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Optimization of experimental conditions in studies on metabolism of 4-nitrobiphenyl

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Abstract—The metabolites of 4-nitrobiphenyl (4-NBP) were studied using S-9 under anaerobic conditions. Dependence of concentrations of metabolites on incubation time and S-9 amounts were followed. Ten metabolites were isolated and tentatively identified by high-performance liquid chromatography/atmospheric pressure chemical ionizaiton/mass spectrometry (HPLC/APCI/MS) and HPLC/UV. Among them, 4-aminobiphenyl and hydroxylaminobiphenyls were found to be the major metabolites, and 4-acetylaminobiphenyl (4-AABP), N-hydroxy-4-acetylaminobiphenyl (4-AABP-N-OH), x-OH-4-nitrobiphenyl (4-NBP-x-OH), biphenylene and N-formyl-4-aminobiphenyl (N-formyl-4-ABP) were the minor metabolites.

Keywords: 4-NBP; metabolism; reductive; carcinogen; HPLC/APCI/MS.

1 Introduction

The direct-acting mutagenicities of nitropolynuclear aromatic hydrocarbons (NO_2-PAHs) have been of great concern in recent years, because of their ubiquitous existence in the environment. Furthermore, some NO_2-PAHs have been proved to be carcinogenic. The mechanism of carcinogenesis is thought to be mediated by the metabolic reduction of these nitro compounds to reactive intermediates. Thus, it is important to develop efficient techniques for isolation and identification of these active intermediates and to elucidate the mechanisms of enzymatic reduction.

4-Nitrobiphenyl (4-NBP) was the first nitro aromatic compound shown to be a bladder carcinogen (Deichmann, 1958). In contrast to ample metabolic studies of other NO_2-PAHs, there are relatively few and initial studies on 4-NBP. In preliminary studies, metabolites of 4-NBP in vitro found by any of the authors were not more than two in number, and the conclusions drawn by different authors were inconsistent. 4-aminobiphenyl (4-ABP) and 4-nitrosobiphenyl (4-NOBP) were identified as reductive metabolites by TLC and UV-absorption (Uehleke, 1967). With a gas-liquid chromatographic system, the slow formation of 4-ABP under anaerobic condition by rat and mouse liver fractions was observed (Poirier, 1974).

In this study, the reductive metabolism of 4-NBP was investigated, and the new technique, high-performance liquid chromatography/atmospheric pressure chemical ionization/mass spectrometry (HPLC/APCI/MS) has been applied probably for the first time to identify metabolites of NO_2-PAHs, the environmental carcinogens. Ten metabolites of 4-NBP (more then those reported in any of the previous papers) have been tentatively identified successfully, and

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among them, several new metabolites which have not been reported previously are reported.

2 Materials and methods

2.1 Chemicals

4-NBP, NADPH and DMSO were obtained from Sigma Chemical Company. 4-ABP was obtained from Aldrich Chemical Company.

Liver S-9 fraction was kindly supplied by Institute of Occupational Medicine, Chinese Academy of Preventive Medicine.

2.2 Metabolism studies

The anaerobic metabolism of 4-NBP was carried out in a 10 ml Erlenmeyer flask containing 5 ml S-9 mix. The latter contained 0.5 ml S-9 fraction, 1μmol NADPH, 2μmol G-6-P, 2μmol $MgCl_2$, 0.225 mmol Tris-HCl buffer (containing 1.15% KCl, pH 7.4) and was bubbled for 20 min with nitrogen gas before use. The incubation was then started by addition of 0.5μmol 4-NBP (dissolved in DMSO), and was carried out at 37℃ for a certain period of time, while air had been excluded throughout the incubation. It was then stopped by the addition of 0.5 ml methanol. The metabolites were then extracted with ethyl acetate (3 × 3 ml), and the ethyl acetate layer was removed following centrifugation, dried (Na_2SO_4), filtered (0.22μm filters) and evaporated under a nitrogen stream. The residue was redissolved in acetonitrile/methanol and analyzed by HPLC/UV and HPLC/APCI/MS.

Control incubations were performed similarly as described above, except that heat-denatured S-9 fraction was used.

2.3 HPLC analyses

HPLC/UV analyses were performed on a Shimadzu LC-5A chromatograph fitted with a Zorbax ODS 5μm column (25.0 cm × 4.6 mm i.d.). The mobile phase was acetonitrile-water or methanol-water with different proportions at a flow rate of 1.0 ml/min. UV peaks were monitored by absorbance at 254 nm.

HPLC/DAD analyses were performed on a Shimadzu LC-10A chromatograph fitted with a Shimpack ODS 5μm column (15.0 cm × 4.6 mm i.d.), with which UV spectrum and purity of each UV peak were obtained.

HPLC/MS analyses were performed on a HEWLETT PACKARD 1050, VG PLATFORM II, HPLC/APCI/MS system, and a Phenomenex ODS 5μm column was used.

HPLC/DAD and HPLC/MS chromatographic conditions were the same as described above in HPLC/UV analyses.

3 Results and discussion

3.1 Dependence of concentrations of metabolites on incubation time

The kinetics of disappearance of 4-NBP and appearance of the major metabolites were studied. The results (Fig. 1) illustrated that the amounts of metabolites A, B, D, F, I increased

continuously with extending incubation time up to a certain time (approximately 60 min). After that, they did not change obviously with time, except metabolite H, which decreased slightly. Metabolites E and G decreased after a short incubation time. The rate of nitroreduction for 4-NBP also became slower after 60 minutes. Therefore, it might be considered that carcinogen 4-NBP tended to be the most activated since an incubation time of 60 minutes, and most of the metabolites reached their maximum concentration.

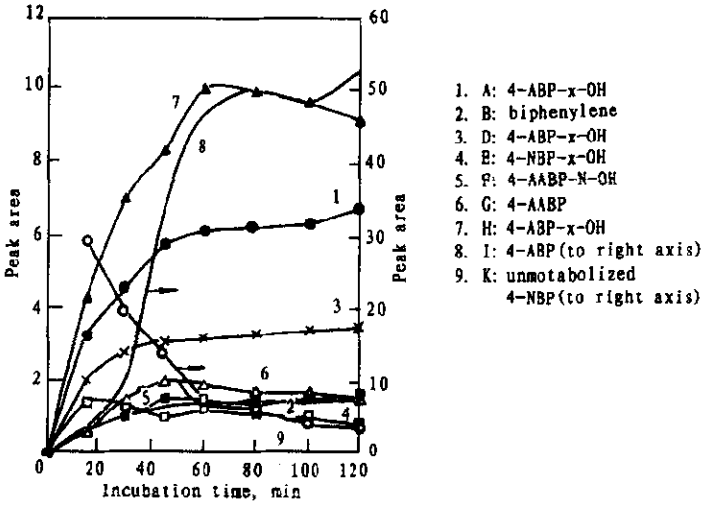

Fig. 1 Dependence of concentration of major metabolites on incubation time

A, B, D-I and K: according to Table 1

3.2 Dependence of concentration of metabolites on S-9 amounts

As can be seen in Fig. 2, the concentrations of the major metabolites increased with S-9

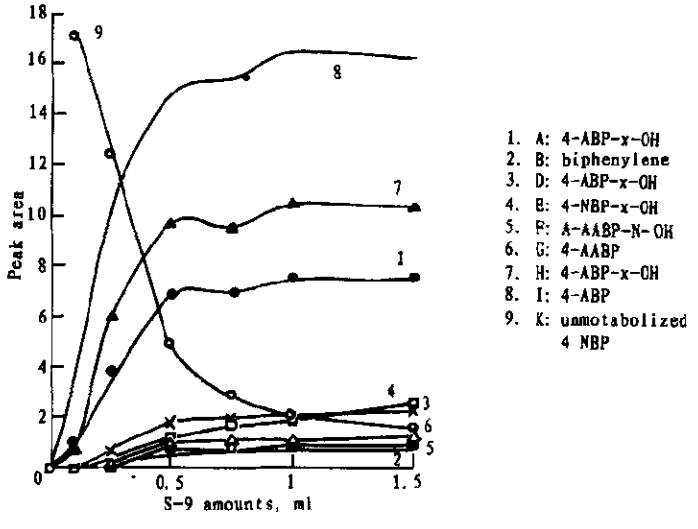

Fig. 2 Dependence of concentrations of major metabolites on S-9 amounts

A, B, D-I and K: according to Table 1

amounts in a range of 0—0.5 ml, and after that, more S-9 amounts did not contribute obviously to the formation of the major metabolites. In addition, handling larger S-9 amounts was troublesome during the extraction. Therefore, we chose 0.5 ml S-9 as the appropriate volume for this experiment.

3.3 The identification of metabolites

HPLC and HPLC/MS were used to identify the metabolites. Fig. 3A and Fig. 3B (Song, 1997) show the HPLC chromatogram of the metabolites of 4-NBP and the control respectively. Table 1 summarized the data of MS and HPLC of these metabolites. Based on comparison of Fig. 3A and Fig. 3B, and results of MS (not shown), peak a, b, c were assigned as polar compounds in S-9 fraction, and K was the unmetabolized 4-NBP.

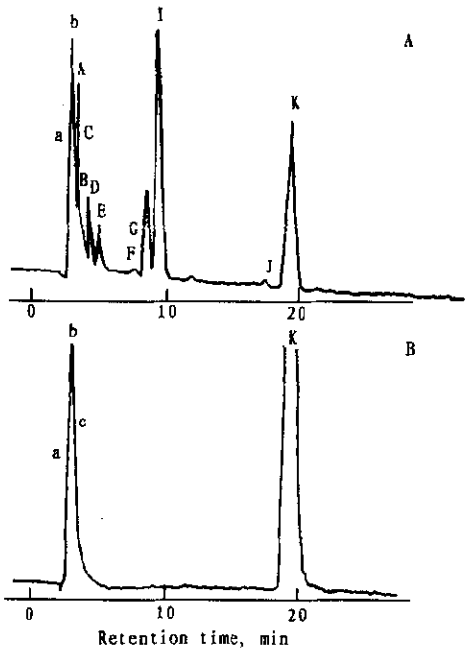

Fig. 3 HPLC chromatogram of metabolites of 4-NBP

In this study, 10 metabolites have been tentatively assigned, 4-ABP and hydroxylaminobiphenyls (including 3 isomers) are found to be the major metabolites, and minor 4-AABP, 4-AABP-x-OH, 4-NBP-x-OH, biphenylene and N-formyl-4-ABP metabolites are also identified.

Table 1 MS and HPLC data of metabolites of 4-NBP

HPLC		MS					
Peak No.*	t_r, min	AP^+/AP^-	MW	Compound	Chemical formula	Fragmention	Intensity
A	2.80	−	185	4-ABP-x-OH	$C_{12}H_{11}NO$	169	$4.70e^4$
B	3.18	−	152	Biphenylene	$C_{12}H_8$		$1.10e^4$
C	NS**	+	197	N-formyl-4-ABP	$C_{13}H_{11}NO$	186	$1.68e^3$
D	3.68	−	185	4-ABP-x-OH	$C_{12}H_{11}NO$	169	$1.88e^4$
E	4.40	−	215	4-NBP-x-OH	$C_{12}H_9NO_3$	199, 185, 169, 141	$1.26e^4$
F	6.82	−	227	4-AABP-N-OH	$C_{14}H_{13}NO_2$	211, 185, 169	$1.14e^4$
G	7.10	+	211	4-AABP	$C_{14}H_{13}NO$	169	$1.05e^4$
H	7.98	−	185	4-ABP-x-OH	$C_{12}H_{11}NO$	169	$1.05e^5$
I	9.10	+	169	4-ABP	$C_{12}H_{11}N$		$1.54e^5$
J	17.21	+	169	x-ABP	$C_{12}H_{11}N$		$1.09e^4$
K	19.59	−	199	4-NBP	$C_{12}H_9NO_2$	183, 169	$1.56e^4$

* : according to Fig. 1 ** : not shown

4-ABP is of special interest, since it is a recognized human urinary bladder carcinogen (Radomski, 1979), and has been studied in recent years (Stillwell, 1986; Kadlubar, 1991). In this study 4-ABP is the major metabolite.

Previous studies showed that hydroxyl nitrofluorenes were associated with high-acting mutagenicity (Moller, 1988). In accordance with the similar structure, 4-ABP-x-OH might be expected to be active in mutagenicity and carcinogenicity. 4-ABP-N-OH was reported to be the

most potent of the aromatic amine bladder carcinogens in dogs and 4-AABP-N-OH was also a strong (Stronger than 4-AABP) mammary carcinogen (Jack, 1973; Miller, 1961).

Mutagenicity studies with *S. typhimurium* strains provided evidence that the N-formyl derivatives of N-hydroxy-2-aminofluorene were stronger mutagens than were its parent compounds and N-acetyl derivatives (King, 1978; Weeks, 1980). Thus, N-formyl-4-ABP tentatively identified might be a mutagen and carcinogen, and more active than 4-NBP.

Another novel finding is the identification of biphenylene, which has not been reported in previous studies. Further confirmation and research about its mutagenicity and carcinogenicity are required.

Previous studies had proved that 4-NOBP was an intermediate of 4-NBP (Uehleke, 1967). In present study, 4-NOBP is not detected. We found that there was a big difference of detecting sensitivity for some different kinds of compounds during HPLC/APCI/MS analyses. There are two possible reasons for the failure of detecting 4-NOBP: the amount of 4-NOBP formed is not enough for its sensitivity or the life of existence of 4-NOBP formed is too short to be detected.

In conclusion, we wish that these studies of metabolism in rat fractions in this paper might make some contribution in investigating mechanisms of carcinogenesis of some environmental pollutants in man.

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