which were packed with Capcell-Pak Silica (particle diameter: 5 μ m) and purchased from Shiseido Co.

The column for separation pretreatment was a 46 (i.d.) $\times$ 250 mm tempered glass column packed with Sep-Pak C₁₈ (Millipore Co.) and prepared by us for low pressure liquid chromatography. The MS spectrometer was a Finnigan MAT INCOS 50 quadrupole mass-spectrometer with a direct inlet system. The NMR spectrometer was a JEOL JNM-GSX-400 Fourier transform spectrometer (¹H 399.65 MHz, ¹³C 100.40 MHz).

Reagents

Reagents were as follows: Dansylhydrazine (5-dimethylaminonaphthalene-1- sulfonylhydrazine; Aldrich Chemical Co.), 2 g/l in acetonitrile, stored at 4°C until

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use; acetate buffer, 0.2 mol/l, pH 5.1; sodium hydroxide 0.25 mol/l; β -D-glucuronidase from calf livers (Wako Chemical Co.).

To prepare the standard stock solution, we dissolved the following steroids separately in 10 ml of ethanol to give a concentration of 100 mg/l: 1 mg of 11 β -hydroxyandrosterone (11-OHA), 11 β -hydroxyetiocholanolone (11-OHE), and 16 α -hydroxyandrosterone (16-OHA; Sigma Chemical Co.). We then combined one volume of each of these solutions (three volumes in all) with seven volumes of ethanol to give a final concentration of 10 mg/l for each steroid, and stored this mixture at 4°C. Working mobile-phase solvents were as follows: (A) dichloromethane/2-hexanol/2-pentanol/2-butanol/water = 500/1/20/1/5 by volume for the analysis of US; (B) dichloromethane/ethanol/water = 500/4/2; (C) benzene/dichloromethane/2-propanol/water = 15/500/15/5 by volume for the separation. The organic layers after mixing of these solutions were used for HPLC. All other reagents and solvents were of analytical reagent grade or HPLC grade and were purchased from commercial sources.

Procedures

(a) Extraction of US in urine

Pretreatment of urine sample

Urine samples from patients with Cushing's syndrome were centrifuged at 1,000 g for 20 minutes. The supernatant (500 ml) was loaded on the column for separation pretreatment. The column was washed with water and then eluted with methanol. A dark brown band that descended from the upper layer of the column and flowed out from the lower end of the column was collected (150–200 ml) and evaporated under reduced pressure.

Hydrolysis of 17-KS glucuronides and extraction of free 17-KS

The residue after evaporation was dissolved in acetate buffer (50 ml), incubated with β -D-glucuronidase (250,000 units) at 37°C for 20 hours, shaken with dichloromethane (150 ml) for 1 minute, then centrifuged at 700 g for 10 minutes. The separated dichloromethane layer was shaken with sodium hydroxide solution (15 ml), and the resulting dichloromethane layer was further shaken with distilled water (30 ml) two times. The dichloromethane layer was subsequently dehydrated with anhydrous sodium sulfate,

and the solvent was removed by evaporation.

Separation and purification of US

First HPLC:

The residue after evaporation was dissolved in dichloromethane (500 μ l), and injected in 5 divided volumes (100 μ l each) into HPLC using a column for US separation and mobile phase solvent B, at a column temperature of 25°C, and flow rate of 1 ml/min. Fractions were obtained every 5 minutes. One hundred μ l of each fraction was evaporated, and the procedure below for the measurement of US (dansylation and determination for HPLC) was performed. The US-containing fractions were combined, and the solvent was evaporated.

Dansylation and determination for HPLC:

The residue after evaporation (100 μ l each) was mixed with dansylhydrazine solution (80 μ l) and then with acetic acid (20 μ l), heated at 50°C for 1 minute, evaporated under reduced pressure at the same temperature, and heated again at the same temperature for 20 minutes. Similarly, the working standard solution (50 μ l; each steroid, 50 ng) was evaporated and dansylated. The dansylated substance was dissolved in dichloromethane (100 μ l), and 10 μ l was injected into HPLC using a column for analysis and mobile phase solvent A at a column temperature of 25°C, a flow rate of 1 ml/min, an excitation wavelength of 330 nm, and a fluorescence wavelength of 540 nm. The peak that appeared following 11-OHA and before 16-OHA was regarded as that of the US (11-OHE appeared followed 16OHA). The US content was expressed as 11-OHA equivalents (1).

Second HPLC:

The residue after evaporation of the solvent in the US-containing fraction from the first HPLC was injected into HPLC under the same conditions as those in the first HPLC. US was measured by a method similar to that in the first HPLC to confirm the US-containing fraction. The US-containing fractions were combined, and the solvent was evaporated.

Third HPLC:

The residue after evaporation of the solvent from the US-containing fraction obtained in the second HPLC was dissolved in dichloromethane (300 μ l) and injected in 3 divided volumes (100 μ l each) into HPLC us-

ing a column for purification and mobile phase solvent C under the same conditions as those in the first HPLC. The fractions corresponding to each peak on chromatogram were obtained, and the US was measured. The US peak fractions were combined, and the solvent was evaporated.

Fourth HPLC:

The residue after evaporation of the solvent from the US peak fraction obtained in the 3rd HPLC was dissolved in dichloromethane (200 μ l) and injected into HPLC under the same conditions as those in the 3rd HPLC. The fraction corresponding to the US peak was obtained. The US peak fractions were combined, the US was measured, and the solvent was evaporated.

Fifth HPLC:

The residue after evaporation of the solvent from the US peak fraction obtained in the 4th HPLC was dissolved in dichloromethane (200 μ l) and injected in 2 divided volumes (100 μ l each) into HPLC using the column for purification and mobile phase solvent C under the same condition as those in the 3rd HPLC.

The US peak fractions were combined, the US was measured, and the solvent was evaporated. The residue after evaporation was dissolved in a small amount of ethanol, filtered through a filter (Millipore, type: SJFH013NS) for a sample of HPLC, and the filtrate was evaporated.

(b) Spectral measurements

^1H , ^{13}C , and 2D NMR spectra were recorded as 6.7 μM solution in CDCl_3 (CEA 99.99%-d) with tetramethylsilane as an internal standard in a 5 mm tube. A JEOL JNM-GSX-400 spectrometer (^1H 399.65 MHz, ^{13}C 100.40 MHz) was used at 25°C, the ambient temperature.

Typical spectral parameters for ^1H and ^{13}C one-dimensional spectra consisted of 64 and 50,000 transients collected over a spectral width of 4,400 Hz and 25,000 Hz each with 32,768 time domain points and a relaxation delay of 5 and 2 seconds, respectively. Apodization, baseline correction, and peak picking were performed using the JEOL standard software.

Data acquisition of 2D NMR was carried out according to JEOL standard pulse programs (RSX system). The conditions for ^1H - ^1H DQFCOSY and NOESY on the phase sensitive mode were: spectral width, 3680 Hz, data points, t2 1024 $\times$ t1 256 each with 128 accu-

mulations, pulse repetition time 2 s, NOESY mixing time 500 ms and 750 ms.

Data processing of DQFCOSY and NOESY was performed on NMR2 software by NMRi, where a 30° phase shifted sine bell was applied in both dimensions prior to Fourier transformation, and the final transformed matrix after zero filling was 2,048 by 2,048 points.

^1H - ^{13}C shift correlation by HMQC or HMBC was obtained on JEOL standard software for acquisition and data processing. The conditions for HMQC and HMBC were: spectral width t2 3,680 Hz $\times$ t1 22,624 Hz and data points, t2 2,048 $\times$ t1 256 each with 512 accumulations, which were zero filled and Fourier-transformed to f2 2,048 $\times$ f1 512 points, pulse repetition time 2 s, and a long range coupling parameter at HMBC, 5 Hz.

Results and Discussion

We used a UV detector to check the purity of the US when the US-containing fraction was collected by HPLC, because we have previously established a system for separating US from the other components in urine.

We used 11OHA as the standard for measuring US by HPLC after dansylation of 17-ketosteroids, because we did not obtain sufficient quantity of US in this experiment, and we assumed the chemical structure of US to be similar to 11OHA (the peak position of US on chromatogram was always located near that of 11OHA in various conditions of HPLC). Furthermore, 11OHA is more stable than US, and easily obtained from commercial sources.

The extracted, refined US demonstrated a single peak, and mixture of other urine-derived substances was not observed on the chromatograms of HPLC. However, NMR demonstrated a residue of 2-propanol, a component of the mobile phase solution used in HPLC, and we estimated a content of approximately 5% impurities by molar ratio.

The quantity of refined US we obtained for chemical structure analysis was 1.12 mg. To determine chemical structure from such a small sample, we first obtained NMR spectrometry data, and then recovered the sample to determine molecular weight and molecular formula by MS spectrometry.

US was found to be 3 α , 11 β -dihydroxyandrost-4-

en-17-one as described below. The structure and ^1H -NMR spectrum for US are shown in Fig. 1 and 2, respectively. The same structure for US has been reported by Fukushima *et al.* in 1966 [3–5], but its identity could not be compared from the literature because Fukushima *et al.* did not perform a chemical structure analysis using the modern NMR techniques that we employed.

The direct EI mass spectral analysis showed the molecular ion at m/z 304, whose molecular composition was suggested to be $\text{C}_{19}\text{H}_{28}\text{O}_3$. The numbers of hydrogen and carbon were recognized from ^1H and ^{13}C NMR spectral analysis together with the three Oxygenated functions, which were two alcohols and one carbonyl, as listed in Table 1.

^1H -NMR showed two singlet methyls at δ_{H} 1.14 (CH_3 -18) and 1.27 (CH_3 -19), two hydroxy-substituted methine protons at δ_{H} 4.12 (m, H3 β) and 4.48 (m, H11 α), and one olefinic proton at δ_{H} 5.41 (bd, $J = 4.4$, H4) as characteristic signals. The other signals were

Fig. 1. The structure of US

Fig. 2. ^1H -NMR of US at 400 MHz in CDCl_3 . The Fig. shows the assignments below the spectrum. The superimposed italic letters are A, water; B, residual 2-propanol used for purification; C, unknown impurity.

Table 1. The NMR assignments of US

functions ^{a)}			$^1\text{H}[\delta, \text{m}^c, J(\text{Hz})]$	$^{13}\text{C}[\delta]$
1	CH_2	α	1.73 m	31.29
		β	1.79 m	
2	$\text{CH}_2^b)$	α	1.68 m	27.73
		β	1.77 m	
3	CH	β	4.12 m	64.47
3	OH	α	1.38 bs	
4	CH =		5.41 bd $J = 4.4$	119.71
5	C =		—	150.54
6	CH_2	α	2.05 m	31.54
		β	2.33 m	
7	CH_2	α	1.03 m	32.13
		β	2.0 m	
8	CH	β	2.1 m	31.08
9	CH	α	0.95 dd $J = 11.2, 3.2$	56.96
10	C		—	38.21
11	CH	α	4.48 m	68.44
11	OH	β	1.07 bd $J = 3.2$	
12	CH_2	α	1.46 dd $J = 14.4, 3.3$	40.85
		β	1.94 dd $J = 14.4, 2.7$	
13	C		—	46.89
14	CH	α	1.22 m	52.69
15	CH_2	β	1.62 m	21.71
		α	1.98 m	
16	CH_2	α	2.17 m	35.33
		β	2.49 dd $J = 18.1, 8.2$	
17	C = O		—	219.47
18	CH_3	β	1.14 s	15.91
19	CH_3	β	1.27 s	22.24

a) The α/β assignment shows the configuration of each proton.

b) α/β assignment exchangeable

c) The multiplicity of couplings is presented by the first-order. Abbreviations are s: singlet, bs: broad singlet, d: doublet, bd: broad doublet, dd: double doublet, and m: multiplet.

too complicated to be assigned.

In ^{13}C -NMR, the functional types of all carbons were differentiated by chemical shifts and INEPT analysis. There were two methyls, seven methylenes, five methines, two quaternaries, a protonated olefin, a substituted olefin, and a carbonyl. Two of the methine carbones (C3 and C11) were substituted by an alcohol as determined from their δ_{C} as well as from the ^1H -NMR spectra.

^1H - ^1H shift correlation was obtained by double

^1H - ^{13}C one-bonded shift correlations were measured by HMQC. Long-range coupled ^1H - ^{13}C shift correla-

The whole NOE correlations are shown in Fig. 3 and in the upper right of Table 2. Both CH₃-18 and CH₃-19 showed axial β -configuration. Two methine protons at the 3 and 11 positions, respectively, were recognized to be equatorial by the NOE correlations.

Table 2. Summary of DQFCOSY (bottom-left) and NOESY (upper-right) of US

[illegible]

Table 3. Summary of proton-carbon long range coupling correlation (HMBC) of US

H C	1 α	1 β	2 α	2 β	3 β	3 OH	4	6 α	6 β	7 α	7 β	8 β	9 α	11 α	11 OH	12 α	12 β	14 α	15 α	15 β	16 α	16 β	18 Mc	19 Mc
1				○									○											
2							○																	
3		○	○																					
4								○	○															
5		○						○	○		○													
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10		○		○			○			○			○											○
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13																○		○	○				○	
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15												○						○			○	○		
16																			○	○	○			
17																○	○		○			○	○	
18																○	○	○						
19	○												○

Fig. 3. NOE correlation of US shown by arrows

Accordingly, the two hydroxyl functions at positions 3 and 11 were both in axial configuration, which is unusual for a metabolite.

Thus, the whole structure of US was elucidated to be 3 α ,11 β -dihydroxyandrost-4-en-17-one as shown in Fig. 2.

Fukushima *et al.* identified 3 α , 11 β -dihydroxyandrost-4-en-17-one as a human metabolite of 11 β -hydroxyandrost-4-en-3,17-dione [3]. They collected urine after administration of isotope-labeled 11 β -hydroxyandrost-4-en-3,17-dione to hypothyroidism subjects, hydrolyzed the steroid conjugates in urine to

free type, and performed an extraction with ether. They then isolated the extract with partition chromatography using celite, obtained a fraction including the radioactive metabolites, separated this by paper chromatography, and obtained a fraction containing 3 α , 11 β -dihydroxyandrost-4-en-17-one and 11 β -hydroxyetiocholanolone. Because these substances were not separated, they treated 3 α , 11 β -dihydroxyandrost-4-en-17-one as a 4,5-oxidoacetate derivative and separated it from 11 β -hydroxyetiocholanolone by paper chromatography. They separately synthesized a 4,5-oxidoacetate derivative of 3 α , 11 β -dihydroxyandrost-4-en-17-one, mixed the separated 4,5-oxidoacetate derivative and the synthesized 4,5-oxidoacetate derivative, and identified these as matching by the fact that the melting point was not lowered and by comparison of the IR spectra of the separation product and the synthesis product.

Fukushima *et al.* reported a great increase of the above steroid in the urine of hypothyroidism subjects administered 11 β -hydroxyandrost-4-en-3,17-dione [5]. However, to our Japanese patent publication in 1995 [2], there was no report concerning the steroid in the urine of Cushing's syndrome patients.

In a prior publication, we published the chemical structure of US as 3 α ,11 β -dihydroxyandrost-4-en-17-one. In that publication, however, we primarily described the method for measuring US [2]. Further-

more, we forecasted a report on clarification of the chemical structure of US by NMR spectrometry in the previous report [1]. The work we have presented here is the forecasted report.

References

1. Iwata J, Morita H, Yasuda K, Kuwayama A, Suzuki T, Demura H (1995) Daily excretion levels of an unidentified ketosteroid in the urine of patients with Cushing's syndrome and healthy subjects measured by a new method. *Endocr J* 42: 449–453.
2. Iwata J (Applicant: EIKEN CHEM CO LTD, publication of application) (1995) Measuring method of 3 α , 11 β -dihydroxy-4-androstene-17-one. *Japanese Patent Publication* No.280811 (in Japanese); *Patent abstracts of Japan* (the same patent No., in English).
3. Fukushima DK, Dobriner S, Bradlow HL, Zumoff B, Hellman L, Gallagher TF (1966) New metabolites of 11 β -hydroxy- Δ^4 -androstene-3,7-dione. *Biochemistry* 5: 1779–1783.
4. Bradlow HL, Zumoff B, Fukushima DK, Hellman L, Gallagher TF (1966) Metabolism of 11 β -hydroxy- Δ^4 -androstene-3,7-dione, effect of thyroid hormone. *J Clin Endocrinol Metab* 26: 949–954.
5. Fukushima DK, Dobriner H, Bradlow HL (1966) Synthesis and reactions of 3,11 β -dihydroxy- Δ^4 -androstene-17-one. *Biochemistry* 5: 1783–1789.
6. Schneider HJ, Buchheit U, Becker N, Schmidt G, Siehl U (1985) ^1H NMR analyses, shielding mechanism, coupling constants, and conformations in steroids bearing hydrogen, hydroxy, oxo groups, and double bonds. *J Am Chem Soc* 107: 7027–7039.