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Determination of hemoglobin adducts formed in rats exposed orally with 3,3'-dichlorobenzidine by GC/MS-SIM

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3,3'-dichlorobenzidine (DCB) can be metabolically N-acetylated and/or N-oxidized, and can form hemoglobin adducts. Gas chromatography/mass spectrometry-selected ion monitoring detection mode (GC/MS-SIM) could be a good analytical method to detect them. 4-Aminobiphenyl and phenanthrene-d₁₀ were used as internal standards, and standard metabolites of DCB were synthesized from DCB. Pyridine is a promoter and acetic acid is a controller in the acetylation of DCB during titrating with acetyl chloride. After washing with acetone, the purity of N-acetyl DCB and N,N'-diacetyl DCB were 98.72% and 98.82%, respectively.

The maximum detection limits (MDLs) by GC/MS-SIM were 0.5 µg/L in DNA adduct and 1.0 ng/g in hemoglobin for DCB and N-acetyl DCB. The base peaks of their fragmentation pattern were 252 *m/z* at the peak of DCB, 252 *m/z* and 294 *m/z* at the peak of N-acetyl DCB, and 252 *m/z*, 294 *m/z*, and 336 *m/z* at the peak of N,N'-diacetyl DCB. This analytical method was applied to determine hemoglobin adducts formed in young female Sprague–Dawley rats orally exposed with 20, 30, and 40 mg DCB/kg/day for three weeks. Two adducts were detectable by GC/MS-SIM after alkaline hydrolysis of hemoglobin samples and extraction. The structure of these adducts could be assigned to DCB and N-acetyl DCB by co-chromatography with the synthetic standards.

After the first week of treatment, the total amount of hemoglobin adducts determined was 837.5 ~ 2501 ng/g hemoglobin. The adduct levels were increased up to 1203.3 ~ 2605.4 ng/g after the second week, and slightly decreased after the third week. The ratio of DCB and N-acetyl DCB was nearly similar in all treatment groups at the third week, such as 4.28 ~ 4.78. Three different treatments (20, 30, and 40 mg DCB/kg) of rats resulted in dose-proportional increases in the total and DCB amount of hemoglobin adducted formed for three weeks. The relative contribution of DCB and N-acetyl DCB to the total hemoglobin adduct level was strongly dose dependent. The results show that GC/MS-SIM is suitable for the biological monitoring of humans exposed to DCB or DCB-containing products *Toxicology and Industrial Health* 2002; 18: 191–199.

Key words: *biological monitoring; GC/MS-SIM; N-acetyl DCB; hemoglobin adducts; 3,3'-dichlorobenzidine (DCB)*

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Introduction

3,3'-dichlorobenzidine (DCB) has been shown to be carcinogenic in several animals (Pliss, 1959; Sellakumar *et al.*, 1969; Stula *et al.*, 1975; 1978; IARC, 1982). It is used in the production of pigments for printing inks, textiles, plastics and enamels, paints, leather, and rubber (DHHS, 1998).

DCB is absorbed readily through the skin, and it is essential in occupational health and for risk assessment to have fast and reliable methods to monitor the exposure of humans. Blood is an invasive surrogate for biological monitoring of persons exposed with DCB. Hemoglobin adducts are suitable biomarkers for the exposure of aromatic amines. They are used as marker for internal exposure, the metabolic activation of carcinogenic amines, and the dose in the target organ. The presence of adducts indicates that the potential toxic or genotoxic metabolites are bioavailable in target organs (Sannioni and Beyerbach, 2000).

Like other aromatic amines, DCB can be metabolically N-acetylated and/or oxidized to the corresponding N-hydroxyl amines. N-acetylation appears to be the major path for the metabolism of DCB in mammals (Tanaka, 1981). A 24-hour urine sample of rats given a single oral dosage of DCB (50 mg/kg/day) contained unchanged DCB, N,N'-diacetyl 3,3'-dichlorobenzidine (di-acDCB), and N-acetyl 3,3'-dichlorobenzidine (acDCB) in a ratio of 1:3:10 (Tanaka, 1981). Indirect evidence for the formation of nitro derivatives was found in a study in which DCB was administered to female Wistar rats by gavages (Birner *et al.*, 1990). The nitroso compounds of DCB are known to react with sulfhydryls in cysteine residues of hemoglobin to give a stable sulfuric acid amide (Albrecht and Neumann, 1985; Birner *et al.*, 1990; Eyer and Gallemann, 1996)

So we firstly synthesized the metabolites of DCB, such as di-acDCB and acDCB. Pyridine and acetic acid are the promoter and controller, respectively, in acetylation of DCB (Lee and Lee, 2002). We analysed DCB and its metabolites adducted with hemoglobin in the blood of rats exposed orally with DCB by gas chromatography/mass spectrometry-selected ion monitoring (GC/MS-SIM). At neutral pH, the sulfuric acid amides of hemoglobin adducts are stable, but they are readily hydrolyzed under

basic conditions, releasing the parent aromatic amine (Neumann, 1984).

Materials and methods

Chemicals

3,3'-dichlorobenzidine 2HCl (DCB 2HCl) was obtained from Sigma (St. Louis, MO, USA). Analytical grades of 4-aminobiphenyl, phenanthrene-d₁₀, potassium carbonate, potassium hydroxide, potassium bishydrogen phosphate, sodium sulfate, pyridine, toluene, acetyl chloride and acetic anhydride (Sigma, St. Louis, MO, USA) were used as reagents. Ethyl ether, methanol, ethanol, acetone and ethyl acetate (E. Merck, Darmstadt, Germany) were used as solvents. All other chemicals were of the highest purity available from Sigma and Merck.

Animals and treatment

Eighty female Sprague–Dawley rats with a body weight of about 220 g, were obtained from Hae-hanbiolink (Chongju, South Korea). They were acclimatized for one week in Maecrlone cages (temperature, 18°C; humidity, 30–70%; illumination time, 06.00 h to 18.00 h), and had free access to tap water and food. Twenty rats were randomly assigned to each group. DCB was orally given to treatment groups daily, in dosages of 20, 30 and 40 mg DCB/kg body weight, for three weeks. Controls were given water-containing citric acid and sucrose orally in the same manner as the treated animals. Because of the low solubility of DCB in water, the dosing solution was dissolved with DCB in water containing citric acid and sucrose (1:1). Heparinized blood samples were collected three times with seven-day intervals from five rats in each group.

Isolation of hemoglobin

Erythrocytes were washed three times with phosphate buffered saline (PBS), and lysed with four volumes of 0.1 mM EDTA, pH 7.5 at 35–41°C in an ultrasonic bath. Cell debris was removed by centrifugation (10 000g). Hemoglobin was isolated by precipitation with acetonitrile and washed twice

with acetonitrile/water, and finally with 80% ethanol, 96% ethanol, ethanol/diethyl ether and diethyl ether. Dried hemoglobin was stored at 4°C and used for samples of hemoglobin adducts (Joppich-Kuhn *et al.*, 1997)

Determination of hemoglobin adducts by GC/MS

200 mg of dried hemoglobin was hydrolyzed for three hours at room temperature with 3 mL of 0.1 M NaOH. DCB and its metabolites released from hemoglobin adducts were extracted with 7 mL ethyl ether by mechanical shaking for 10 minutes. The organic phase was transferred into a 20 mL glass stoppered test tube and dried in an evaporator. It was dissolved with 100 µL ethyl acetate and analyzed by GC/MS.

4-aminobiphenyl and phenanthrene-d₁₀ were added as internal standards for blank and each sample. Standard metabolites of DCB were synthesized from DCB by using pyridine and acetic acid (Lee *et al.*, 2003).

Conditions of gas chromatography-mass spectrometry

All mass spectra were obtained with 6890/5973 GC-MSD (Agilent Technologies; Palo Alto, CA, USA). The ion source was operated in the electron ionization mode (EI: 70 eV, 230°C). Full-scan mass spectra (m/z 40 ~ 800) were recorded for

identification of analysts. Detection modes were nitrogen phosphorous detection (NPD) and selected ion monitoring detection mode (SIM). Column was HP-5MS (30m × 0.25 mm i.d. × 0.25 µm F.T.). Samples were injected in the pulsed split ratio (1/15). The flow rate of the helium was 1.0 mL/minute. The GC operating temperatures were: injector temperature, 280°C; transfer line temperature, 280°C; oven temperature, programmed from 100°C at 20°C/minute to 310°C (held for two minutes) (Lee *et al.*, 2003; Shin *et al.*, 2003).

Results

Identification of DCB and its metabolites

Saturated phenol was used as solvent for identifying DCB, N-acetyl DCB and N,N'-diacetyl DCB with GC/MS-SIM. Figure 1 shows chromatograms of them. Figure 2 shows fragmentation patterns of DCB, N-acetyl DCB and N,N'-diacetyl DCB. The base peak was 252 m/z at the peaks of DCB, N-acetyl DCB, and N,N'-diacetyl DCB.

Synthesis of metabolite of DCB, N-acetyl DCB

Metabolites of hemoglobin adducts were monoacetyl-DCB and diacetyl-DCB. So DCB and mono-

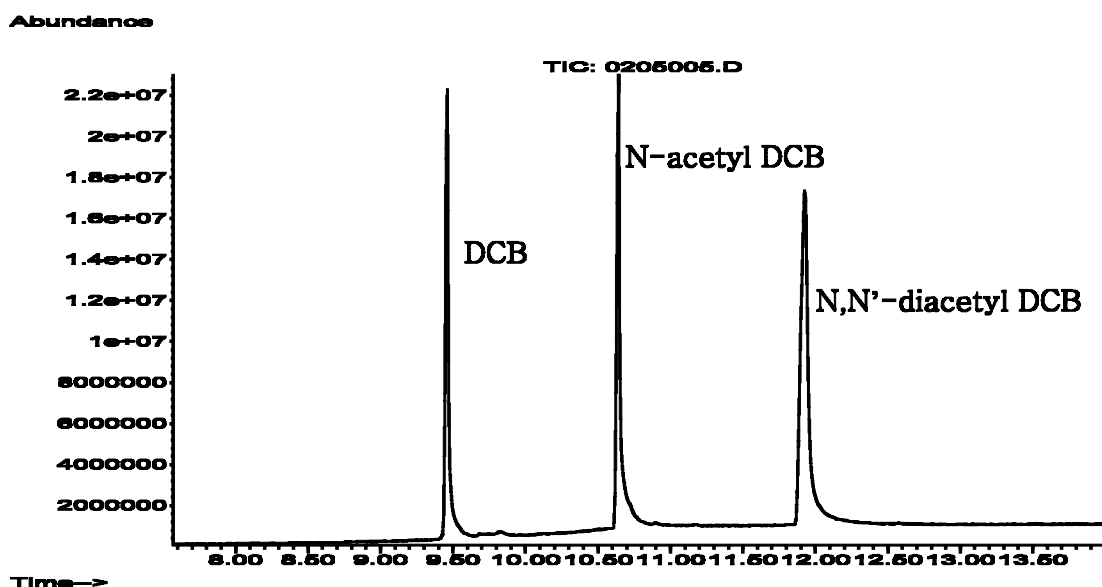


Figure 1. Chromatogram of DCB, N-acetyl DCB and N,N'-diacetyl DCB by using GC/MS-SIM

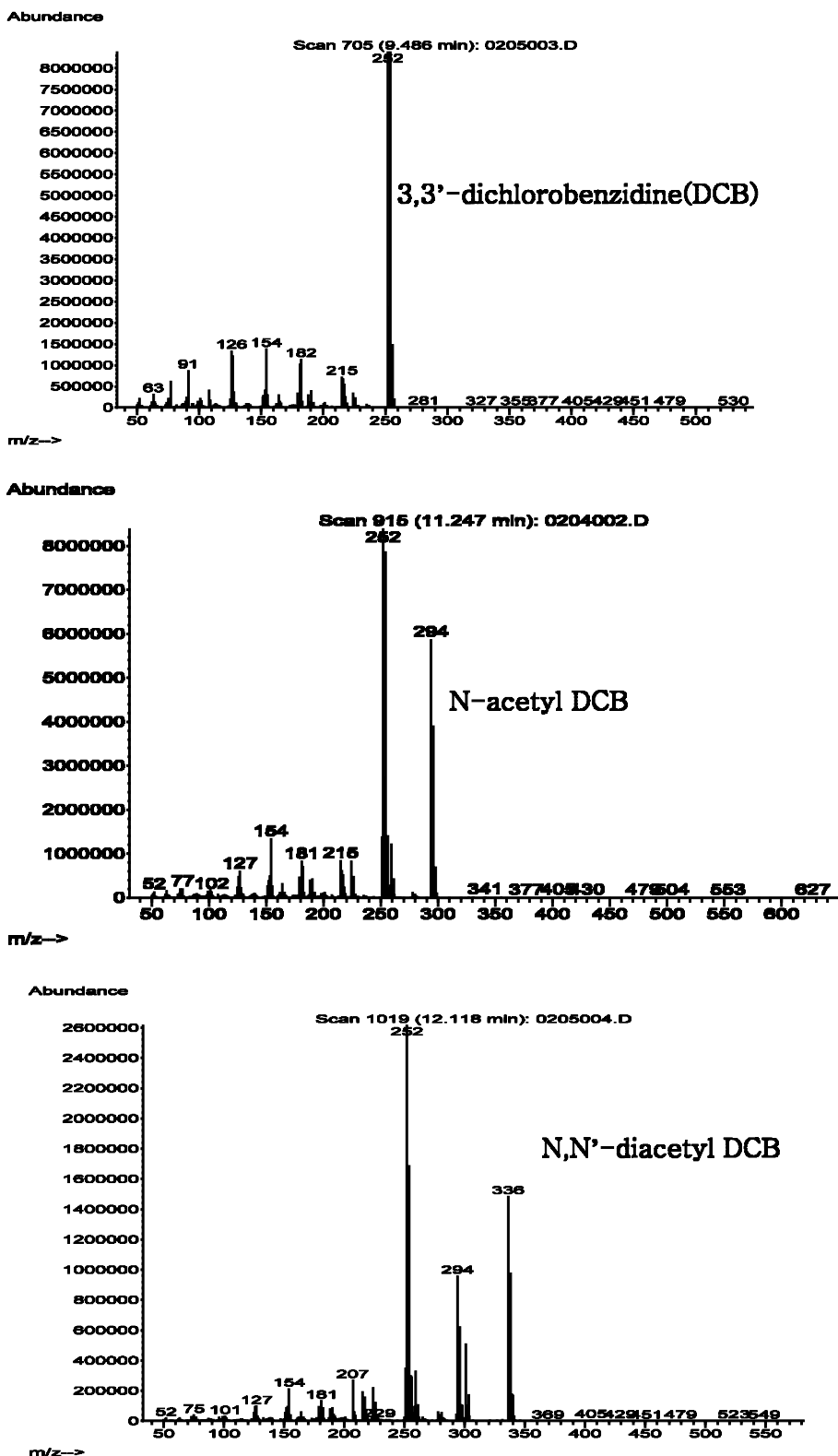


Figure 2. Fragmentation pattern of DCB, N-acetyl DCB and N,N'-diacetyl DCB by using GC/MS-SIM. Their base peaks were 252 m/z at DCB, 252 m/z and 294 m/z at N-acetyl DCB, and 252 m/z, 294 m/z and 336 m/z at N,N'-diacetyl DCB.

acetyl-DCB were measured when its metabolites were separated from hemoglobin adducts.

N-acetyl DCB was synthesized with pyridine and acetic acid, which were promoter and controller, respectively, in acetylation of DCB (Lee *et al.*, 2002). DCB was dissolved in 2 mL of pyridine and 0.3 mL of acetic acid was added to the solution, and was titrated with 21 μ L acetyl chloride (more than three times more than DCB in moles). The supernatant was achieved by filtration and drying.

Figure 3A shows the peaks of pyridine, N-acetyl DCB and N,N'-diacetyl-DCB by using gas chromatography/mass spectrometry-nitrogen phosphorous detection (GC/MS-NPD). For removing diacetyl-DCB from the dried product, it was dissolved to a mixture solvent of 0.5 N NaOH and toluene (1:2), obtained the toluene layer by 2000 rpm (400 g) centrifugation (5 minutes), and dried with nitrogen gas. Figure 3B shows that N,N'-diacetyl-DCB was removed from it, but pyridine

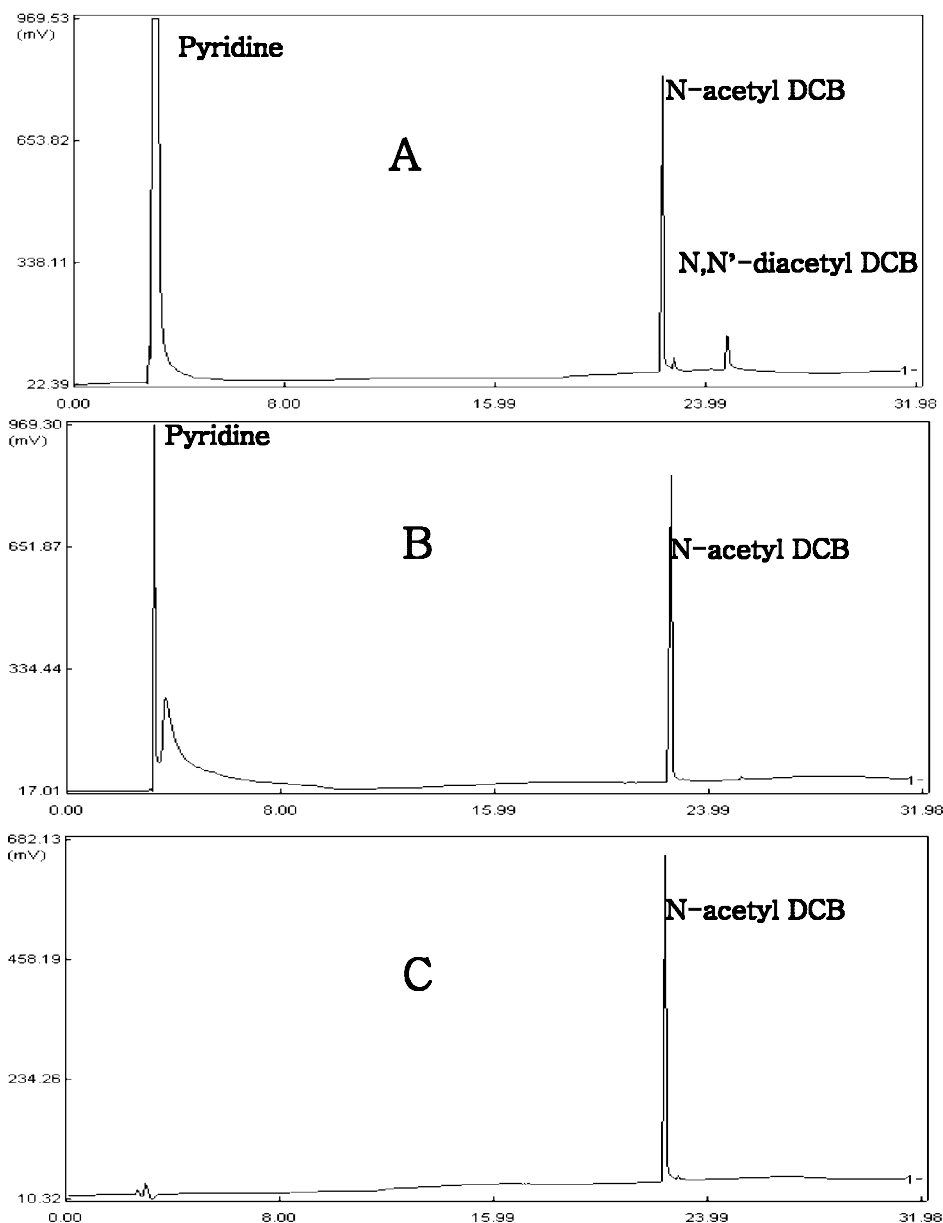


Figure 3. Chromatogram of pyridine, N-acetyl DCB and N,N'-diacetyl-DCB during the isolation procedure of N-acetyl DCB from N,N'-diacetyl DCB and pyridine by using GC/MS-NPD. A, all DCB became N-acetyl DCB and N,N'-diacetyl DCB by using acetic acid (controller of acetylation) in titration procedure; B, Removed N,N'-diacetyl DCB from the mixture, using a mixed solvent of 0.5 N NaOH and toluene (1:2); C, isolated and purified N-acetyl DCB by using acetone.

was not. Pyridine was easily removed from it by washing with acetone. Figure 3C showed the peak of the isolated N-acetyl-DCB by GC/MS-NPD. Finally we identified the purified N-acetyl-DCB by GC/MS-SIM. Its purity was 98.82%.

Chromatogram of standard materials

Figure 4 (B) is a chromatogram of international standards with 4-aminobiphenyl (A) and phenanthrene-d₁₀ (B) by GC/MS-SIM. The retention times of 4-aminobiphenyl (A) and phenanthrene-d₁₀ (B) were 6.73 and 6.96 minutes, respectively. Figure 4 (A) is a chromatogram of standard DCB, N-acetyl

DCB and N,N'-diacetyl DCB by GC/MS-SIM. The retention times of DCB, N-acetyl DCB and N,N'-diacetyl DCB were 10.19, 11.46, and 12.95 minutes, respectively.

Precision of GC/MS measurement

Calibration curves for DCB and N-acetyl DCB were established in Figure 5 after adding 0 ~ 200 ng/mL and 0 ~ 5000 ng/g of standard and 7.5 g of internal standard in DNA solution and 0.1 g hemoglobin. Their regression coefficients (r^2) are 0.9998 and 0.9974. Their maximum detection

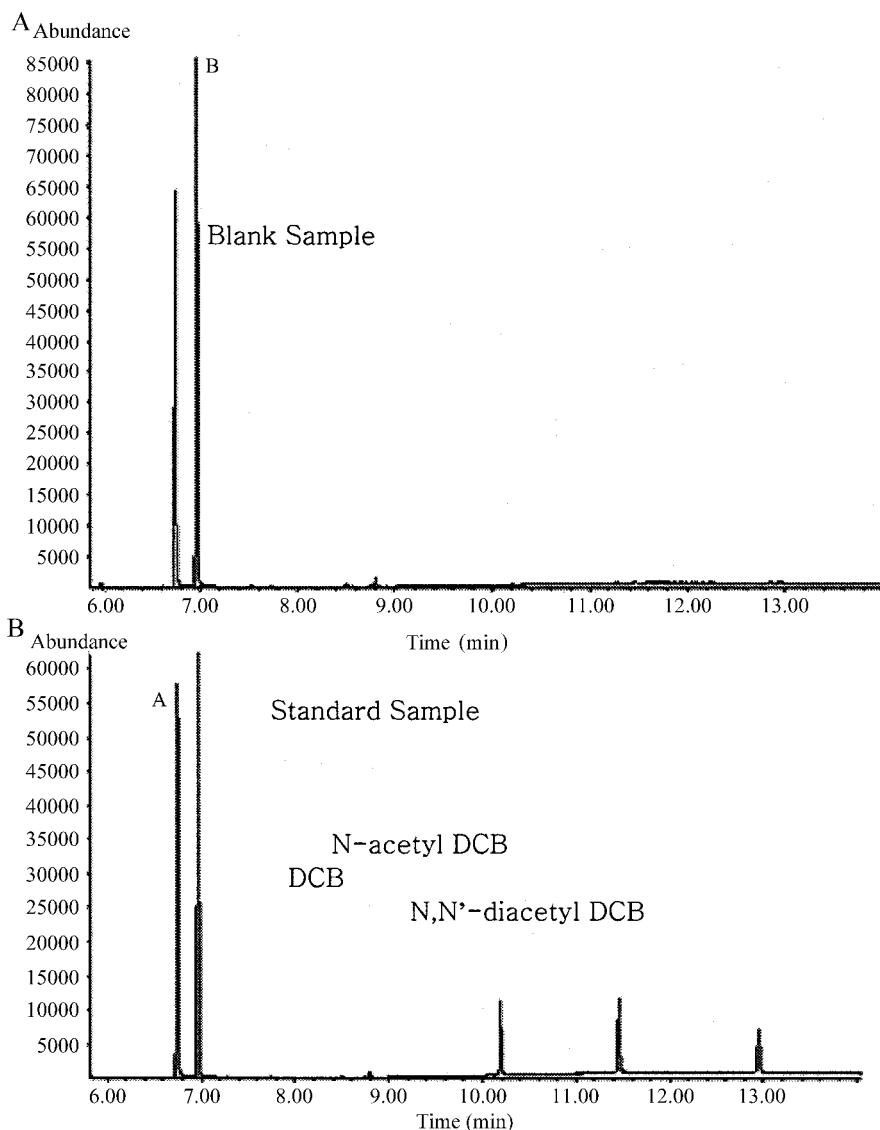


Figure 4. Chromatogram of internal standard with 4-aminobiphenyl (A) and phenanthrene-d₁₀ (B), and standard metabolites, with DCB, N-acetyl DCB, and N,N'-diacetyl DCB by using GC/MS-SIM

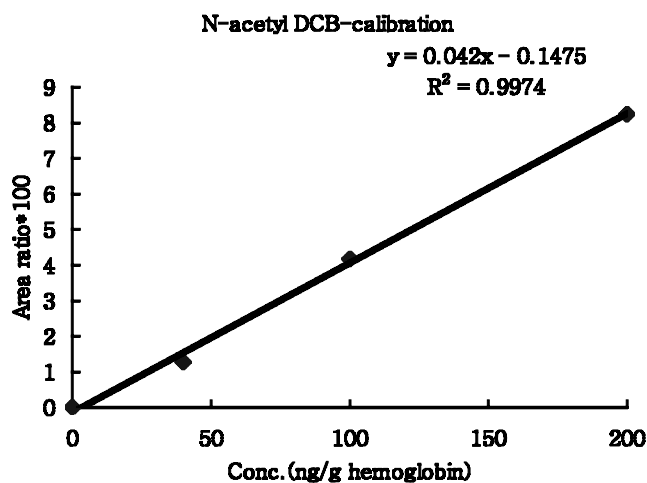
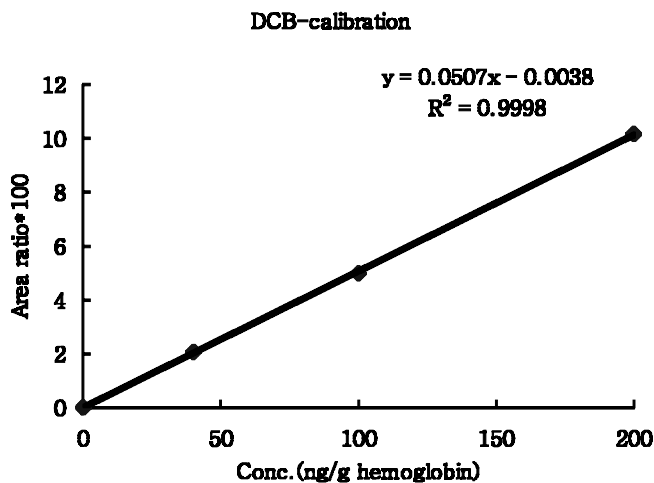


Figure 5. Calibration curves of standard DCB and N-acetyl DCB with peak area by using GC/MS-SIM

Table 1. The concentration of DCB and N-acetyl DCB separated from hemoglobin adducts in female Sprague-Dawley rats during oral treatment for three weeks with 20, 30, and 40 mg/kg DCB/kg body weight

Dosage per day (mg/kg body)	First week	Second week	Third week
DCB			
20	692.7±150	906.0±145	868.0±157
30	1382.5±160	1598.2±180	1409.8±199
40	2220.4±220	2120.4±280	1966.2±250
N-acetyl DCB			
20	144.8±39	297.3±45	202.7±62
30	202.2±57	386.5±89	325.5±77
40	280.6±80	485.0±78	421.4±95
Total			
20	837.5±189	1203.3±190	1070.7±219
30	1584.4±217	1984.7±269	1735.3±276
40	2501.0±300	2605.4±358	2387.6±345

Unit: ng/g hemoglobin

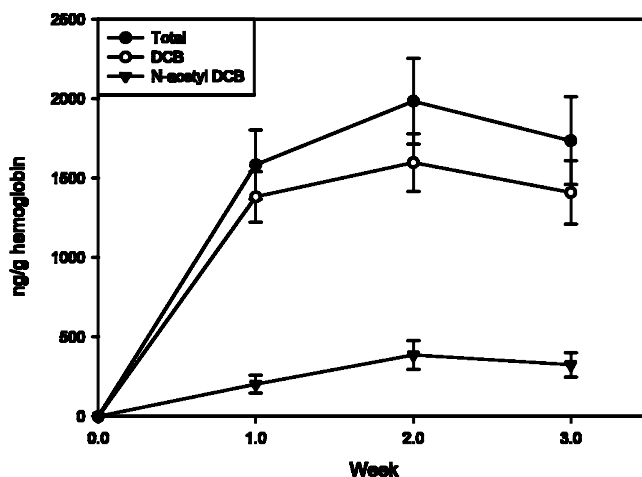


Figure 6. Accumulation of hemoglobin adducts obtained from GC/MS-SIM analysis of hemoglobin sample isolated from female Sprague-Dawley rats during oral treatment for three weeks with 30 mg DCB/kg body weight

limits (MDLs) were 0.5 µg/L in DNA adducts and 1.0 ng/g in hemoglobin.

Hemoglobin adduct levels

Table 1 is the accumulation of hemoglobin adducts in three different groups (20, 30 and 40 mg DCB/kg body weight per day) for three weeks. The level of hemoglobin adducts determined was increased after the second week, but slightly decreased after the third week. The concentration difference between DCB and N-acetyl DCB was 4.28~4.67 at the third week. Figure 6 is the accumulation of hemoglobin adducts in 30 mg DCB/kg/day treatment group. The dose-proportional increases of hemoglobin adducts in three different treatments (20, 30 and 40 mg DCB/kg/day) of rats during three weeks result in Figure 7.

Discussion

DCB is a suspected human carcinogen, and the major pathways of its metabolism are N-acetylation and oxidation (Tanaka, 1981). So, metabolites of DCB, such as N-acetyl DCB and N,N'-diacetyl DCB, can form adducts in target cells. DCB dissolved in pyridine can be easily acetylated by titration of acetyl chloride in a laboratory. A mixture of acetylated DCB was obtained with a mixture solvent of pyridine and acetic acid. The former was the promoter and the latter was the

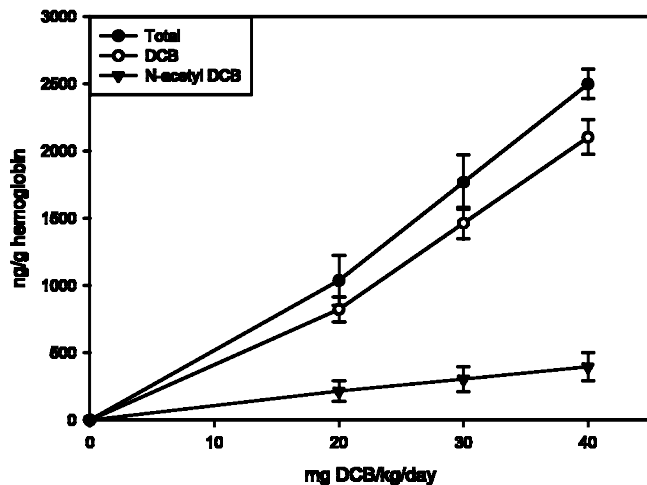


Figure 7. Hemoglobin adduct levels obtained from GC/MS-SIM analysis of hemoglobin samples isolated from female Sprague–Dawley rats during oral treatment for three weeks with three different doses of DCB

controller in the acetylation of DCB (Lee *et al.*, 2003). N-acetyl DCB can be extracted from them with a mixture solvent of 0.5 N NaOH and toluene (1:2), because it can dissolve DCB and N, N'-diacetyl DCB, but cannot dissolve N-acetyl DCB. Acetone is a good solvent for washing N-acetyl DCB (Figure 3).

After hydrolysis of hemoglobin samples isolated from DCB-treated rats and extraction, two adducts were detectable by GC/MS-SIM without derivatization. Because of transacetylation occurring during derivatization, the extent of N-acetyl DCB has been a critical issue (Bailey *et al.*, 1990). The results of this study show their MDLs were 0.5 µg/L in DNA adducts and 1.0 ng/g in hemoglobin. This sensitivity was enough for hemoglobin adducts monitoring. Joppich-Kuhn *et al.* (1997) attained the result that the limit of detection was below 0.1 ng/g hemoglobin for DCB and N-acetyl DCB by using GC/MS-NCI with derivatization of HFB (hepatafluorobutryl).

Hemoglobin is bound to only about 0.02% of the administered dose of DCB within the first 24 hours (Sagelsdorff *et al.*, 1990). But the hemoglobin adducts are stable *in vivo*, and their elimination is related to the life span of the erythrocytes (Joppich-Kuhn *et al.*, 1997). This persistence allows a retrospective estimation of total exposure, which took place over a period of several weeks. Hemo-

globin adducts could be used as a significant biomarker of DCB exposure monitoring.

The relative contribution of DCB and N-acetyl DCB to the total hemoglobin adduct level was treatment time-dependence. After the first week of treatment, the total amount of hemoglobin adducts determined was 837.5 ~ 2501 ng/g hemoglobin. The adduct levels were increased up to 1203.3 ~ 2605.4 ng/g after the second week, and slightly decreased after the third week. The ratio of DCB and N-acetyl DCB was nearly similar in all treatment groups at the third week, such as 4.28 ~ 4.78. Joppich-Kuhn *et al.*, 1997 reported that they were gradually increased and reached constant levels at the third week in hemoglobin adducts in female Wistar rats during treatment for four weeks with 0.006% DCB in the drinking water. Also they reported the ratio of DCB and N-acetyl DCB in hemoglobin adducts was about 3:1 at the third week. It was related to the metabolizing rate of DCB. Tanaka (1981) reports that a 24 hour urine sample of rats given a single oral dosage of DCB (50 mg/kg/day) contained N-acetyl DCB and N,N'-diacetyl DCB in a ratio of 10:3.

Three different treatments (20, 30 and 40 mg DCB/kg) of rats during three weeks results in dose-proportional increase in the total and DCB amount of hemoglobin adducts formed (Figure 7). The relative contribution of DCB and N-acetyl DCB to the total hemoglobin adduct level was strongly dose dependent. At low DCB dose, the DCB and N-acetyl DCB adducts were formed at moderately different levels, whereas at the high dose the DCB adduct was predominant. This proportional formation of the acetylated adduct indicates saturation of the acetylation pathway at high doses (Joppich-Kuhn *et al.*, 1997).

The results show that GC/MS-SIM has good selectivity and sensitivity for analysing DCB and its metabolites, and is suitable for the biological monitoring of humans exposed to DCB or DCB-containing products.

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