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A Study on Its Metabolites Separated from DNA Adducts of Blood Lymphocytes in Rats Exposed Orally with 3,3'-dichlorobenzidine(DCB) by GC/MS-SIM

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Abstract : 3,3'-Dichlorobenzidine(DCB) has be shown carcinogenic in several animals, and the development of non-invasive biomonitoring method in workers exposed with it is a very important subject. DNA adduct is a good biomarker for biomonitoring about carcinogens exposure, and lymphocytes is a good non-invasive samples. So we studied to analyze metabolites in blood lymphocytes of female Sprague-Dawley rats exposed orally with DCB(20, 30, and 40 mg/kg wt.) for 3 weeks. For analysis of them, we isolated DNA adducts from blood lymphocytes by using the enzymes method in ^{32}P-postlabeling, and measured them by using gas chromatography/mass spectrometry-selected ion monitoring(GC/MS-SIM). 4-aminobiphenyl and phenanthrene-d_{10} were added as internal standard for blank sample. Standard metabolites of DCB were synthesized with using pyridine and acetic acid which were promoter and controller in acetylation of DCB. And they were used for calibration curve. Our results showed two kinds of metabolites in DNA adducts of blood lymphocytes. They were N-acetyl 3,3'-dichlorobenzidine(acDCB) and N,N'-diacetyl 3,3'-dichlorobenzidine(di-acDCB). They were combined with DNA at the same time as an acetyl of it was removed. So we measured DCB and acDCB for two kinds of metabolites in DNA adducts of blood lymphocytes. Our results showed the levels of DCB were 1.46~2.26 times more than that of acDCB. And also the levels of metabolites in 20, 30 and 40 mg/kg wt. were gradually increased with going days from 1st to 3rd week. They are 1.66, 1.38 and 0.90 times in total metabolites, 1.76, 1.49 and 1.02 times in DCB, and 1.51, 1.22 and 1.28 times in acDCB. In conclusion, the results of this study showed DCB exposed to rats formed DNA adduct in blood lymphocytes after acetylated to N-acetyl 3,3'-dichloro benzidine(acDCB) and N,N'-diacetyl 3,3'-dichlorobenzidine(di-acDCB), and they could be analyzed by using gas chromatography/mass spectrometry-selected ion monitoring(GC/MS-SIM).

Keywords : 3,3'-dichlorobenzidine(DCB), N-acetyl 3,3'-dichlorobenzidine(acDCB), N,N'-diacetyl 3,3'-dichlorobenzidine(di-acDCB), blood lymphocytes, DNA adducts, GC/MS-SIM

Introduction

3,3'-Dichlorobenzidine(DCB) has been shown to be carcinogenic in several animals(Pliss G.B., 1959 Sellakumar, *et al.*, 1969; Stula E.F., 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 exposure of humans. Blood is a non-invasive surrogate for biological monitoring of DCB exposure in persons. Lymphocytes have nuclei among blood cells, and its life is some hours to years(Lee, 1992). So lymphocyte adducts are suitable biomarkers for the internal exposure of aromatic amines, the metabolic activation of carcinogenic amines, and the dose in the target organ. The presence of their 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

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corresponding N-hydroxylamines. N-acetylation appears to be the major path for the metabolism of DCB in mammals(Tanaka, 1981). A 24 hr urine sample of rats given a single oral dose of 3,3'-dichlorobenzidine(50 mg/kg/day) contained unchanged 3,3'-dichlorobenzidine(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). And indirect evidence for the formation of nitro derivatives was found in a study in which DCB was administered to female Wistar rats by gavage(Birner *et al.*, 1990).

So we firstly synthesized the metabolites of DCB, which were N,N'-diacetyl 3,3'- dichlorobenzidine (di-acDCB) and N-acetyl 3,3'-dichlorobenzidine (acDCB). Pyridine and acetic acid are promoter and controller in acetylation of DCB(Lee, *et al.*, 2002). And we analyzed its metabolites formed DNA adduct of blood lymphocyte in rats exposed orally with 3,3'-dichlorobenzidine(DCB) by gas chromatography/mass spectrometry-selected ion monitoring(GC/MS-SIM). DNA adduct from blood lymphocytes was isolated by using the enzymes methods in ^{32}P-postlabeling. And then its metabolites formed DNA adducts of blood lymphocytes were excised by using enzyme, N-glycosylase MutY (Zharkov *et al.*, 2000).

This study is a new trial to analyze lymphocyte DNA adducts without radioactive material, ^{32}P[*r*-ATP], but with GC/MS. So the results will be a good information to investigate the non-invasive biomonitoring of DNA adducts for preventing workers from exposure of carcinogens.

Materials and Methods

Chemicals

3,3'-dichlorobenzidine 2HCl(DCB · 2HCl) and Ficoll-Plaque(for lymphocyte separation) were obtained from Sigma(St. Louis, Mo. USA) and Pharmacia Biotech), respectively. Analytical grade of RNAse A, RNAse T_1, Proteinase K, N-glycosylase MutY, 4-aminobiphenyl, phenanthrene-d_{10}, 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, aceton and ethylacetate(E. Merck, Darmstadt, Germany) were used as solvents. All other chemicals were of the highest purity available from Sigma and Merk (Darmstadt, Germany).

Animals and Treatment

Twenty five female Sprague-Dawley rats with a body weight of about 220 g, were obtained from Haehanbiolink(Chongju, South Korea). They were acclimatized for one week in Maecrlone cages (temp. of 18°C, humidity of 30~70%, illumination time from 6 a.m. to 6 p.m.), and had free access to tap water and food. DCB was orally given daily to treatment group with a dose of 20, 30 and 40 mg/kg body weight, for 3 weeks. Because of the low solubility of DCB in water, the dosing solution was made emulsion with DCB in water containing citric acid and sucrose(1:1). Heparinized blood samples were collected 3 times with 7 days intervals.

Isolation of DNA Adduct from Blood Lymphocytes

Lymphocytes were separated from heparinized blood with 1 vol. of Ficoll-Paque. 15~20 m*l* blood gives 150~350 g DNA from the lymphocytes. This lymphocytes were suspended in 0.5 m*l* of cold 1 mM $MgCl_2$, 20 mM Tris HCl, pH 8.0, and added 30 *l* of 10% Triton X-100. Washed it by centrifuging for 5 min at 6,000 rpm in refrigerated centrifuge.

For isolating DNA from the lysed lymphocytes, firstly removed RNA and protein with 60 *l* RNAse A, 20 *l* RNAse T_1 and 60 *l* Proteinase K through incubating at 37°C, respectively, then purified it with phenol, phenol/savage and savage, and finally washed and dried it with 60 *l* of 4 M sodium acetate, cold ethanol, and 70% ethanol. DNA of lymphocytes was hydrolyzed with Micrococcal Nuclease Spleen Phosphodiesterase (MN SPD). And DNA adduct was extracted from them with n-butanol and 10 mM tetrabutylamonium (TBA)

Determination of Lymphocyte Adduct by GC/MS

DNA adduct extracted from lymphocytes was hydrolyzed for 20 hr at 37°C with N-glycosylase MutY(Zharkov *et al.*, 2000). And its metabolites released from DNA adducts of lymphocytes were extracted with 7 m*l* ethyl ether by mechanical shaking for 10 min. The organic phase was

transferred into a 20 m*l* glass stoppered test tube and dried in evaporator. It was dissolved with 100 μ*l* ethyl acetate and analyzed by GC/MS.

4-aminobiphenyl and phenanthrene-d_{10} were added as internal standard for blank sample. Standard metabolites of DCB were synthesized by using pyridine and acetic acid which were promoter and controller in acetylation of DCB (Lee, *et al.*, 2002). And they were used for calibration curve.

Conditions of Gas Chromatography-mass Spectrometry

All mass spectra were obtained with 6890/5973 GC-MSD(Hewlett-Packard Co.). 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 analysts identification. Detection mode was an ion monitoring detection mode (SIM). Column was HP-5MS(30 m × 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 m*l*/min. The GC operating temperatures were: injector temperature, 280°C; transfer line temperature, 280°C oven temperature, programmed from 100°C at 20°C/min to 310°C (held for 2 min).

Results

Identification of DCB and Its Metabolites by GC/MS

Metabolites of DCB(3,3'-dichlorobenzidine) formed lymphocyte DNA adducts were N,N'-diacetyl-DCB(di-acDCB) and N-acetyl-DCB(acDCB). We synthesized them and isolated from each other by using both pyridine as promoter and acetic acid as controller of the acetylation of DCB, respectively. Residual pyridine was removed by using aceton (Lee, *et al.*, 2002).

Chromatogram of internal standard with 4-aminobiphenyl(A) and phenanthrene-d_{10}(B) by GC/MS-SIM was Fig. 1. Their retention times were 6.73 at 4-aminobiphenyl(A) and 6.96 at phenanthrene -d_{10}(B), respectively. Fig. 2 was chromatogram of standard DCB and its metabolites(acDCB and di-acDCB) by GC/MS-SIM. And their retention time were 10.19 at DCB, 11.46 at acDCB and 12.95 at di-acDCB, respectively.

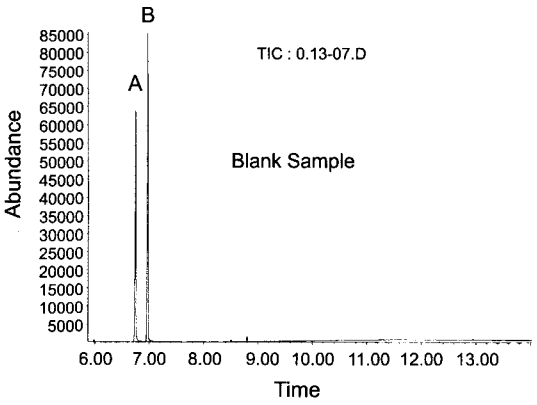

Fig. 1. Chromatogram of internal standard with 4-aminobiphenyl(A) and phenanthrene-d_{10}(B) by using gas chromatography/mass spectrometry-selected ion monitoring detection mode(GC/MS-SIM).

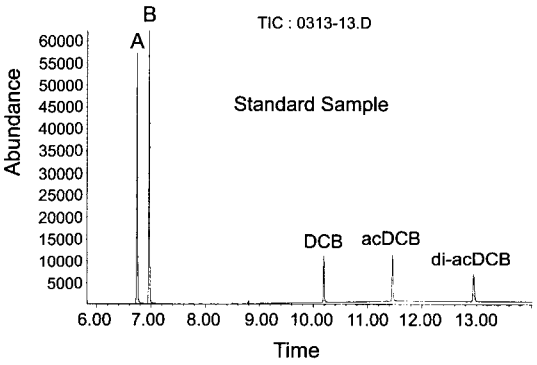

Fig. 2. Chromatogram of standard 3,3'-dichlorobenzidine (DCB) and its metabolites(N-acetyl and N,N'-diacetyl 3,3'-dichlorobenzidine(acDCB anddi-acDCB) by using gas chromatography/mass spectrometry-selected ion monitoring detection mode(GC/MS-SIM).

Precision of GC/MS Measurement

Fig. 3 shows calibration curves for 3,3'-dichlorobenzidine(DCB), N-acetyl 3,3'-dichloro benzidine (acDCB), and N,N'-diacetyl 3,3'-dichlorobenzidine (di-acDCB). They were established after adding 0~200 ng/m*l* of standard and 7.5 g of internal standard in DNA solution. Their regression coefficients(r^2) were 0.9972~0.9998. And their maximum detection limits(MDL) were 0.5 μg/*l* in DNA adduct solution.

DNA Adducts of Lymphocytes by GC/MS

Table 1 was the concentration of 3,3'-

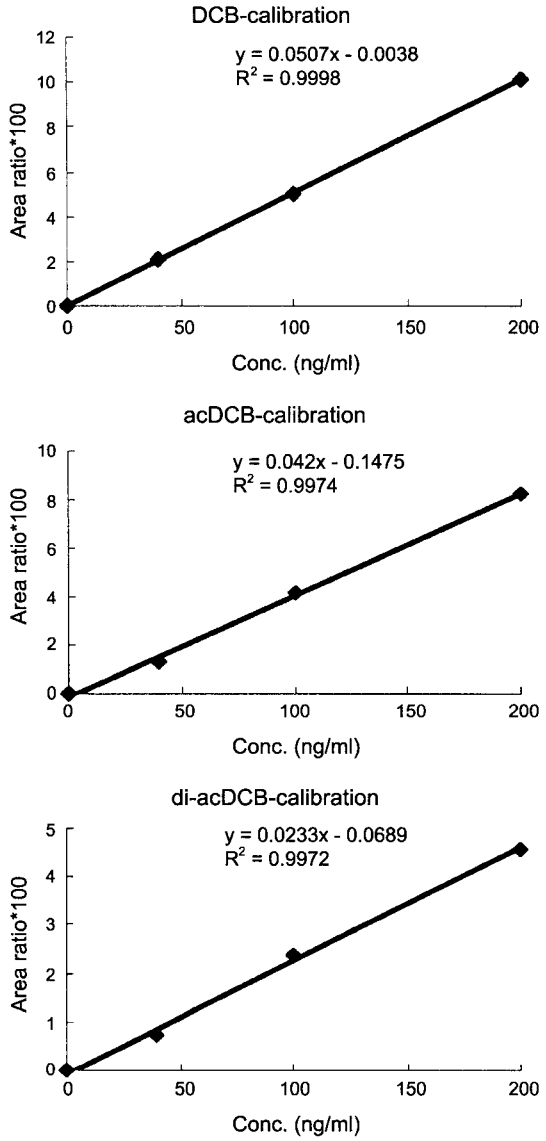

Fig. 3. Calibration curves of standard DCB and its metabolite(N-acetyl 3,3'-dichlorobenzidine, acDCB) with peak area by gas chromatography/mass spectrometry-selected ion monitoring detection mode(GC/MS-SIM).

dichlorobenzidine(DCB) and its metabolites in lymphocytes DNA adducts of rats exposed orally DCB for 3 weeks. Total level of metabolites in 20, 30 and 40 mg/kg wt. were 9.2±3.0 ng/g DNA, 20.4±4.3 ng/g DNA and 35.2±6.0 ng/g DNA on 1st week, and then increased gradually to 15.3± 5.9 ng/g DNA, 28.2±5.6 ng/g DNA and 38.8±7.2

Table 1. The concentration of 3,3'-dichlorobenzidine(DCB) and N-acetyl DCB separated from lymphocytes adducts in female Sprague-Dawley rats during oral treatment for 3 weeks with 20, 30, and 40 mg DCB/kg body wt

Unit : ng/g DNA

	Week		
Dose per day	1st	2nd	3rd
3,3-dichlorobenzidine(DCB)			
20 mg/kg body wt.	5.5±1.5	7.6±2.1	9.7±3.3
30 mg/kg body wt.	12.3±2.4	16.9±2.5	18.3±3.5
40 mg/kg body wt.	24.4±3.3	25.1±4.2	24.9±5.4
N-acetyl DCB			
20 mg/kg body wt.	3.7±1.5	4.6±1.3	5.6±2.6
30 mg/kg body wt.	8.1±1.9	8.5±2.6	9.9±2.1
40 mg/kg body wt.	10.8±2.7	13.3±1.9	13.9±1.8
Total DCB			
20 mg/kg body wt.	9.2± 3.0	12.2±4.4	15.3±5.9
30 mg/kg body wt.	20.4±4.3	25.4±5.1	28.2±5.6
40 mg/kg body wt.	35.2±6.0	38.4±6.1	3.8.8±7.2

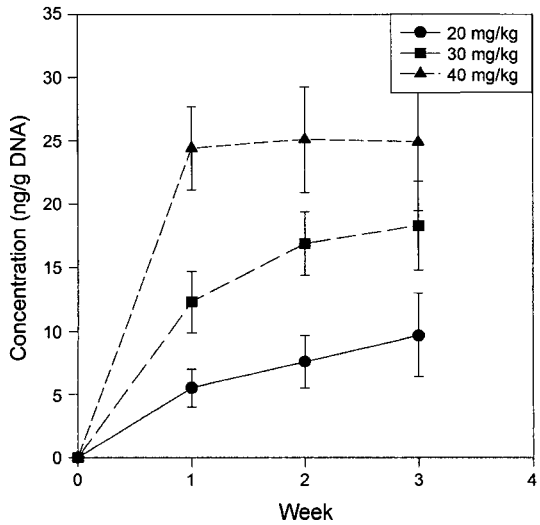

Fig. 4. The levels of DCB isolated from DNA adducts of blood lymphocytes in female Sprague-Dawley rats during oral treatment for 3 weeks with 20, 30 and 40 mg/kg wt. 3,3'-dichlorobenzidine(DCB).

ng/g DNA on 3rd week, respectively. So Total metabolites in 20, 30 and 40 mg/kg wt. on 1st week were 1.66, 1.38 and 0.90 times more than on 3rd week, respectively. And the levels of DCB

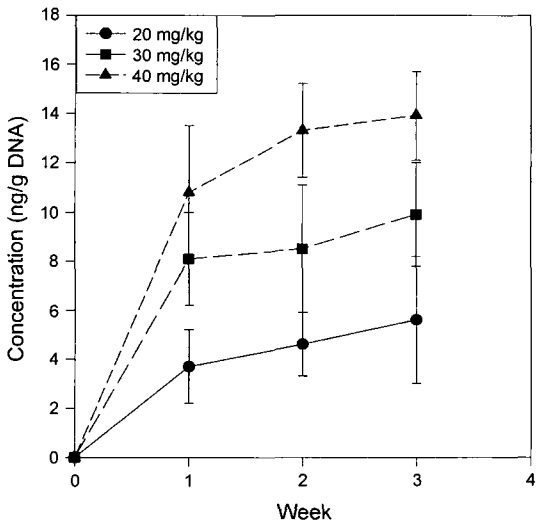

Fig. 5. The levels of acDCB isolated from DNA adduct of blood lymphocytes in female Sprague-Dawley rats during oral treatment for 3 weeks with 20, 30 and 40 mg/kg wt. 3,3'-dichlorobenzidine(DCB).

were 1.46~2.26 times more than the level of acDCB.

The trend of DCB concentration for 3 weeks was Fig. 4. The levels of DCB in 20, 30 and 40 mg/kg wt. were 5.5±1.5 ng/g DNA, 12.3±2.4 ng/g DNA and 24.4±3.3 ng/g DNA on 1st week, and then increased gradually to 9.7±3.3 ng/g DNA, 18.3±3.5 ng/g DNA and 24.9±5.4 ng/g DNA on 3rd week, respectively. So the level of DCB 20, 30 and 40 mg/kg wt. on 1st week were 1.76, 1.49 and 1.02 times more than on 3rd week, respectively.

Fig. 5 shows the trend of acDCB concentration for 3 weeks. The levels of acDCB in 20, 30 and 40 mg/kg wt. were 3.7±1.5 ng/g DNA, 8.1±1.9 ng/g DNA and 10.8±2.7 ng/g DNA on 1st week, and then increased gradually to 5.6±2.6 ng/g DNA, 9.9±2.1 ng/g DNA and 13.9±1.8 ng/g DNA on 3rd week, respectively. So the levels of DCB 20, 30 and 40 mg/kg wt. on 1st week were 1.51, 1.22 and 1.28 times more than on 3rd week, respectively.

Discussion

DNA adduct in target organs is a very important biomarker for biologically monitoring workers exposed to carcinogens. And lymphocyte DNA adduct is a marker from surrogate tissues, which was used to asses the risk to the target organ (Talaska *et al.*, 1995), because lymphocytes have only nuclei among the blood cells, and its life is some hours to years(Lee, 1992). Reh *et al.*(2000) reported that occupational nitrosamine exposure a rubber vehicle seal were compared with the peripheral blood lymphocyte concentrations of two nitrosamine-related DNA adducts.

We experiment with female Sprague-Dawley rats for investigating the metabolites formed DNA adducts of blood lymphocytes in rats exposed orally with 3,3'-dichlorobenzidine(DCB).

Like other aromatic amines, DCB can be metabolically N-acetylated and/or oxidized to the corresponding N-hydroxylamines. N-acetylation appears to be the major path for the metabolism of DCB in mammals(Tanaka, 1981). So DCB was acetylated to be N-acetyl 3,3'-dichlorobenzidine and N,N'-diacetyl 3,3'-dichlorobenzidine. And then they formed DNA adducts at various target organs include lymphocytes(Reh *et al.*, 2000). We also separated DNA adducts from blood lymphocytes, and analyzed two kinds of its metabolites by using gas chromatography/mass spectrometry-selected ion monitoring(GC/MS-SIM). They were 3,3'-dichlorobenzidine(DCB) and N-acetyl 3,3'-di chlorobenzidine(acDCB) because each metabolite was combined with DNA at the same time as an acetyl of it was removed(Reid *et al.*, 1984). And our results showed the DCB concentration were 1.46~2.26 times more than acDCB concentration. Tanaka(1981) also reported the similar result. He analyzed a 24 hr urine sample of rats given a single oral dose of 3,3'-dichlorobenzidine(50 mg/kg/day), and reported the ratio of unchanged 3, 3'-dichlorobenzidine(DCB), N,N'-diacetyl 3,3'-dichl-orobenzidine(di-acDCB), and N-acetyl 3,3'-dichlorobenzidine(acDCB) was 1:3:10(Tanaka, 1981). And indirect evidence for the formation of nitro derivatives was found in a study in which DCB was administered to female Wistar rats by gavage(Birner *et al.*, 1990).

The results also showed the levels of metabolites in 20, 30 and 40 mg/kg wt. were gradually increased with going weeks from 1st to 3rd. They are 1.66, 1.38 and 0.90 times in total metabolites, 1.76, 1.49 and 1.02 times in DCB, and 1.51, 1.22 and 1.28

times in acDCB. Joppich-Kuhn *et al.*(1997) reported accumulation of hemoglobin adducts were similarly increased for 4 weeks in female Wistar rats treated with 0.006% DCB in th drinking water. In animal, absorption of DCB from the gastrointestinal tract is rapid. Hsu & Sikka(1982) were reported that, following a dose of 40 mg/kg, the plasma level of unchanged DCB attained a peak concentration of 1.25 μg/m*l* at 4hrs in Sprague Dawley rats, and about 90% of the administered DCB was excreted in feces and urine within 72 hrs largely as metabolites. This results indicated a high bioavailability of DCB. Hsu & Sikka(1982) also reported that the elimination of DCB was two phases, with half-lives of 6 hours and 14 hours in plasma for rapid and slow phases, respectively.

In conclusion, the results of this study showed DCB exposed to rats formed DNA abduct in blood lymphocytes after acetylated to N-acetyl 3,3'-dichloro benzidine(acDCB) and N,N'-diacetyl 3,3'-dichlorobenzidine(di-acDCB), and they could be analyzed by using gas chromatography/mass spectrometry-selected ion monitoring(GC/MS-SIM).

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