Daily Excretion Levels of an Unidentified Ketosteroid in the Urine of Patients with Cushing's Syndrome and Healthy Subjects Measured by a New Method

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Abstract. We developed a new method for measuring an unidentified ketosteroid glucuronide (US-G) detected by the method of Iwata et al. for measuring 17-ketosteroid glucuronides by reversed phase HPLC on a Capcell-Pak C8 column with three kinds of mobile phase solutions (Iwata method; Clin Chem 35: 795-799, 1989). The Iwata method inadequately separated US-G and two hydroxy 17-ketosteroids, 11β-hydroxyetiocholanolone and 11β-hydroxyandrosterone, and it exhibits insufficient sensitivity for measuring traces of US-G in the urine of healthy subjects. We solved these problems by developing a new method which measures US-G in urine, as a free type by hydrolyzing the glucuronide type enzymatically, by normal phase HPLC on a Capcell-Pak Silica column with one kind of mobile phase solution. By this method, the levels of US excreted as a glucuronide in the urine of healthy subjects and of patients with Cushing's syndrome were determined as proportions of the levels of 11β-hydroxyandrosterone. The average daily urinary excretion of US was 971 µg (125-4,995 µg) in patients with Cushing's syndrome (n=22: two males and 20 females aged 26 to 65 years), and 34 µg (0-141 µg) in healthy subjects (n=63: 49 males, and 14 females aged 21 to 54 years), and the differences were clearly significant. However, there were no differences between the urinary US levels of patients with pituitary adenoma and patients with adrenal adenoma. Furthermore, no US was detected in the urine of patients with aldosteronism (two males and eight females aged 34 to 61 years). The daily level of urinary US excretion in two of the patients with polycystic ovary syndrome was 159 and 142 µg, but no US was detected in the other two patients.

Key words: Unidentified ketosteroid glucuronide, 17-Ketosteroid glucuronides, Cushing's syndrome

WHEN 17-ketosteroid glucuronides (17KS-Gs) in the urine of patients with Cushing's syndrome were determined by a direct method without hydrolysis, by high-performance liquid chromatography (HPLC) with fluorometric detection under normal phase conditions, devised by Iwata et al. (Iwata method [1-4], a large peak of unidentified Ketosteroid glucuronide (US-G) appeared between the 11β-hydroxyetiocholanolone glucuronide (11OHE-G) peak and the 11β-hydroxyandrosterone glucuronide (11OHA-G) peak on the chromatograms. The US peak was not detected, or the levels yielded very small peaks in the urine of the healthy subjects [1, 5].

Furthermore, we tried hydrolyzing dansyl-US-G with β-D-glucuronidase and hydrolyzing the resultant dansyl-US with a strong acid to derive US,
and we found that US was swiftly decomposed by the strong acid.

While most of the commonly employed methods of measuring the principal constituent of urinary 17KS-G use a strong acid in the measurement process, the Iwata method does not. We think this is a primary factor in the discovery of US.

However, this method is unsuitable for measuring US, as it inadequately separates 11OHE-G, US-G and 11OHA-G peaks on the chromatogram, and its sensitivity is too low to be used in measuring urinary US-G in healthy subjects. To solve this problem, we have developed a new method suitable for measuring the US level. We have used the new method to measure the amount of US excreted in urine, and have compared the levels of patients with Cushing's syndrome and of healthy subjects.

Materials and Methods

Apparatus

The HPLC (model: 655A-11; Hitachi, Tokyo), was equipped with a controller (model: L-5000 LC), fluorescent spectrophotometer (model: F-1000), recorder (model: D-2000 chromato-integrator), and column oven (model: 655A-52).

The column for the HPLC was a 4.6 (i.d.) x 250 mm stainless-steel column packed with Capcell-Pak Silica (particle size: 5 µm in diameter), purchased from Shiseido Co., Tokyo.

Reagents

The reagents were as follows: dansylhydrazine (2 g/l) in acetonitrile, stored at 4 °C until use, acetate buffer (0.2 mol/l; pH 5.1), sodium hydroxide solution (0.25 mol/l), and β-D-glucuronidase extracted from calf liver (Wako Pure Chemical Co., Osaka).

To prepare the standard stock solution, we dissolved separately, each reagent in 10 ml of ethanol, 1 mg of 11-ketoandrostosterone (11COA), 16α-hydroxydehydroepiandrosterone (16OHDHEA), 11-ketoepiandrosterone (11COE), 11β-hydroxyandrosterone (11OHA), 16α-hydroxyandrostosterone (16OHA), and 11α-hydroxyetiocholanolone (11OHE) (Sigma Chemical Co.) to give a steroid concentration of 100 mg/l. We then combined one part of each of these solutions (six parts in all), with four parts of ethanol to give a final concentration of 10 mg/l for each steroid, and stored this mixture at 4 °C.

The working mobile-phase solvent was the organic layer separated from the mixture dichloromethane/2-hexanol/2-pentanol/2-butanol/water (500/1/20/1/5 by vol.) after shaking for 30 sec.

Procedure

We added 0.4 ml of acetate buffer to 0.6 ml of urine, then added 1,000 units of β-D-glucuronidase to the mixture and incubated it at 37 °C for 20 h.

After the incubation, we added 6 ml of dichloromethane, then shook the mixture for 1 min. The isolated dichloromethane layer was transferred to another test tube, to which 1 ml of sodium hydroxide solution was added. After shaking the mixture for 30 sec, the aqueous layer was removed, and the residue was washed in 2 ml of water. The dichloromethane layer was then transferred to another test tube, and 1 g of anhydrous sodium sulfate was added. After dehydration, 5 ml (fraction extracted from 0.5 ml of urine) of the dehydrated dichloromethane layer was evaporated.

The residue was mixed with 80 µl of dansylhydrazine solution followed by 20 µl of acetic acid, then stirred. After heating at 50 °C for one minute, the mixture was evaporated under reduced pressure at the same temperature, then heated at the same temperature for another 20 min. Similarly, 50 µl of the standard solution for determination (50 ng per steroid) was evaporated for dansylation.

The individual dansylated 17K5s were dissolved in 100 µl of dichloromethane, and 5–10 µl of each solution was injected into the chromatograph for determination under the following conditions for chromatography: column temperature of 25 °C flow rate of 1 ml/min, excitation wavelength of 330 nm, and emission wavelength of 540 nm.

Results

Figure 1 shows the chromatograms of four oxy-17KS-Gs produced by the Iwata method, and Fig. 2 shows the chromatograms of six oxy-17KS-Gs
produced by the new method for standard solutions and urine samples from healthy subjects and patients with Cushing’s syndrome. Each peak of the standard in Fig. 1 was obtained with 315 ng of 17KS-G, which was equivalent in volume to 200 ng each of 17KS, and that in Fig. 2 was obtained with 7 ng of 17KS. The fluorescence intensity values (peak height) for 11OHA and 11OHE obtained by the new method were 28.5 times and 29.6 times those obtained by Iwata’s method under the same sensitivity of the fluorescent spectrophotometer.

The new method yielded a recovery rate of 95.4% to 102.9% (n=10), and the within-day coefficient of variation (CV) determined by ten repeated mea-

**Fig. 1.** Chromatograms of four urinary oxy-17KS glucuronides produced by the Iwata method. a: standard, 315 ng each of 17KS glucuronides, which is equivalent to 200 ng each of 17KS. b: healthy man, 51 years-old. c: man with Cushing’s syndrome (adrenal adenoma), 29 years-old. d: woman with Cushing’s disease, 42 years-old. U, unidentified 17KS glucuronide; 1, 11β-hydroxyetiocholanolone glucuronide; 2, 11β-hydroxyandrostenedione glucuronide; 3, 11-ketoetiocholanolone glucuronide; 4, 11-ketoandrostenedione glucuronide.

**Fig. 2.** Chromatograms of six urinary oxy-17KS glucuronides produced by the new method. A: standard, 7 ng each of 17KS. B: healthy woman, 51 years-old. C: man with Cushing’s disease (adrenal adenoma), 53 years-old. U, unidentified 17KS; 1, 11β-hydroxyetiocholanolone; 2, 11β-hydroxyandrostenedione; 3, 11-ketoetiocholanolone; 4, 11-ketoandrostenedione; 5, 16α-dehydroepiandrostenedione; 6, 16α-hydroxyandrostenedione.
The average level for the 22 patients with Cushing’s syndrome was 971 µg (SD: 1,184 µg; 126–4,995 µg), the average level for the 17 patients with pituitary adenoma was 993 µg (SD: 1,333 µg; 126–4,995 µg), and for the five patients with pituitary adenoma, it was 900 µg (SD: 493 µg; 460–1,720 µg).

There was clearly a significant difference between the US excretion levels in the urine of the 63 healthy persons and the urine of the 22 patients with Cushing’s syndrome (critical region at α=0.01, f=81: T<sub>1</sub>=2.64, T<sub>c</sub>=7.60).

Two of the four patients with polycystic ovary syndrome associated with high urine 17KS levels had a US-G excretion level of 159 and 142 µg/day, although no US-G was detected in the urine of the other two.

No US-G was detected in the urine of any of the ten patients with aldosteronism (two males and eight females aged between 34 and 61 years).

**Discussion**

The Iwata method, by which US was detected, is a direct method for determining four sulfates and seven glucuronides of 17KS (four 17KS-Ss and seven 17KS-Gs) in urine, with the sulfate type and glucuronide type by HPLC.

The outline is as follows: After pretreatment of urine samples with a Sep-Pak C<sub>18</sub> cartridge, four 17KS-Ss and seven 17KS-Gs in the pretreated urine samples are reacted with tetraptammonium ions to form ion pairs. The ion-paired 17KS-Gs are extracted with benzene. By adding sodium sulfate to the remaining sample, we can then extract ion-paired 17KS-Gs with dichloromethane. Each extract is labeled with dansylhydrazine in an acetic acid-acetonitrile solution. The labeled steroids are separated by reversed phase HPLC on a Capcell-Pak C<sub>8</sub> (silicon-polymer-coated silica gel modified with octyl groups) column with three kinds of mobile phase solutions. However, it was difficult to quantitatively measure US-G in urine by Iwata’s method because of inadequate separation of US-G from 11OHE-G and 11OHA-G, and its low sensitivity (Fig. 1).

We tried to measure US liberated by hydrolyzing US-G with β-D-glucuronidase, which takes 20 h, to solve this problem because the fluorescence intensities of dansylated 17KSs in organic solvent were higher than those of dansylated 17KS-Gs in
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aqueous solution.

The new method developed by us is a method which measures US-G in urine, as a free type by hydrolyzing the glucuronide type with \( \beta \)-D-glucuronidase, by HPLC. It is a method for measuring six oxy-17KS-G (Fig. 2).

The outline is as follows: US-G in urine samples are hydrolyzed with \( \beta \)-D-glucuronidase, and free US liberated by hydrolysis is extracted with dichloromethane. The extract is labeled with dansylhydrazine in an acetic acid-acetonitrile solution. The labeled US is separated by normal phase HPLC on a Capcell-Pak silica (silicon-polymer-coated silica gel) column with one kind of mobile phase solution.

The new method yields improved separation of US-G from the two hydroxy-17KS-Gs (Fig. 2) as compared with the Iwata method (Fig. 1). Furthermore, since its sensitivity is about 30 times higher than that of the Iwata method, it permits researchers to measure the urinary US level in healthy individuals.

Given the satisfactory results of recovery and the simultaneous reproducibility tests, we conclude that the new method is highly reliable.

We determined the daily US excretion levels in the urine of the patients with Cushing's syndrome and the healthy subjects by the new method, and compared the levels of the patients with those of the healthy subjects. Figure 3 shows a marked difference between the daily US excretion levels in the urine of the patients with Cushing's syndrome and those of the healthy subjects. The marked increase in excretion in patients with Cushing's syndrome is clearly related to the etiology of the disease.

The 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 adenosomas. This suggests that the pituitary gland is not the site of US production.

Two of the four patients with polycystic ovary syndrome associated with high 17KS excretion levels had maximal urinary US excretion levels of 159 and 142 \( \mu \)g/day, although no US was detected in the urine of the other two patients.

It is also interesting that no US was detected in any of the patients with aldosteronism. The excretion level in the urine of the healthy subjects in their 20s tended to be higher than that in the urine of those in their 50s.

The urinary US excretion level in the patients with Cushing's syndrome was much higher than that in the healthy subjects, indicating that urinary US excretion levels may serve as a useful indicator for the diagnosis of Cushing's syndrome.

We are now preparing a report on clarification of the chemical structure of US by NMR spectrometry.

References