Urinary Monitoring Method of 3,3’-Dichlorobenzidine (DCB) and Its Metabolites, N-Acetyl-DCB and N,N’-Diacetyl-DCB

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Abstract: 3,3’-dichlorobenzidine (DCB) is suspected to be carcinogenic in experimental animal and human. Several studies have investigated excretion of metabolites in urine, hemoglobin adduction and cancer incidence among workers exposed occupationally to DCB. In these researches, metabolites of DCB had a very important role. The purpose of this study was to develop the urinary monitoring method of its metabolites from rats exposed with DCB, by easily synthesizing them in the laboratory. N,N’-diacetyl-DCB was easily synthesized with DCB in pyridine by adding sufficient acetyl chloride or acetic anhydride. N-acetyl-DCB was isolated from the supernatant, which made by adding 21µl acetyl chloride (more 3 times than DCB in moles) to 32 mg DCB in 2 ml pyridine and 0.3 ml acetic acid. Gas chromatography/mass spectrometry (GC/MS) was used for the identification, gas chromatography nitrogen phosphorous detection (GC-NPD) for the quantification and gas chromatography flame ionization detection (GC-FID) for the determination of purity. The base peak of DCB, N-acetyl-DCB and N,N’-diacetyl-DCB was 252 m/z. The other main peaks were 294 m/z for N-acetyl-DCB, and 294 and 336 m/z for N,N’-diacetyl-DCB. The purities of N-acetyl-DCB and N,N’-diacetyl-DCB were identified as 98.82 and 98.72% by GC-FID, respectively. After treatment orally to rats with 20mg DCB/kg body weight for 2 weeks, the urinary excretion amount of DCB was nearly constant at range of 0.11–0.18 mg/L for 2 weeks. But excretion of N-acetyl-DCB was continually increased from 1.30 mg/L on 1st day to 4.15 mg/L on 14th day. And level of N,N’-diacetyl-DCB in urine was sharply increased from 2.13 mg/L on 1st day to 11.00 mg/L on 14th day.

Key words: 3,3’-Dichlorobenzidine (DCB), N-acetyl-DCB, N,N’-diacetyl-DCB, Promoter and controller of the acetylation of DCB, GC/MS, NPD, FID

Introduction

3,3’-dichlorobenzidine (DCB) is an important intermediate in the production of diarylide azo pigments10 and a known animal carcinogen11. Since Rinde and Troll12 observed reductive cleavage of the azo bond of benzidine-based azo dyes in vivo, it has been hypothesized that metabolic liberation of DCB, a known animal carcinogen13, from these pigments could pose a hazard to animals and human. In analogy to other aromatic amines, DCB can be metabolically N-acetylated and/or oxidized to the corresponding N-hydroxylamine. N-Hydroxylamine undergoes covalent interaction with DNA14 and therefore, DCB is suspected to be a genotoxic carcinogen.

Recently Joppich-Kuhn et al.6 determined the DCB-hemoglobin adducts by GC/MS with negative chemical
ionization (NCI). And Zwirner-Baier & Neumann monitored biologically the acetylation and the deacetylation in the metabolic activation of aromatic amines (benzidine and DCB) as determined by hemoglobin binding. They needed the metabolites of DCB, which were very important role, and required high purity for their exact determination. Many toxicological researchers about DCB also require to easily get the standard metabolites of DCB in their laboratory.

This study investigated the urinary monitoring method of the DCB exposed to rats by easily synthesizing its metabolites (N-acetyl-DCB and N,N’-diacetyl-DCB) in the laboratory.

Materials and Method

Chemicals

3,3’-dichlorobenzidine · 2HCl (DCB · 2HCl) was obtained from Sigma (St. Louis, Mo. USA). Analytical grade of 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 (Darmstadt, Germany).

Animals and treatment

Seven 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 daily orally given to treatment group with a dose of 20 mg/kg body weight, for 2 weeks. Because of the low solubility of DCB in water, the dosing solution was made by dissolving DCB in water containing citric acid and sucrose (1:1). Urine samples were collected daily.

Urinary monitoring of DCB and its metabolites

All GC experiments were performed with a Hewlett-Packard (HP) 5890A gas chromatography with a nitrogen-phosphorus detector or a flame ionization detector for the determination of the purity. The carrier gas (helium) flow rate was 1.2 ml/min and detector make-up gas (helium) flow rate was 25 ml/min. The injection port temperature was 300°C, the detector temperature 310°C, and the oven temperature was programmed from 100 to 310°C at 20°C/min. 2 µl aliquot of the final solution was injected in the split mode (split ratio; 1:15).

All mass spectra were obtained with a Agilent 6890/5973 N instrument. 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 the identification of analysts at high concentration. Quantitative analysis modes were selected ion monitoring detection mode (SIM). Columns for GC were HP-5 capillary column (50 m × 0.32 mm i.d. × 0.17 µm F.T.). Samples were injected in the pulsed split mode (split ratio, 1:15). The flow rate of the helium was 1.0 mL/min. The GC operating temperature were : injector temperature, 300°C; transfer line temperature, 300°C; oven temperature, programmed from 100°C at 20°C/min to 310°C (held for 2 min).

Results

Synthesis of N,N’-diacetyl-3,3’-DCB

We dissolved 32 mg DCB with 2 ml pyridine, and then added sufficient acetyl chloride or acetic anhydride for the acetylation of DCB. White precipitation was obtained by filtration. The dried product was dissolved with the saturated phenol again, and identified to N,N’-diacetyl-3,3’-DCB with GC/MS. Figure 1 shows the chromatogram and mass spectrum. The base ion was 252 m/z and the other main ions were 294 and 336 m/z. And its purity was determined as 98.72 % by GC-FID.

Synthesis of N,N’-diacetyl-3,3’-DCB

We dissolved 32 mg DCB in 2 ml pyridine, added 0.3 ml acetic acid, and added 21 µL acetyl chloride (more 3 times than DCB in moles). And we got the supernatant by filtration with paper filter on 7,000 rpm centrifugation, and dried it with nitrogen gas. Figure 2A shows the peaks of pyridine, N-acetyl-DCB and N,N’-diacetyl-DCB by using gas
For removing N,N'-diacetyl-DCB from the dried product, we dissolved it with a mixture solvent of 0.5N NaOH and toluene (1:2), got the toluene layer by 2,000 rpm centrifugation (5 min.), and dried it with nitrogen gas. Figure 2B shows N,N'-diacetyl-DCB peak removed from it, but pyridine was not removed. Pyridine was easily removed from it by washing with acetone. Figure 2C shows the peak of the isolated N-acetyl-DCB by GC-NPD. Finally we identified the purified N-acetyl-DCB by GC-MS. Figure 3 shows the chromatogram and fragmentation pattern of it. The base peak was 254 m/z and the other main peak was 294 m/z. And its purity was determined as 98.82 % by GC-FID.

Urinary monitoring of DCB and its metabolites

The reproducibility of the assay was very good, as shown in Table 1. For five independent determination at 0.1, 5.0 and 10.0 ng/mL, the coefficient of variation was less than 12%. Detection limits were 0.01 ng/mL for DCB and N-acetyl DCB, and 0.05 ng/mL for N,N'-diacetyl DCB based upon an assayed urine (1 ml). Detections of limit were defined by a minimum signal-to-noise of 3 and coefficients of variation for replicate determination (n=5) of 15% or less.

The daily concentrations of DCB, N-acetyl-DCB and N,N'-diacetyl-DCB in urine of rats orally treated with 20 mg/kg body weight DCB for 2 weeks are shown in Table 2. The excretion amount of DCB was nearly constant at 0.11–0.18 mg/L for 2 weeks. But level of N-acetyl-DCB was continually increased from 1.30 mg/L on 1st day to 4.15 mg/L on 14th day after treatment. And also, level of N,N'-diacetyl-DCB was sharply increased from 8th day. Urinary excretion amount of N,N'-diacetyl-DCB was increased from 2.13 mg/L on 1st day to 11.00 mg/L on 14th day after treatment.

Discussion

DCB is water-insoluble chemical compound. Many kinds of solvents were applied to dissolve DCB. Solvents of DCB in experimental animal studies were Tween 20/water (1:200) by Kellner et al.9), Emulphor/ethanol/water (1:1:8) by Decad et al.8), corn oil by Iba10) and Iba & Thomas13), and propanediol/ethanol (1:1) by Zwirner-Baier & Neumann7). Morales, et al.11) used 0.2% triethylamine in methanol as extraction solution for air sample of benzidine, DCB, and its salts. And Roberts & Rossano12) dissolved in 70% acetic acid after making mixtures of citric acid containing less than 1% DCB. We found the partially dissolving solvents were benzene, ether, ethanol and methanol, and the completely dissolving solvents were 70% acetic acid on mixtures of citric acid containing less than 1% DCB, pyridine, mixture of 0.5N NaOH and toluene (1:2), phenol saturated with 20 mM TRIZA base.

Pyridine is a good solvent for many compounds, and is used for synthetic intermediate in laboratory. Decad, et al.9) reported DCB monoacetate and diacetate were prepared by acetylation with acetyl chloride in pyridine. We also made N,N'-diacetyl-DCB with this method such as precipitation, and identified that its base peak was 252 m/z and other main peaks were 294 and 336 m/z with GC/MS. We found the
supernatant was composed of DCB, N-acetyl-DCB and N,N'-diacetyl-DCB, and the composition of precipitation was N,N'-diacetyl-DCB. And also found acetic acid could decreased the concentration of N,N'-diacetyl-DCB in both supernatant and precipitation. When the volume of pyridine and acetic acid was same as 3 ml in DCB solution, N,N'-diacetyl-DCB was hardly formed in both supernatant and precipitation, but both DCB and N-acetyl-DCB were only detected in solution. The reason of these results is that pyridine accelerated the reaction between the acetyl and each side N of DCB, but acetic acid partly blocked this reaction. So we found that pyridine was a good promoter, and acetic acid was a good controller, for the acetylation of DCB.

Then, for the isolation of N-acetyl-DCB from supernatant, we checked the solvents that had the highly different solubility about DCB, N-acetyl-DCB and N,N'-diacetyl-DCB. And we found DCB and N-acetyl-DCB were dissolved at the same solvent, but N,N'-diacetyl-DCB was dissolved at the different solvent. A mixture of 0.5 N NaOH and toluene (1:2) was a good case. It dissolved DCB and N-acetyl-DCB, but not dissolved N,N'-diacetyl-DCB. So we made that all DCB became to be N-acetyl-DCB and N,N'-diacetyl-DCB with adding excessive acetyl chloride in pyridine and acetic acid, such as a promoter and a controller of acetylation, as
DCB solvent mixture. We dissolved 32 mg DCB in 2 ml pyridine (promoter), added 0.3 ml acetic acid (controller), and added 21 µL acetyl chloride (more than 3 times DCB in moles). And we obtained the supernatant by filtration with paper filter on 7,000 rpm centrifugation, and dried it with nitrogen gas. For isolating N-acetyl-DCB from the dried product, we dissolved it with a mixture of 0.5N NaOH and toluene (1:2), got the toluene layer by 2,000 rpm centrifugation (5 min.), and dried it again with nitrogen gas. Pyridine resided in the dried product with N-acetyl-DCB. But we got the purified N-acetyl-DCB by washing with acetone. The more repeatedly washing it with acetone made the more pure N-acetyl-DCB.

With using this standard N-acetyl-DCB and N,N'-diacetyl-DCB, we analyzed the urinary metabolites from rats, which were orally treated with 20 mg DCB/kg body weight for 2 weeks. In our study, the urinary excretion rate of DCB was nearly constant at range of 0.11–0.18 mg/L for 2 weeks. But excretion of N-acetyl-DCB and N,N'-diacetyl-DCB were continually increased day by day, especially the latter was sharply increased from 8th day after treatment. Level of N-acetyl-DCB was increased from 1.30 mg/L on 1st day to 4.15 mg/L on 14th day, and that of N,N'-diacetyl-DCB was sharply increased from 2.13 mg/L on 1st day to 11.00 mg/L on 14th day in our results. Tanaka\textsuperscript{14}) reported the urinary metabolites of DCB in rats were 0.30 mg/L as N-acetyl-DCB, and 1.13 mg/L as N,N'-diacetyl-DCB, respectively, on 24 hr after oral treatment of DCB. The ratios of N,N'-diacetyl-DCB and N-acetyl-DCB were 3.76 in Tanaka’s 24 hrs study, and 1.6–2.6 during our 2 weeks study.

**Conclusion**

DCB was partially dissolved in benzene, ether, ethanol and methanol, and completely dissolved in 70% acetic acid on mixtures of citric acid containing less than 1% DCB, pyridine, a mixture of 0.5 N NaOH and toluene (1:2), and phenol saturated with 20 mM TRIZA base.

N,N'-diacetyl-DCB was easily synthesized with DCB in pyridine by adding sufficient acetyl chloride or acetic anhydride. N-acetyl-DCB was isolated from the supernatant, which made by adding 21 µL acetyl chloride (more than 3 times than DCB in moles) to 32 mg DCB in 2 ml pyridine and 0.3

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**Table 1.** Within-run precision and accuracy of DCB, N-acetyl-DCB and N,N'-diacetyl-DCB in urine (n=5)

<table>
<thead>
<tr>
<th>Added concentration (ng/mL)</th>
<th>Found concentration (ng/mL)</th>
<th>Mean ± SD (CV %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DCB</td>
<td>N-acetyl DCB</td>
</tr>
<tr>
<td>0.10</td>
<td>0.11 ± 0.02 (8.2)</td>
<td>0.13 ± 0.02 (7.8)</td>
</tr>
<tr>
<td>5.00</td>
<td>4.88 ± 0.21 (3.5)</td>
<td>4.98 ± 0.32 (3.6)</td>
</tr>
<tr>
<td>10.0</td>
<td>10.2 ± 0.50 (1.5)</td>
<td>9.78 ± 2.10 (2.1)</td>
</tr>
</tbody>
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SD: standard deviation, CV : coefficient of variation.
ml acetic acid.

Gas chromatography/mass spectrometry (GC/MS) was used for the identification, gas chromatography nitrogen phosphorous detection (GC-NPD) for the quantification and gas chromatography flame ionization detection (GC-FID) for the determination of purity. The base peak of DCB, N-acetyl-DCB and N,N’-diacetyl-DCB were 252 m/z. The other main peaks were 294 m/z for N-acetyl-DCB, and 294 and 336 m/z for N,N’-diacetyl-DCB.

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Acknowledgement

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