Letter

Identification of an *N*-acetylcysteine conjugate in the urine after oral administration of 2,4-dichloro-1-nitrobenzene to rats

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ABSTRACT — Oral administration of 2,4-dichloro-1-nitrobenzene (2,4-DCNB) causes kidney tumors in the rat. The objective of the present study was to identify the chemical structure of 2,4-DCNB metabolites in urine. Urine from 2,4-DCNB fed rats was more yellow than urine from control rats, exhibiting a broad UV-spectrum around a peak wavelength of 360 nm; the control urine did not have an absorbance. The yellow component was extracted and analyzed. The optical properties of the yellow component were the same as the *N*-acetylcysteine conjugate of 1,4-dichloro-2-nitrobenzene (1,4-DCNB); 1,4-DCNB is secreted in urine as an *N*-acetylcysteine conjugate. LC-MS/MS analyses of this yellow component demonstrated its chemical structure to be the *N*-acetylcysteine conjugate of 2,4-DCNB. Nuclear overhauser effect and LC-MS/MS analyses revealed the structural isomer of this 2,4-DCNB metabolite as *N*-acetyl-*S*-(5-chloro-2-nitrophenyl)-*L*-cysteine. We also discuss the possibility that the *N*-acetylcysteine conjugate identified in this study plays a role as a proximate carcinogen in the formation of kidney tumors.

Key words: 2,4-dichloro-1-nitrobenzene, Metabolites, *N*-acetylcysteine, Rats, NOE, LC-MS/MS

INTRODUCTION

Toxic effects of aromatic compounds containing chlorine and nitro groups have recently attracted a great deal of attention (Kano *et al.*, 2006; Matsumoto *et al.*, 2006a, 2006b; Yamazaki *et al.*, 2006). Kano *et al.* (2006) reported that 2,4-dichloro-1-nitrobenzene (2,4-DCNB) in the diet induced kidney tumors in rats. Bray *et al.* (1957) studied the metabolism of 2,4-DCNB in rabbits and reported that it is metabolized to *N*-acetyl-*S*-(5-chloro-2-nitrophenyl)-*L*-cysteine (A5C2NPC). However, identification of metabolite of 2,4-DCNB in the urine of rats has not yet formally been made. The objective of the present study was to identify this metabolite.

Dietary administration of 1,4-dichloro-2-nitrobenzene (1,4-DCNB), which causes liver tumors in rats (Yamazaki *et al.*, 2006), results in a distinctive yellowing of the urine of the test animal, and this yellowing is caused by an *N*-acetylcysteine conjugate of 1,4-DCNB, *N*-acetyl-*S*-(4-

chloro-3-nitrophenyl)-*L*-cysteine (A4C3NPC) (Ohnishi *et al.*, 2004). Similarly, dietary administration of 2,4-DCNB to rats also results in a distinctive yellowing of urine of the test animal. In this report, we demonstrate that the yellowing is caused by an *N*-acetylcysteine conjugate of 2,4-DCNB in rats, A5C2NPC.

MATERIALS AND METHODS

Chemicals

2,4-DCNB, high performance liquid chromatography (HPLC)-grade acetonitrile and distilled water were obtained from Wako Pure Chemical Ind. (Osaka, Japan).

Animals and their maintenance

Male F344/DuCrj rats (SPF) were purchased at 8 weeks of age from Charles River Japan, Inc. (Kanagawa, Japan). The animals were housed in polycarbonate metabolic cages. Room temperature and relative humidity were controlled.

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led at $23 \pm 2^\circ\text{C}$ and $55 \pm 15\%$, respectively. The animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). The present study was approved by the ethics committee of the Japan Bioassay Research Center.

Diet preparation

A diet containing 2,4-DCNB was prepared by mixing 2,4-DCNB into CRF-1 powdered diet (Oriental Yeast Co., Ltd., Chiba, Japan) in a spiral mixer for 20 min. A group of 3 male rats was fed the diet containing 1.0% 2,4-DCNB for 2 days.

Sample collection of urine

Urine samples were collected from 3 rats fed the 2,4-DCNB-containing diet in polycarbonate metabolic cages for 24 hr, and then were pooled as one sample.

Sample preparation for UV analysis of urine

The urine from the rats fed 2,4-DCNB and control urine were separately dissolved in distilled water (DW). The UV spectra of these samples were obtained by measurement with a UV spectrometer.

Sample preparation for $^1\text{H-NMR}$ and LC-MS/MS analyses of urine

The pH of 8 ml of urine was adjusted to 1.2–1.3 by adding 2 ml of 10% trifluoroacetic acid. The yellow component was extracted using Oasis HLB columns from Waters Corp. (Milford, CT, USA), which were pretreated with 3 ml acetonitrile and then 3 ml DW. The water in the column was removed by centrifugation ($400 \times g$ for 10 min), and the yellow component was subsequently eluted by 3 ml acetonitrile. The elute was evaporated under vacuum at 40°C to dryness. The residue dissolved in the mobile phase of LC-MS/MS was used for LC-MS/MS analyses of the 2,4-DCNB conjugates. Residue dissolved in deuterated methanol was used for $^1\text{H-nuclear magnetic resonance (NMR)}$ analyses of the 2,4-DCNB conjugates.

UV and $^1\text{H-NMR}$ analysis

UV analysis was performed using a UV-2400PC (Shimadzu, Kyoto, Japan). $^1\text{H-NMR}$ analysis was performed using a ECA400 (JEOL, Ltd., Tokyo, Japan) by the nuclear overhauser effect (NOE) method (Breyer-Pfaff *et al.*, 1987; Kaburagi *et al.*, 2000) for the 2,4-DCNB conjugates.

LC-MS/MS analysis

LC-MS/MS analyses were performed using an HP 1090 (Hewlett Packard, Waldbronn, Germany) and a TSQ

7000 (Thermo Quest, San Jose, CA, USA). HPLC was carried out using flow injection at a flow rate of 0.2 ml/min. The mobile phase was acetonitrile–0.02% acetic acid (3 : 2, v/v). The mode of ionizing in the mass spectrometer was executed with Electro Spray Ionization. The mass spectrometer was operated in negative ion mode with a potential of 4.5 kV applied to the electro spray needle. Dried nitrogen gas was used as the sheath gas (70 psi) and auxiliary gas (5 units) to assist with nebulization. The capillary temperature was held at 200°C .

RESULTS AND DISCUSSION

Urine from the 2,4-DCNB fed rats was more yellow than urine from control rats. The UV spectra obtained for urine from 2,4-DCNB fed rats and control urine. Urine from the 2,4-DCNB fed rats exhibited a broad UV-spectrum at around a peak wavelength of 360 nm, whereas the control urine did not have an absorbance. 1,4-DCNB fed rats show an absorbance peak at a wavelength of 383 nm (Ohnishi *et al.*, 2004), suggesting the possibility that major metabolites of 2,4-DCNB found in urine of 2,4-DCNB fed rats is the same species, an *N*-acetylcysteine conjugate, as that found in urine of 1,4-DCNB fed rats (Ohnishi *et al.*, 2004). Also, it is highly likely that halogenonitrobenzenes, such as 2,4-DCNB are conjugated with glutathione in the liver and secreted in the urine as a mercapturic acid (Barnes *et al.*, 1959). Therefore, the yellow component in the urine of 2,4-DCNB fed rats was extracted and analyzed initially as an *N*-acetylcysteine conjugate.

We then analyzed the urinary metabolite using LC-MS/MS. In rats fed 1,4-DCNB, a characteristic fragment of the *N*-acetylcysteine conjugate of 1,4-DCNB could be clearly observed at m/z 187 after fragmentation of the precursor ion at m/z 317 (Ohnishi *et al.*, 2004). This characteristic fragment can also be observed in the MS spectrum of the urinary metabolite of rats fed 2,4-DCNB (Fig. 1). The urine from the 2,4-DCNB fed rats exhibited a metabolite peak by LC-MS/MS, whereas the control urine did not have a peak. This fragment is in agreement with the calculation value of the *N*-acetylcysteine moiety of A5C2NPC.

Fig. 2 shows the NOE spectrum of the component and the chemical structure with proton numbering. The metabolite shows the presence of three protons which suggest hydrogens of an aromatic ring: H-a (8.14 ppm; doublet), H-b (7.68 ppm; doublet), and H-c (7.35 ppm; double doublet), upper axis in Fig. 2. Two NOE peaks were observed at the crossing position between protons H-b and proton H-d: proton H-d showed doublet and double doublet at

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Fig. 1. LC-MS/MS spectrum of the yellow components in urine from rats fed a diet containing 2,4-dichloro-1-nitrobenzene.

3.32 ppm and at 3.62 ppm, side axis in Fig. 2. Another NOE peak was observed at the crossing between protons H-b and H-e: proton H-e showed a double doublet peak at 4.66 ppm, side axis in Fig. 2. These results indicate that the protons H-d and H-e are located near the position of proton H-b. Taken together, these results are entirely consistent with proton H-d and H-e residing in the *N*-acetylcysteine moiety of *N*-acetylcysteine conjugated 2,4-DCNB. Finally, our results suggest that the *S*-conjugated chlorine is not located at the 4-position but at the 2-position of the benzene ring.

The difference between the urine metabolites of 2,4-DCNB and 1,4-DCNB is that the *N*-acetylcysteine and the nitro groups are in the *ortho* position on the benzene ring of the 2,4-DCNB metabolite and in the *meta* position on the benzene ring of the 1,4-DCNB metabolite: *N*-acetylcysteine is in the 2-position and the nitro group is in the 1-position in A4C3NPC while *N*-acetylcysteine is in the 4-position and the nitro group is in the 2-position in A5C2NPC. One important aspect of this finding is highlighted by the fact that in humans, β -naphthylamine

induces urinary bladder tumors whereas α -naphthylamine does not. While the mechanism behind this difference is beyond the scope of the present study, we can speculate on the metabolic mechanism behind 2,4-DCNB induced renal toxicity. Nash *et al.* (1984) and Dekant *et al.* (1986) studied the toxic effects of chlorinated aliphatic compounds. Nash *et al.* (1984) reported that nephrotoxic and nephrocarcinogenic effects were induced by hexachlorobutadiene (HCB) and that the effects were caused by the glutathione conjugate of HCB being degraded to its equivalent cysteine conjugate which was subsequently cleaved by renal β -lyase to produce a toxic thiol which caused localized kidney damage. Similarly, Dekant *et al.* (1986) reported that trichloroethylene was metabolized to the *N*-acetylcysteine conjugate 4-acetyl-*S*-(1,2-dichlorovinyl)-*L*-cysteine through the action of renal β -lyase to induce its nephrotoxic effects. Therefore, we propose the scheme of metabolic mechanism shown in Fig. 3 as the pathway by which ingested 2,4-DCNB is metabolized to A5C2NPC and induces renal cancers.

Fig. 2. NOE spectrum of the yellow components in urine from rats fed a diet containing 2,4-dichloro-1-nitrobenzene.

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Fig. 3. Proposed metabolism mechanism by which 2,4-DCNB exerts renal carcinogenicity.

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