한국환경위생학회지, 제29권 제2호(2003)
Kor. J. Env. Hlth., Vol. 29, No. 2, pp 50~55(2003)

Synthesis and Isolation of Monoacetyl-DCB and Diacetyl-DCB from 3,3'-dichlorobenzidine(DCB)

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(Received April 14, 2003; Accepted May 19, 2003)

디클로로벤지딘으로부터 대사물질의 합성과 분리방법에 대한 연구

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ABSTRACT

3,3-dichlorobenzidine is suspected to be cancinogenic in experimental animal and human. Several studies have investigated excretion of metabolites in urine, hemoglobin adduction and cancer incidence among workers occupationally exposed to 3,3'-dichlorobenzidine. In these researches, metabolites of 3,3'-dichlorobenzidine had a very important role, and were required as highly purity. The purpose of this study was synthesis and isolation of its metabolites from 3,3'-dichlorobenzidine. 3,3'-dichlorobenzidine 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 0.5N NaOH and toluene(1:2), and phenol saturated with 20 mM TRIZA base. DCB, monoacetyl-DCB and diacetyl-DCB were measured by using gas chromatography/mass spectrometry(GC/MS). Detection for checking them was nitrogen phosphorous detection mode(NPD), and for identifying them was selected ion monitoring mode(SIM). The base peaks were 252 m/z in DCB, 252, and 294 m/z in monoacetyl-DCB, and 252, 294 and 336 m/z in diacetyl-DCB, respectively. Diacetyl-DCB was synthesized by titrating DCB solution of pyridine with sufficient acetyl chloride. Precipitation was diacetyl-DCB, which was purity of 98.7%. And its supernatant was composed of DCB, monoacetyl-DCB and diacetyl-DCB. By using acetic acid as controller of acetylation, monoacetyl-DCB was isolated from diacetyl-DCB. And residual pyridine was removed by using acetone. The purity of monoacetyl-DCB was 98.8%.

Keywords: Dichlorobenzidine(DCB), Monoacetyl-DCB, Diacetyl-DCB, Controller of acetylation, GC/MS, NPD(nitrogen phosphorous detection), SIM(selected ion monitoring detection mode)

요 약

3,3'-디클로로벤지딘(DCB)는 실험동물에 발암물질로 밝혀졌고, 사람에게 암을 유발시킬 수 있는 발암물질로 의심되고 있다. 많은 연구자들이 사업장에서 DCB에 폭로된 근로자들을 대상으로 뇨중에 배설된 대사물질, 헤모글로빈 부가체, 그리고 암 발생율 등에 대하여 연구를 하고 있다. 이러한 연구를 하기 위해서는 표준물질로 되어 있는 DCB의 대사물질이 꼭 필요하다. 따라서 본 연구의 목적은 DCB를 이용하여 이들의 대사물질을 합성하여 표준물질로 사용코자 합니다. DCB는 벤젠, 에테르, 에탄올, 메탄올 등에 부분적으로 용해되지만, 구연산이 1% 이하로 함유된 70% 아세트산, 피리딘, 0.1N NaOH와 톨로엔이 1:2로 섞인 혼합물, 20 mM TRIZA염으로 포화된 페놀 등에는 완전히 용해되기 때문에 본 연구에서는 DCB를 피리딘에 녹여서 사용하였다. DCB와 대사물질인 mono-acetyl-DCB 및 diacetyl-DCB는 가스크로마토그래피(GC/MS)로 분석하였고, 검출기는 NPD와 SIM를 사용하였다. DCB의 기본피크는 252 m/z이었고, mono-acetyl-DCB의 기본피크는 252와 294 m/z로 구성되어 있었으며, diacetyl-DCB의 기본피크는 252, 294, 336 m/z로 구성되어 있었다. Discetyl-DCB는 피리딘에 용해된 DCB에 염소아세틸를 충분히 적정하여 합성하였다. 이렇게 얻은 diacetyl-DCB의 순도는 98.7%이었다. 침전물위에 있는 용해물질 속에는 DCB, mono-acetyl-DCB, diacetyl-DCB가 함유되어 있었는데, 아세트산을 아세틸화를 조절하는 물질로 사용하여 DCB를 모두 아세틸화시키었고, diacetyl-DCB로부터 mono-acetyl-DCB를 분리하여 추출하였다. 추출된 mono-acetyl-DCB는 아세톤으로 세척하여 98.8%의 순도를 얻었다.

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I. Introduction

3,3'-dichlorobenzidine(DCB) is an important intermediate in the production of diarylide azo pigments[1] and a known animal carcinogen.[2,4] Since Rinde and Troll[3] observed reductive cleavage of the azo bond of benzidine-based azo dyes in vivo, it has been hypothesized that metabolic liberation of DCB 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 undergo covalent interaction with DNA,[5] therefore, DCB is suspected to be a genotoxic carcinogen. Recently Joppich-Kuhn *et al.*[6] determined the dichlorobenzidine-hemoglobin adducts by GC/MS with negative chemical ionization (NCI). Zwirner-Baier & Neumann[7] was also monitoring the acetylation and the deacetylation in the metabolic activation of aromatic amines(benzidine and 3,3'-dichlorobenzidine) as determined by hemoglobin binding. High purity of the metabolites of DCB, which are believed to play very important role as high purity in these studies, is essential for toxicological research. The purpose of this study was to investigate an method for the synthesis and isolation of metabolites of DCB in the laboratory. Monoacetyl-DCB and diacetyl-DCB are the important metabolites among them.

II. Materials and Method

1. Chemicals

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

2. Gas chromatography-mass spectrometry

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 ml/min. The GC operating temperature were : injector temperature, 300°C; transfer line temperture, 300°C; oven temperature, programmed from 100°C at 20°C/min to 310°C(held for 2 min).

III. Results

1. DCB solution

We tried to dissolve DCB with many kinds of solvents. We found the partly dissolving solvents

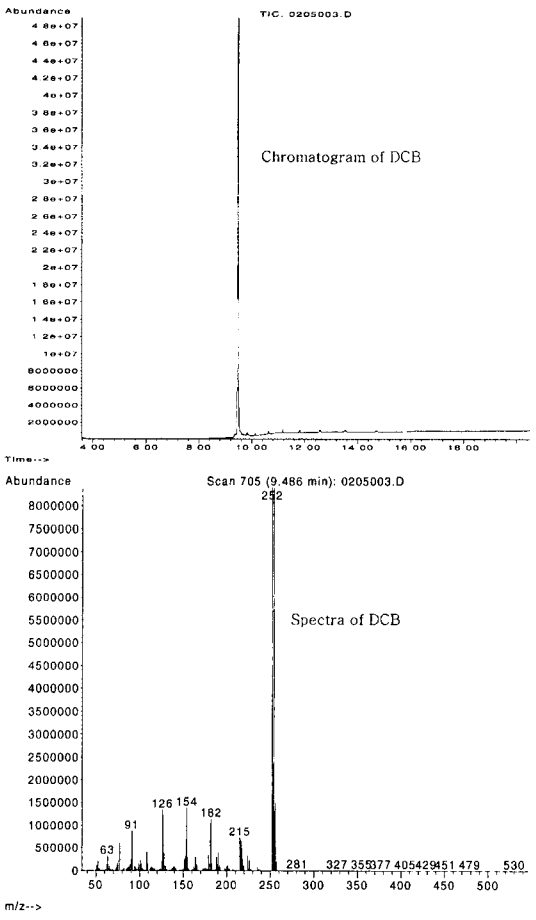

Fig. 1. Chromatogram(above) and fragmentation pattern(below) of DCB by using gas chromatography/mass spectrometry-selected ion monitoring detection mode (GC/MS-SIM).

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,[8] a mixture of 0.5N NaOH and toluene(1:2), and phenol saturated with 20 mM TRIZA base. Saturated phenol was used as solvent for identifying DCB with GC/MS-SIM. Fig. 1 showed the chromatogram and fragmentation pattern of DCB. Its base peak was 252 m/z.

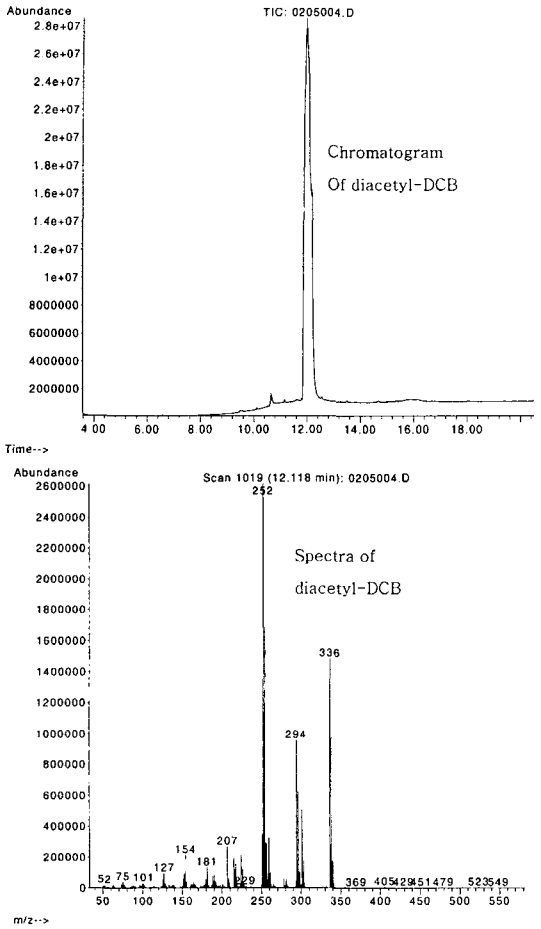

Fig. 2. Chromatogram(above) and fragmentation pattern(below) of diacetyl-DCB by using gas chromatography/mass spectrometry-selected ion monitoring detection mode(GC/MS-SIM).

2. Synthesis of diacetyl-DCB

We dissolved DCB with pyridine, and then added it with sufficient amount of acetyl chloride or acetic anhydride for acetylation of DCB. White precipitation could be got from the solution by filtration. Dried product was dissolved with the saturated phenol again, and identified to diacetyl-DCB with GC/MS. Fig. 2 showed the chromatogram and fragmentation. Its base peaks were 252, 294 and 336 m/z. The purity was identified as 98.72% by using FID.

3. Controller of acetylation of DCB

We found the composition of supernatant were DCB, monoacetyl-DCB and diacetyl-DCB. Table 1 showed the level of diacetyl-DCB was decreased as the acetic acid added more, and was almost zero when acetic acid and pyridine were the same volume of 3 m*l*. Fig. 3 showed the differential peak patterns. Thus, we found pyridine was promoter, and acetic acid was controller of acetylation of DCB

4. Synthesis and isolation of monoacetyl-DCB

The mixture solvent of 0.5N NaOH and toluene(1:2) dissolved DCB and monoacetyl-DCB, but couldn't dissolved diacetyl-DCB. Thus, we could made all DCB to be monoacetyl-DCB and

Table 1. levels of monoacetyl-DCB and diacetyl-DCB in the supernatant and sedimentation by adding acetic acid to DCB in pyridine

Added volume of acetic acid(m*l*)	Peak area counts of supernatant measured by GC/MS			Precipitate
	DCB	N-acetyl-DCB	N,N'-diacetyl-DCB	N,N'-diacetyl-DCB
0.1	7154.8	4317.7	595.3	Yes
0.2	5610.3	7088.2	1200.1	Yes
0.3	2796.2	7891.2	1375.8	Yes
0.5	5595.2	5576.8	148.089	No
1.0	6037.0	7433.0	464.9	No
1.5	5537.7	5503.1	377.0	No
3.0	3985.8	2345.0	4.1	No

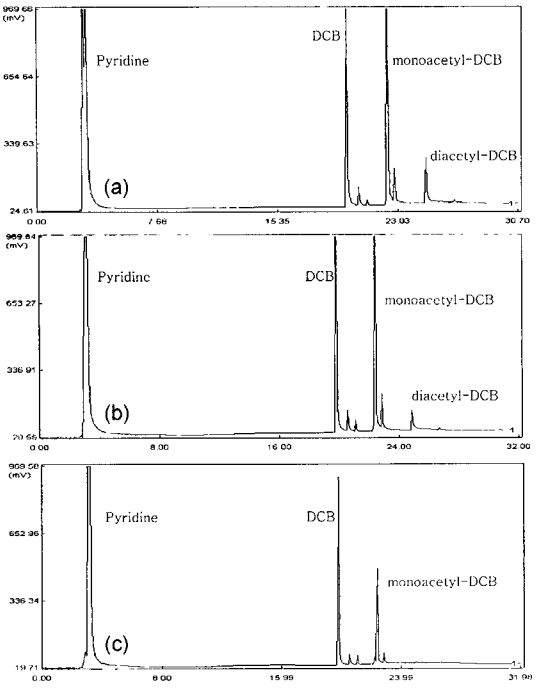

Fig. 3. Chromatogram of pyridine, DCB, monoacetyl-DCB and diacetyl-DCB in the supernatant of which the acetic acid(controller of acetylation) added to pyridine as the solvent of DCB solution in titration with acetyl chloride by using gas chromatography/mass spectrometry-nitrogen phosphorous detection mode(GC/MS-NPD). (a): added 0.3 m/ acetic acid, (b): added 1.0 m/ acetic acid, (c): added 3.0 m/ acetic acid

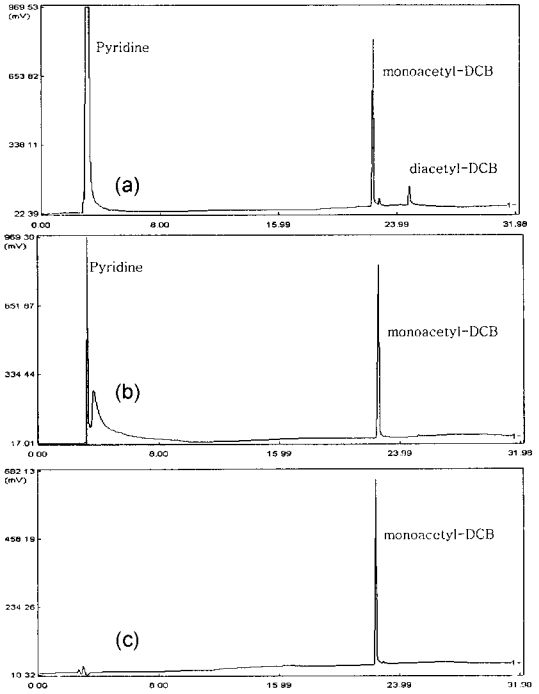

Fig. 4. Chromatogram of pyridine, monoacetyl-DCB and diacetyl-DCB in isolation procedure of monoacetyl-DCB from diacetyl-DCB and pyridine by using gas chromatography/mass spectrometry-nitrogen phosphorous detection mode(GC/MS-NPD). (a): all DCB became to be monoacetyl-DCB and diacetyl-DCB by using acetic acid(controller of acetylation). (b): Removed diacetyl-DCB by using a mixture solvent of 0.5N NaOH and toluene(1:2), (c): isolated monoacetyl-DCB by using acetone.

diacetyl-DCB with using acetic acid(controller of acetylation). We dissolved 32 mg DCB in 2 m/ pyridine, and added 0.3 m/ acetic acid and 21 μ/ acetyl chloride(more 3 times than DCB in moles). The supernatant was obtainable by filtration with paper filter or 7,000 rpm centrifugation, and by dryness it with nitrogen gas. Fig. 4(a) showed the peaks of pyridine, monoacetyl-DCB and diacetyl-DCB by using GC/MS-NPD. For removing diacetyl -DCB from the dried product, we dissolved it with a mixture solvent of 0.5N NaOH and toluene(1:2), separate the toluene layer by 2,000 rpm centrifugation (5 min.), and dried it with nitrogen gas. Fig. 4(b) showed diacetyl-DCB was removed from it. Pyridine was easily removed by washing with acetone. Fig. 4(c) showed the peak of the isolated monoacetyl-DCB. Finally we identified it was the purified monoacetyl-DCB with GC/MS. Fig. 5 showed the chromatogram and fragmentation pattern. Its base peaks were 254 and 294 m/z. The purity was identified as 98.82% by using FID.

IV. Discussion

DCB is water-insoluble chemical. 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 Iba[10] and Iba & Thomas,[11] and propanediol/ethanol (1:1) by Zwirner-Baier & Neumann.[7] Morales, *et*

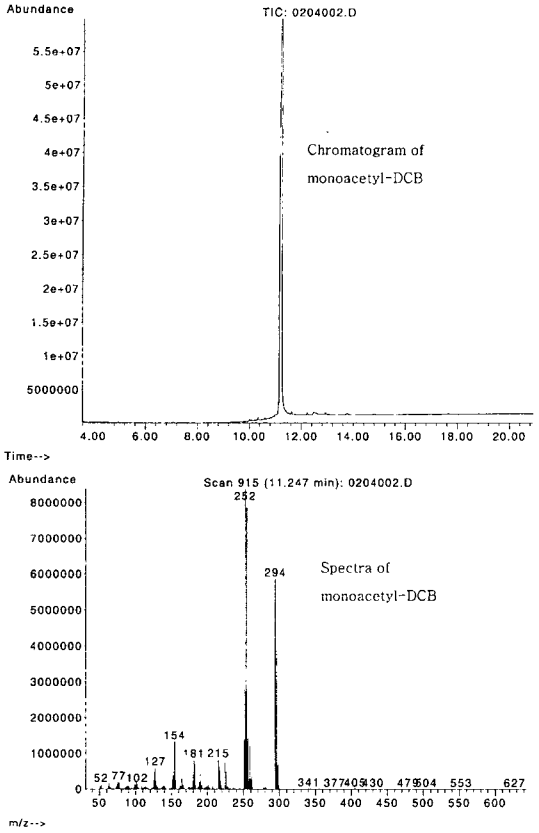

Fig. 5. Chromatogram(above) and fragmentation pattern(below) of monoacetyl-DCB by using gas chromatography/mass spectrometry-selected ion monitoring detection mode(GC/MS-SIM).

al.[12] used 0.2% triethylamine in methanol as extraction solution for air sample of benzidine, dichlorobenzidine, and its salts. Roberts & Rossano[13] dissolved in 70% acetic acid after making mixtures of citric acid containing less than 1% DCB. We found the partly 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 used for synthetic intermediate in laboratory. Decad, *et al.*[8] reported 3,3'-dichlorobenzidine monoacetate and diacetate were prepared by acetylation with acetyl chloride in pyridine. We also synthesized diacetyl-DCB with this method, i.e., precipitation, and identified that its base peak were 252, 294 and 336 m/z with GC/MS. We found the composition of supernatant were DCB, monoacetyl-DCB and diacetyl-DCB, and its of sedimentation was diacetyl-DCB. It was found that acetic acid could decrease the concentration of diacetyl-DCB in supernatant and precipitation. When the volume of pyridine and acetic acid was same as 3 m*l*, diacetyl-DCB was almost not formed, and only DCB and monoacetyl-DCB was detected in solution. We found pyridine accelerated the reaction between the acetyl and each side N of DCB, but acetic acid partly blocked this reaction. It was believed that acetic acid is a good controller in acetylation of DCB.

For isolation of monoacetyl-DCB from supernatant, we check the solvents about DCB and monoacetyl -DCB. We found they were dissolved at the same solvent, but not diacetyl-DCB. A mixture of 0.5N NaOH and toluene(1:2) was a good case. It was dissolved DCB and monoacetyl-DCB, but not dissolved diacetyl-DCB. Thus, we made that DCB became to be monoacetyl-DCB and diacetyl-DCB by adding excessive acetyl chloride in pyridine and acetic acid, as promoter and controller of acetylation, as DCB solvent mixture. We dissolved 32 mg DCB in 2 m*l* pyridine, and added 0.3 m*l* acetic acid(controller of acetylation) and 21 μ*l* acetyl chloride(more 3 times than DCB in moles).

V. 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 0.5N NaOH and toluene(1:2), and phenol saturated with 20 mM TRIZA base. DCB, monoacetyl-DCB and diacetyl-DCB were checked and identified by using GC/MS. The base peaks were 252 m/z in DCB, 252, and 294 m/z in monoacetyl-DCB, and 252, 294 and 336 m/z in diacetyl-DCB, respectively.

Diacetyl-DCB was synthesized with DCB in pyridine by adding sufficient acetyl chloride. Precipitation was diacetyl-DCB, which purity was 98.7%. Its supernatant was composed of DCB, monoacetyl-DCB and diacetyl-DCB. By using

acetic acid as controller of acetylation, monoacetyl -DCB was isolated from diacetyl-DCB. Residual pyridine was removed by using aceton. The purity of monoacetyl-DCB was 98.8%.

Acknowledgement

This work was supported by grant R02-2000-00203 from the Basic Research Program of the Korea Science & Engineering Foundation.

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