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Non-invasive Biological Monitoring of DNA Adducts Formed at Workers Handling 3,3'-Dichlorobenzidine(DCB) by Using GC/MS

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Abstract : We examine the metabolites(DCB and acetyl DCB) extracted from exfoliated urothelial cells of 33 workers who employed DCB-handling industries. The characteristics of workers submitted urine, whose age, working years and smoking persons were 41.9 ± 11.1, 8.7 ± 5.5 and 25(32.0%), respectively. DNA adduct was isolated from the exfoliated urothelial cells by applying ^{32}p-postlabeling procedure. Metabolites(DCB and acetyl DCB) were extracted from DNA adducts by hydrolyzing and N-glycosylase. Concentrations of DCB and acetyl DCB were 28.6 ± 5.25 ng/g DNA, and 17.0 ± 3.73 ng/g DNA, respectively. The regression between DCB level and exposure years of workers is $y = 1.668 + 2.588x$($p = 0.005$, $r^2 = 0.394$). The regression between acetyl DCB level and exposure years of workers is $y = 8.071 + 1.325x$($p = 0.076$, $r^2 = 0.222$). Smoking workers are significantly higher than non-smoking workers on DCB and acetyl DCB level($p = 0.065$ and 0.021, respectively). DCB level was 33.9 ± 7.14 ng/g DNA on smokers, and 23.1 ± 9.97 ng/g DNA on non-smokers. Acetyl DCB was 25.1 ± 5.27 ng/g DNA on smokers, and 8.92 ± 7.22 ng/g DNA on non-smokers.

Keywords : 3,3'-dichlorobenzidine(DCB), acetyl DCB, GC/MS, DNA adduct Exfoliated urothelial cells

Introduction

Incidences of bladder cancer among employees in aromatic amine industry were reported as early as 1895[1]. Since then evidence has accumulated from animal experiments and human observation documenting the carcinogenicity of these compounds[2,3]. 3,3'-Dichlorobenzidine(DCB) has been shown to be carcinogenic in several animals and epidemic studies[3-6]. It is used in the production of pigments for printing inks, textiles, plastics and enamels, paints, leather, and rubber[2].

DCB is absorbed readily through the skin, and the target organ is urinary bladder. 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[9]. A 24 hr urine sample of rats given a single oral dose of 3,3'-dichlorobenzidine(50 mg/kg/day) contained unchanged DCB, N,N'-diacetyl 3,3'-dichlorobenzidine, and N-acetyl 3,3'-dichlorobenzidine in a ratio of 1:3:10[9]. The indirect evidence for the formation of nitro derivatives was found in a study in which DCB was administered to female Wister rats by gavages[10].

Urinary bladder is an internal organ. So we need the surrogate tissues for biological monitoring of workers' target organ. The epithelial cells(urothelium) of urinary bladder could be a good non-invasive biological samples, because of regularly exfoliating and being voided with urine[7,8]. These cells have long been used for cytological studies to determine the presence of the malignant phenotype.

DCB and its metabolites in urine and hemoglobin adducts were analyzed with chromatography method[8,11,12]. Mass spectrometry has the potential to become a standard analytical tool for detecting DCB and its metabolites.

In this study, we measure the metabolites of DCB extracted from DNA adduct of exfoliated urothelial cells isolated from urine of workers handling DCB by using GC/MS.

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Materials and Method

Chemicals

3,3'-dichlorobenzidine · 2HCl(DCB) was obtained from Sigma(St. Louis, Mo.). All other chemicals were of the highest purity available from Sigma and Merck(Darmstadt, Germany).

Isolation of exfoliated urothelial cells and DNA

The urine samples were submitted from 33 workers at 3 dyes industries handling DCB It were filtered through a 62 μm sieve and centrifuged for 10 min at 1800 rpm in a refrigerated centrifuge. Urine was decanted and the resulting pellet was washed at least three times with sucrose buffer. It was exfoliated urothelial cells for non-invasive biological monitoring.

For DNA isolation, 1 m*l* of 20 mM Tris-HCl buffer(pH 7.4) containing 1% sodium deodecyl sulfate(SDS) was added to the final washed pellet. 18U of ribonuclease A and 40U of ribonuclease T_1 were added, and the samples were incubated for 1hr at 37°C. Proteinase K(1.2U) was then added, and the samples were incubated at 37°C for an additional 30 min. Samples were subsequently solvent extracted as described by Gupta[13]. Following the final extraction, 0.1 volumes of the 4.5 M LiCl and 30 μg of glycogen were added. DNA was co-precipitated with glycogen by addition of 2 volumes of ice-cold ethanol. Samples were cooled to −80°C for 30 min and then centrifuged at 7000 rpm in a refrigerated centrifuge. The pellet was washed three times with 70% ethanol and the DNA was dissolved in a small volume of 1.5 mM NaCl-0.1 mM sodium citrate.

Isolation of DNA adducts

4 μ*l* of DNA sample was hydrolyzed for 3 hr with 0.21U of micrococcal nuclease (MN) and 2.1 μg of spleen phosphodiesterase at 37°C. 10 mM of tetrabutylammonium(TBA), 0.1 M NH_4 formate (pH 3.5) were added, and extract DNA adducts with butanol. DNA adducts were dissolved in 10 mM bicine(pH 9.6).

Gas chromatography-mass spectrometry

DCB-DNA adducts were hydrolyzed for 20 hr at 37°C with N-glycosylase. Metabolites(DCB and acetyl DCB) 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 using GC/MS.

All mass spectra were obtained with 5890/5971 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 modes were nitrogen phosphorous detection(NPD), selected ion monitoring detection mode(SIM) and flame ionization detection(FID). Columns for them were HP-5 capillary column(50 m × 0.32 mm i.d. × 0.17 μm F.T.) and ultra 2 capillary column (30 m × 0.2 mm i.d. × 0.33 μm F.T.), respectively. 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 temperature were : injector temperature, 300°C; transfer line temperature, 300°C; oven temperature, programmed from 100°C at 20/min to 310°C(held for 2 min)(Table 1).

Table 1. Analytical conditions of GC/MS-SIM for analyzing metabolites of DCB

Subjects	Analytical Conditions
Column	HP-5MS(cross-linked 5% phenylmethylsiloxane 50 m × 0.25 mm I.D. × 0.25 μm F.T.)
Carrier gas	He at 0.8 m*l*/min
Split ratio	1/30
Injection port temp.	280°C
Transfer line temp.	300°C
Oven temp. program	Initial temp. and time : 80°C and 0 min Rate : 20°C/min Final temp. and time : 300°C and 5 min
Scan mode (Solvent delay : 2.0 min)	Mass range : 405~500 m/z
SIM mode (Solvent delay : 2.0 min)	Group : 1 Start time : 2.0 min Selected ions m/z : 279, 376

Table 2. Demographic characteristics of workers submitted the urine samples

Mean ± S.D

Industry	No. of Workers	Age	Smoking		Sex		Working Years	Unine Vol (m*l*)
			Yes	No	Mr	Mrs		
A	7	31.7 ± 8.0	6	1	7	0	5.3 ± 4.1	262 ± 115
B	18	47.7 ± 7.2	12	6	17	1	10.7 ± 5.4	328 ± 109
C	8	39.5 ± 11.5	6	2	8	0	7.3 ± 5.4	204 ± 80
Total	33	41.9 ± 11.1	24	9	32	1	8.7 ± 5.5	284 ± 114

Results

Demographic characteristics of workers submitted urine samples

The characteristics of workers submitted urine samples, whose age, working years and smoking persons were 41.9 ± 11.1, 8.7 ± 5.5 and 25(32.0%), respectively, such as Table 2.

Identification of DCB and its metabolites of by GC/MS

Metabolites of DCB were N,N'-diacetyl-DCB and N-acetyl-DCB. They were synthesized and isolated from each other by using both pyridine as promoter and acetic acid as controller of the acetylation of DCB, respectively[14].

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 times were 10.19 at DCB, 11.46 at acDCB and 12.95 at di-acDCB, respectively.

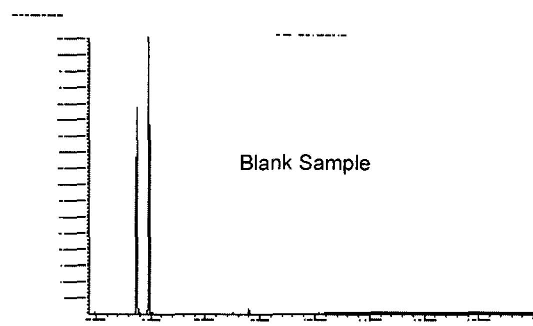

Fig. 1. Chromatogram of internal standard with 4-aminobiphenyl(A) and phenanthrene-d_{10}(B) by using gas chromatography/mass spectrometry-selected ion monitoring detection mode(GC/MS-SIM).

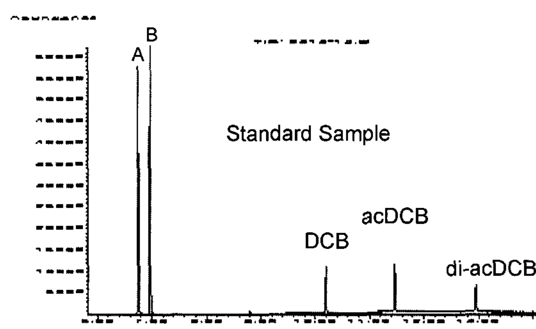

Fig. 2. Chromatogram of standard 3,3'-dichlorobenzidine (DCB) and its metabolites(N-acetyl and N,N'-diacetyl 3,3'-dichlorobenzidine) by using gas chromatography/mass spectrometry-selected ion monitoring detection mode(GC/MS-SIM).

The calibration curves for 3,3'-dichlorobenzidine and N-acetyl DCB 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) are 0.9974 and 0.9998. And their maximum detection limits(MDL) were 0.5 μg/*l* in DNA adduct solution.

Levels of metabolites combined with DNA adducts

Fig. 3 is the metabolites(DCB and acetyl DCB) extracted from DNA adduct in exfoliated urothelial cells isolated from urine of workers handling DCB. Concentrations of DCB and acetyl DCB were 28.6 ± 5.25 ng/g DNA, and 17.0 ± 3.73 ng/g DNA, respectively.

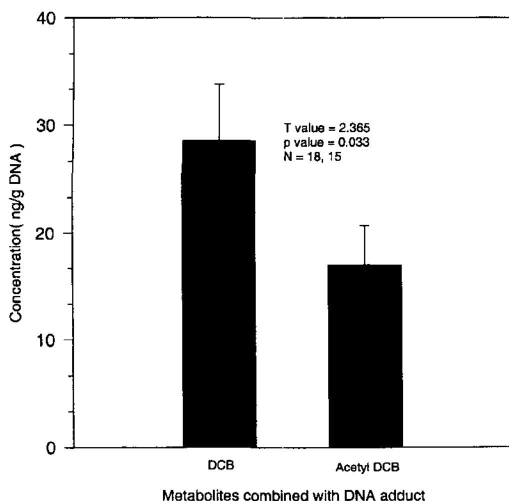

Fig. 3. Concentration of metabolites(DCB and acetyl DCB) extracted from DNA-adducts in exfoliated urothelial cells of workers exposured 3,3'-dichlorobenzidine(DCB). Error bars show standard errors.

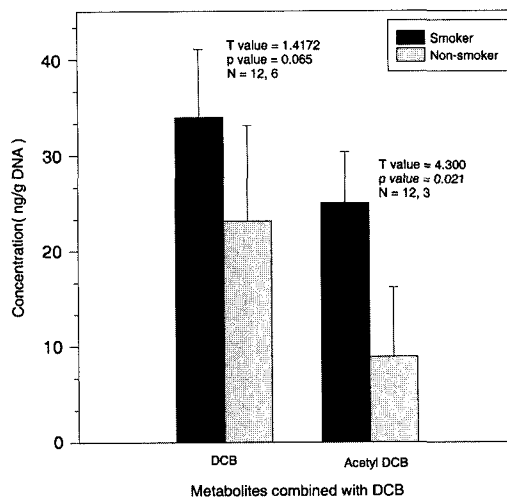

Fig. 5. Comparison between smokers and non smokers about DCB and acetyl DCB levles extracted from DNA adducts in exfoliated urothelial cells of workers handling DCB. Error bars show standard errors.

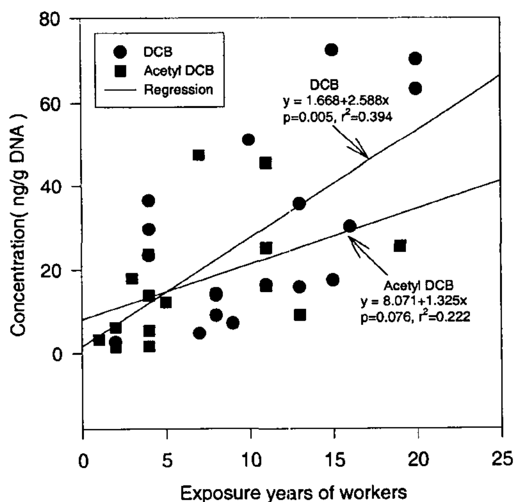

Fig. 4. Regression of metabolites(DCB and acetyl DCB) extracted from DNA adducts in exfoliated urothelial cells and exposured years of workers handled DCB.

Regressions between metabolites levels and exposure years

Fig. 4 is regressions between metabolites (DCB and acetyl DCB) extracted from DNA adduct in exfoliated urothelial cells and exposure years of workers handling DCB. The regressions are $y = 1.668 + 2.588x$($p = 0.005$, $r^2 = 0.394$) for DCB level and exposure years of workers, and $y = 8.071 + 1.325x$($p = 0.076$, $r^2 = 0.222$) for acetyl DCB level and exposure years of workers.

Comparison between smokers and non-smokers

Fig. 5 is the comparison between smoker and non-smokers about DCB and acetyl DCB extracted from DNA adducts in exfoliated urothelial cells of workers handling DCB. Smoking workers are significantly higher than non-smoking workers on DCB and acetyl DCB level ($p = 0.065$ and 0.021, respectively). DCB level were 33.9 ± 7.14 ng/g DNA on smoking workers, and 23.1 ± 9.97 ng/g DNA on non-smoking workers. Acetyl DCB were 25.1 ± 5.27 ng/g DNA on smoking workers, and 8.92 ± 7.22 ng/g DNA on non-smoking workers.

Discussion

DCB is the suspected human carcinogen, and the major pathways of its metabolism are N-acetylation and oxidation[9]. So metabolites of DCB, such as N-acetyl DCB and N,N'-diacetyl DCB, can formed adducts in target cells. The target organ of DCB is urinary bladder. 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[9]. So the metabolites combined with DNA are DCB and acetyl DCB[9,11].

Urinary bladder is an internal organ. So we need the surrogate tissues for biological monitoring of workers' target organ. The epithelial cells(urothelium) of urinary bladder could be a good non-invasive biological samples, because of regularly exfoliating and being voided with urine[7,8]. These cells have long been used for cytological studies to determine the presence of the malignant phenotype.

We extracted the same metabolites from DNA adduct of exfoliated epithelial cells, and their concentration are 28.6 ± 5.25 ng/g DNA for DCB, and 17.0 ± 3.73 ng/g DNA for acetyl DCB, respectively. Talaska[7] detected DNA adducts extracted from exfoliated urothelial cells of dogs treated with 4-aminobiphenyl by ^{32}P-postlabeling method.

Longer duration of employment in DCB-exposed job was associated with the increased risk for bladder cancer. In our studies, the regressions are $y = 1.668 + 2.588x$($p = 0.005$, $r^2 = 0.394$) for DCB level and exposure years of workers, and $y = 8.071 + 1.325x$($p = 0.076$, $r^2 = 0.222$) for acetyl DCB level and exposure years of workers. Bi[15] reported that the 25 fold increase of bladder cancer incidence in the benzidine exposed group was related to level of exposure, with the SIR rising from 4.8 for low exposure to 36.2 for medium exposure, and 158.4 for high exposure.

Cigarette use was an independent risk factor for bladder cancer. In our studies, Smoking workers are significantly higher than non-smoking workers on DCB and acetyl DCB level($p = 0.065$ and 0.021, respectively). DCB level were 33.9 ± 7.14 ng/g DNA on smoking workers, and 23.1 ± 9.97 ng/g DNA on non-smoking workers. Acetyl DCB were 25.1 ± 5.27 ng/g DNA on smoking workers, and 8.92 ± 7.22 ng/g DNA on non-smoking workers. Bi[15] reported benzidine-exposed workers who smoked tobacco had a 31 fold risk, while non-smoking workers had an 11-fold risk.

Acknowledgement

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