Characterization of Hydroxy-biphenyl-*O*-sulfates in Urine and Urine Crystals Induced by Biphenyl and $KHCO_3$ Administration in Rats

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In order to obtain information on the relationship between calculi and urine-crystal constituents, component analyses of the biphenyl sulfate conjugates in urine and urine crystals in rats fed a diet containing biphenyl and $KHCO_3$ were performed by LC-MS/MS and FT-IR. LC-MS/MS analysis revealed the presence of biphenyl metabolites, *i.e.*, three isomers of monohydroxy-biphenyl-*O*-sulfate (HBPOS) and five isomers of dihydroxy-biphenyl-*O*-sulfate (DHBPOS), in rat urine. The same results were obtained for the crystals in rat urine. These findings suggested that the metabolism of biphenyl resulting in the formation of sulfate conjugates follows the process: biphenyl → mono- or dihydroxylation → sulfate conjugation. FT-IR analysis of the urine crystals indicated that the major constituent was the potassium salt of 4-hydroxy-biphenyl-*O*-sulfate (4-HBPOS), which is known to be the main constituent of urinary bladder calculi. Consequently, in view of the similarity of the major constituent, the potassium salt of 4-HBPOS, in both the urine crystals and calculi, it is thought that the formation of the calculi can be attributed to the lower solubility of the potassium salt of 4-HBPOS, as compared to the other sulfate conjugates.

Key words —— metabolite, biphenyl, sulfate conjugate, rat urine, LC-MS/MS

INTRODUCTION

Recently, the relationship between calculi induced by chemicals such as biphenyl, uracil, *etc*., and tumors in the urinary bladder of rats has attracted a lot of medical attention.[1–4] We have investigated the physicochemical characteristics of the calculi induced by biphenyl administration in rats. We have reported that the main component of biphenyl-induced calculi in the urinary bladder of male rats is the potassium salt of 4-hydroxy-biphenyl-*O*-sulfate (4-HBPOS).[5] In view of this finding, it was predicted that the potassium salt of 4-HBPOS is apt to crystallize from urine at higher pH. With regard to the rat's urinary features, Lina *et al*.[6] have presented interesting results obtained in studies on the role of the alkalizing salt $KHCO_3$ and the neutral salt KCl in the diet, indicating that a comparable increase in urinary volume and potassium levels was found with both $KHCO_3$ and KCl, but only $KHCO_3$ induced an increase in urinary pH. Moreover, numerous studies concerning urinary metabolites produced from biphenyl in rats have been conducted by GC-MS analysis after treatment with aryl-sulfatase and β-glucuronidase to convert them to their trimethylsilyl ethers.[7,8] Biphenyl has been found to be metabolized *via* multiple pathways resulting in the production of various urinary metabolites such as conjugates (sulfate and glucuronide) of 4-hydroxy-biphenyl (4-HBP), and 4,4'-dihydroxy-biphenyl (4,4'-DHBP) produced as major metabolites, and conjugates of 2-hydroxy-biphenyl (2-HBP), 3-hydroxy-biphenyl (3-HBP), and 3,4-dihydroxy-biphenyl (3,4-DHBP) produced as minor metabolites.[7,8] However, the conventional analytical procedures involving the use of enzymes have been unable to discriminate between sulfate and glucuronide conjugates. Thus, a direct procedure such as LC-MS/MS is useful to analyze and discriminate between these conjugates. So far, we have analyzed the sulfate conjugates in urinary calculi in rats by LC-MS/MS.[9] As a result, it was found that only the sulfate conjugate of 4-

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HBP is significantly involved in the formation of urinary calculi.

In this paper, in order to obtain information on the relationship between calculi and urine-crystal constituents, analyses of the components of sulfate conjugates in urine and urine crystals in rats fed a diet containing biphenyl and $KHCO_3$ were performed by LC-MS/MS and FT-IR, taking into account the results of Lina *et al*.[6]

MATERIALS AND METHODS

Chemicals —— Biphenyl, 2-HBP, 3-HBP, 4-HBP, 2,3-dihydroxy-biphenyl (2,3-DHBP), 4,4'-DHBP and $KHCO_3$ were obtained from Wako Pure Chemical Ind. (Osaka, Japan). 3,4-DHBP was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). 2,5-Dihydroxy-biphenyl (2,5-DHBP) was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Various hydroxybiphenyl sulfate conjugates were synthesized according to the method of Hoiberg and Mumma[10] based on the reaction of the appropriate hydroxybiphenyls and sulfuric acid with dicyclohexyl-carbodiimide. The potassium salt of 4-HBPOS was prepared by adding 20% potassium hydroxide aqueous solution to the reaction solution, whereby it was precipitated from solution. The precipitate was recrystallized from ethanol. It should be noted that potassium salts of the other sulfate conjugates could not be obtained as precipitates by the same method of treatment. On the other hand, precipitates of sodium salts of the following various sulfate conjugates were obtained by a salting-out technique using saturated sodium chloride solution[11]: 4-HBPOS, 4,4'-dihydroxy-biphenyl-*O*-sulfate (4,4'-DHBPOS), 2-hydroxy-biphenyl-*O*-sulfate (2-HBPOS), 3-hydroxy-biphenyl-*O*-sulfate (3-HBPOS), 3,4-dihydroxy-biphenyl-3-*O*-sulfate (3,4-DHBP-3-OS) and 3,4-dihydroxy-biphenyl-4-*O*-sulfate (3,4-DHBP-4-OS). Two isomers of 2,3-dihydroxy-biphenyl-2-*O*-sulfate or 2,3-dihydroxy-biphenyl-3-*O*-sulfate (2,3-DHBPOS) and two isomers of 2,5-dihydroxy-biphenyl-2-*O*-sulfate or 2,5-dihydroxy-biphenyl-5-*O*-sulfate (2,5-DHBPOS) were not obtained by salting-out, and the material in solution was used for the experiment.

Animals and Their Maintenance —— Male F344/DuCrj rats were purchased from Charles River Japan, Inc. (Atsugi, Japan). The animals were housed individually in stainless-steel wire-mesh cages (170 W × 294 D × 176 H; dimensions in millimeters). Room temperature and the relative humidity were controlled at 23 ± 0.2°C and 57 ± 1%, respectively. Fluorescent lighting was provided, with a 12 h light/dark cycle.

Diet Preparation —— The diet containing biphenyl and $KHCO_3$ was prepared by mixing then into CRF-1 powdered diet (Oriental Yeast Co., Ltd., Chiba, Japan) in a stainless-steel ribbon mixer for 20 min. Groups of 5 male rats were fed the diet containing 1.6% biphenyl and 5.0% $KHCO_3$ for 7 d.

Sample Preparation for Analysis of Urine and Urine Crystals —— Urine samples were collected from five rats fed the biphenyl-containing diet in polycarbonate metabolic cages between days 6 and 7, and then pooled as one sample. The precipitates were separated from the urine by centrifugation, and then dissolved in acetonitrile. The acetonitrile solution was centrifuged and the supernatant was evaporated under vacuum at 40°C to obtain the urine crystals. The remaining urine diluted with acetonitrile or the urine crystals dissolved in acetonitrile were used for LC-MS/MS analyses of the biphenyl sulfate conjugates.

LC-MS/MS Chromatography —— LC-MS/MS analysis was performed under the same conditions as those previously reported.[9] Biphenyl metabolites in the urine, urine crystals, and standard samples were separated by LC-MS/MS (Thermoquest TSQ-7000, CA., U.S.A.). The chromatography was carried out using a Tosoh TSK-Gel ODS-80TS column (Tokyo, Japan) (150 × 2.0 mm i.d.) at a flow rate of 0.25 ml/min, with the column at ambient temperature. The mobile phase was acetonitrile-5 mM ammonium acetate (23 : 77, v/v). Monohydroxy-biphenyl-*O*-sulfates (HBPOS) were measured on the basis of the peaks in the MS chromatograms by adjusting the mass of the precursor ions to 249 *m/z* and then mass of the product ions to 169 *m/z* (Figs. 1 and 2A). Dihydroxy-biphenyl-*O*-sulfates (DHBPOS) were measured on the basis of the peaks in the MS chromatograms by adjusting the mass of precursor ions to 265 *m/z* and the mass of the product ions to 185 *m/z* (Figs. 1 and 2, B).

Analysis —— FT-IR analyses of the urine crystals and standard samples (potassium salt of 4-HBPOS, sodium salt of 4-HBPOS) were performed using a Shimadzu FTIR-8200PC (Kyoto, Japan) in the wave number region of 1350–1000 cm^{-1} with a resolution of 4 cm^{-1} by the KBr method. ^{1}H-NMR analysis was performed using a JEOL JNM-LA500 (Tokyo, Japan) for the sodium salts of 3,4-DHBP-3-OS and 3,4-DHBP-4-OS in deuterated methanol.

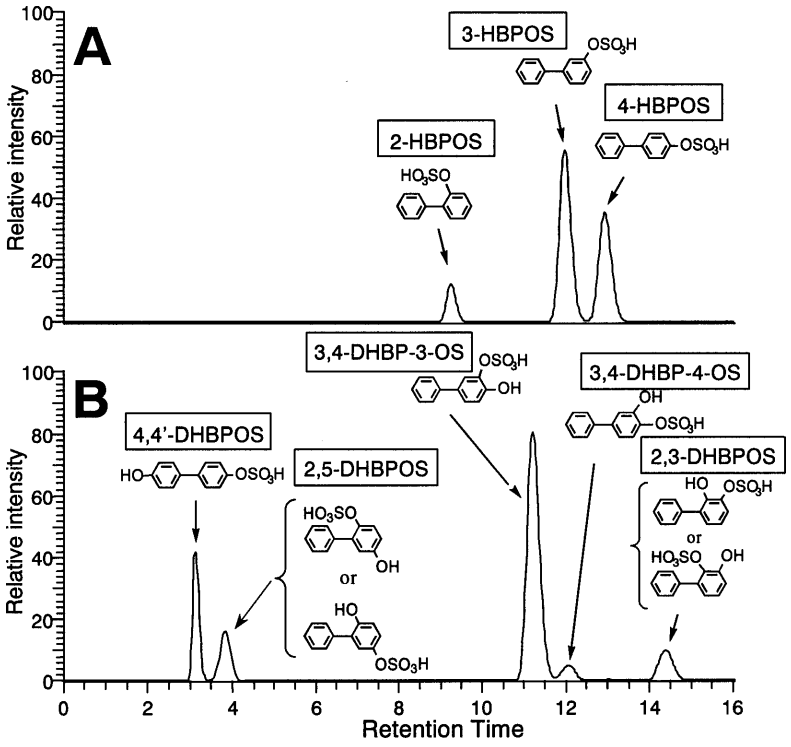

Fig. 1. MS Chromatograms Obtained for Biphenyl Sulfate Conjugates in the Urine of Rats Fed a Diet Containing Biphenyl and $KHCO_3$

(A) MS analysis conditions: the mass of the precursor ion was adjusted to 249 *m/z* and the mass of the product ions was adjusted to 169 *m/z*. (B) MS analysis conditions: the mass of the precursor ion was adjusted to 265 *m/z* and the mass of the product ions was adjusted to 185 *m/z*.

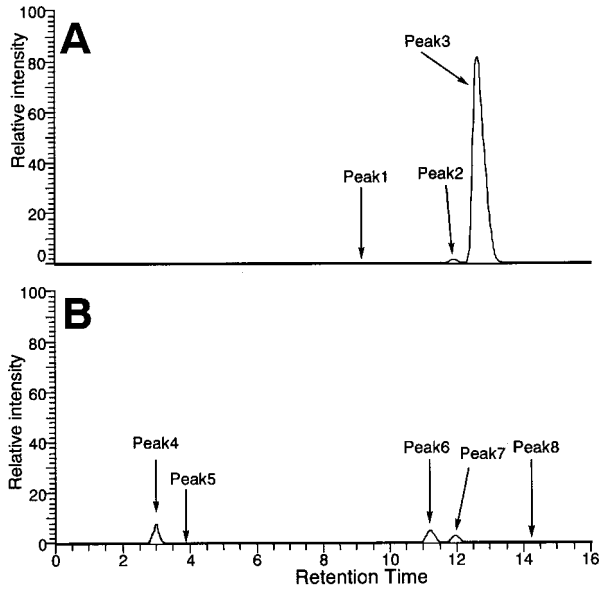

Fig. 2. MS Chromatograms Obtained for Biphenyl Sulfate Conjugates among the Constituents of Urine Crystals in Rats Fed a Diet Containing Biphenyl and $KHCO_3$

(A) MS analysis conditions were such that the mass of the precursor ion was adjusted to 249 *m/z* and the mass of the product ion was adjusted to 169 *m/z*. (B) MS analysis conditions were such that the mass of the precursor ion was adjusted to 265 *m/z* and the mass of the product ion was adjusted to 185 *m/z*. Each of Peaks 1, 5, and 8 had a relative content of less than 1% (see Table 1).

RESULTS AND DISCUSSION

MS Chromatograms Obtained for Sulfate Conjugates in Urine

Figure 1 shows the MS chromatograms obtained for the sulfate conjugates in the urine of rats fed the diet containing biphenyl and $KHCO_3$. The results of analysis showed the presence of biphenyl metabolites, *i.e.*, three isomers of HBPOS and five isomers of DHBPOS, in the rat urine. The three isomers of HBPOS were identified as 2-, 3-, and 4-HBPOS, and the five isomers of DHBPOS as 4,4'-DHBPOS, 3,4-DHBP-3-OS, 3,4-DHBP-4-OS, 2,3-DHBPOS, and 2,5-DHBPOS through comparison with the corresponding standard samples. The chemical structure of each of the isomers is shown in Fig. 1. It should be noted that the chemical structures of the isomers of 3,4-DHBPOS (3,4-DHBP-3-OS and 3,4-DHBP-4-OS) which were obtained by separation through HLPC under the same conditions as in Fig. 1, were analyzed from the ^{1}H-NMR spectra. Table 1 summarizes the levels of the biphenyl sulfate conjugates in rat urine, including those in the urine crystals (described below), where the contents were estimated from the areas of the peaks in the MS chromatograms. Among the biphenyl sulfate conjugates

Table 1. Content of Biphenyl Sulfate Conjugates in Rat Urine, Including Urine Crystals

Biphenyl sulfate conjugates	Urine (%)	Urine crystals (%)
2-HBPOS (Peak 1)	3.32[a)]	0.06
3-HBPOS (Peak 2)	23.37	1.06
4-HBPOS (Peak 3)	11.94	89.45
4,4′-DHBPOS (Peak 4)	7.17	3.11
2,5-DHBPOS (Peak 5)	5.62	0.02
3,4-DHBP-3-OS (Peak 6)	40.88	3.90
3,4-DHBP-4-OS (Peak 7)	2.27	2.28
2,3-DHBPOS (Peak 8)	5.43	0.12

a) The component fraction (%) for each of the sulfate conjugates was estimated from the ratio of the LS-MS/MS peak area of the sulfate to the total area.

present, 3-HBPOS, 4-HBPOS and 3,4-DHBP-3-OS accounted for more than 70% of the total. Consequently, it is evident that the present LC-MS/MS procedures resulted in accurate separation and identification of the various isomers of the biphenyl sulfate conjugates, which could not be definitely identified by the conventional GC-MS procedures using enzymes. The results of analysis of the MS chromatograms to suggested that the metabolism of biphenyl resulting in the formation of sulfate conjugates follows the process: biphenyl → mono- or dihydroxylation → sulfate conjugation.

MS Chromatograms Obtained for Sulfate Conjugates as Urine-Crystal Constituents

Figure 2 shows the MS chromatograms obtained by analysis of the sulfate conjugates as constituents of urine crystals in rats. Three peaks appeared in the MS chromatograms obtained by adjusting the mass of precursor ions to 249 *m/z* and the mass of the product ions to 169 *m/z* (A), whereas five peaks appeared by adjusting the mass of precursor ions to 265 *m/z* and the mass of the product ions to 185 *m/z* (B). From the retention times, the former were consistent with the MS chromatograms corresponding to the three isomers of HBPOS, and the latter were consistent with those corresponding to the five isomers of DHBPOS. As shown in Table 1, in comparison with the results for urine, the major constituent in urine crystals was found to be 4-HBPOS, accounting for about 90% of the total. This result is thought to be attributable to the lower solubility of 4-HBPOS in urine, as compared to the other constituents.

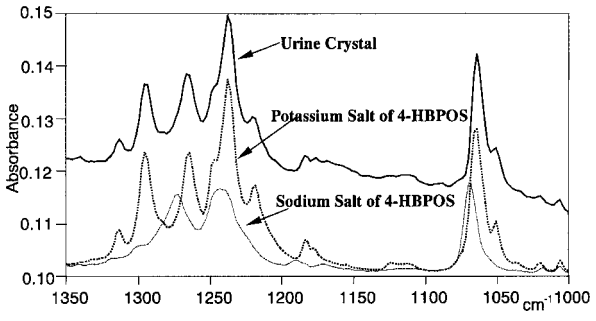

Fig. 3. FT-IR Spectrum Obtained for the Urine Crystals, and, for Comparison, Those Obtained for the Potassium and Sodium Salts of Synthesized 4-HBPOS

FT-IR Spectra Obtained for Urine Crystals in Rats

Figure 3 shows the FT-IR spectrum obtained for the urine crystals, and for comparison, those obtained for the potassium and sodium salts of synthesized 4-HBPOS. The FT-IR spectrum obtained for the urine crystals showed bands in the wavenumber region of 1000–1350 cm^{-1} assigned as the stretching vibration of S–O in the sulfate group. The spectral pattern observed in the case of the urine crystals apparently coincided with that of the potassium salt rather than that of the sodium salt. This result implies that the major component of the urine crystals is the potassium salt of 4-HBPOS, which is known to be the main constituent of urinary bladder calculi.[5)]

Consequently, in view of the conformity of the major constituent (potassium salt of 4-HBPOS), when comparing urine crystals and calculi, it is thought that the formation of calculi is attributable to the lower solubility of the potassium salt of 4-HBPOS, as compared to the other sulfate conjugates.

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