Determination of the Mutagen 3-Nitrobenzanthrone in Rainwater Collected in Kyoto, Japan

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The mutagen 3-nitrobenzanthrone was determined in rainwater. Rainwater was collected at a residential area in Kyoto, Japan. Organic matter containing 3-nitrobenzanthrone in rainwater was extracted using a 3M (St. Paul, MN, U.S.A.) Empore Extraction Disk C_{18}, and the extract was purified on a pyrenylethyl silylated silica gel column. The purified sample was then reduced using a platinum black column heated at 80°C. The reduced product of 3-nitrobenzanthrone, *i.e*. 3-aminobenzanthrone, was analyzed by normal-phase HPLC with fluorescence detection. 3-Nitrobenzanthrone in the rainwater was determined in the range of 0.07–2.6 ng l^{-1}. The concentration was higher from May to July and lower from October to December. 1-Nitropyrene was also determined by reversed-phase HPLC with fluorescence detection after platinum black column reduction. 1-Nitropyrene was detected in the range of 0.0056–0.19 ng l^{-1}, and this concentration range was lower than that of 3-nitrobenzanthrone.

Key words —— nitrobenzanthrone, rainwater, solid-phase extraction, platinum black

INTRODUCTION

Nitroarenes are produced by the incomplete combustion of organic compounds such as fossil fuels,[1–3] and are also produced in the atmosphere from parent arenes and nitrogen oxides.[4–6] Nitroarenes are widespread environmental pollutants[7–9] and some are suspected to be human carcinogens.[10]

3-Nitrobenzanthrone (3-nitro-7*H*-benz[*de*]anthracen-7-one, 3-NBA, Fig. 1) was recently detected in diesel exhaust[11] and airborne particulate matter.[11,12] 3-NBA is highly mutagenic in the Ames Salmonella assay (6290000 revertants $nmol^{-1}$ in *Salmonella typhimurium* YG1024), and its potency is comparable to that of 1,8-dinitropyrene, which is the direct mutagen with the strongest activity (4780000 revertants $nmol^{-1}$ in YG1024) that has been reported thus far in the literature.[11] 3-NBA induced micronuclei in mouse peripheral blood reticulocytes[11] and in a human B-lymphoblastoid cell line.[13] 3-NBA also bound covalently to DNA after metabolic activation, forming multiple DNA adducts both *in vitro* and *in vivo*.[14,15] These facts indicate that 3-NBA in the environment may have an adverse effect on human health. To help estimate the potential risk of 3-NBA to human health, it is important to clarify the levels of 3-NBA in the environment.

We recently developed a sensitive method for determining 3-NBA using normal-phase HPLC with fluorescence detection after hydrazine and Raney nickel reduction, and determined 3-NBA in surface soil collected in the Chubu area, including Nagoya, Japan.[16] In this study, we optimized a pretreatment method for determining 3-NBA in rainwater, and determined 3-NBA as well as 1-nitropyrene (1-NP), a major nitroarene, in rainwater collected at a residential area in Kyoto, Japan.

MATERIALS AND METHODS

Chemicals —— 3-NBA was kindly provided by Dr. Takeji Takamura and Dr. Hitomi Suzuki of Kyoto University (Kyoto, Japan). Platinum black, HPLC-grade acetone and methanol were purchased from Wako Pure Chemical Industries (Osaka, Japan). HPLC-grade *n*-hexane and ethyl acetate were obtained from Kanto Chemicals (Tokyo, Japan). All other reagents were of analytical grade.

Sampling —— A deposit gauge consisted of an 18 cm-diameter glass funnel and an amber glass reservoir on the roof of a 3-story building (Kyoto Pharmaceutical University) in a residential area of Kyoto,

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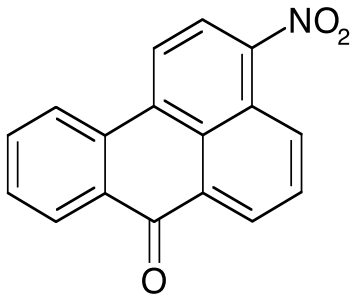

Fig. 1. Structure of 3-Nitrobenzanthrone

Japan. The reservoir was changed every day when it rained and 3-NBA in rainwater was extracted immediately.

Solid-Phase Extraction —— A 3M (St. Paul, MN, U.S.A.) Empore Extraction Disk C_{18} (47 mm diameter) was set on a 3M Vacuum Manifold System, and the extraction disk and the apparatus were washed with 40 ml methanol and 20 ml distilled water, successively. A rainwater sample (less than 1000 ml) was passed through the extraction disk at a flow rate of 70–100 ml min^{-1}. The disk was then dried by passing it under a stream of air for about 20 min, and the analytes of interest on the disk were eluted with 20 ml methanol at a flow rate of about 2 ml min^{-1}. In the case of collected rainwater was more than 1000 ml, rainwater was divided into less than 1000 ml. Each sample was concentrated on one extraction disk, and obtained samples were combined. The extract was evaporated to dryness, and the residue was dissolved in 0.25 ml methanol.

Clean-Up —— The extract (0.2 ml) was applied to a clean-up HPLC system. The clean-up HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-10AS pump, a Rheodyne (Cotati, CA, U.S.A.) 7125 sample injector with a 1-ml loop, a Nacalai Tesque (Kyoto, Japan) Cosmosil 5PYE (4.6 mm i.d. × 15 cm) column, a Shimadzu CTO-10A$_{VP}$ column oven (temperature, 30°C) and a Shimadzu SPD-10AV UV detector (wavelength, 400 nm). The mobile phase was acetone at a flow rate of 1 ml min^{-1}. Effluents at retention times of 2.6–3.4 min (for 1-NP) and 4.9–5.9 min (for 3-NBA) were collected, and these effluents were evaporated to dryness. Each residue was dissolved in 0.25 ml methanol.

Reduction —— The purified sample (0.2 ml) was then applied to a reduction system. The reduction system consisted of a Shimadzu DGU-12A on-line degasser, a Shimadzu LC-6A pump, a Rheodyne 7125 sample injector with a 1-ml sample loop and a reduction column (4 mm i.d. × 1 cm) packed with platinum black, connected in series. The reduction column was stored in a Jasco (Tokyo, Japan) RO-1561 reaction oven (temperature, 80°C). Methanol was used as a carrier at a flow rate of 0.2 ml min^{-1}. Effluent in the elution time of 0–10 min was collected, and this effluent was evaporated to dryness. The residue was dissolved in 0.25 ml ethyl acetate (for 3-NBA) or 0.25 ml methanol (for 1-NP).

HPLC —— The reduced samples (each 0.02 ml) were finally applied to HPLC systems. The HPLC system for the determination of 3-NBA consisted of a Shimadzu LC-10AT$_{VP}$ pump, a Rheodyne 7125 injector, a Tosoh (Tokyo, Japan) TSKgel CN-80Ts (4.6 mm i.d. × 25 cm) cyanopropyl column and a Jasco FP-1520S fluorescence detector. *n*-Hexane/ethyl acetate (3 : 1, v/v) was used as a mobile phase at a flow rate of 1 ml min^{-1}. Detection excitation and emission wavelengths were 490 nm and 560 nm, respectively.

The HPLC system for determining of 1-NP consisted of a Shimadzu LC-10AS pump, a Rheodyne 7125 sample injector, a Nacalai Tesque Cosmosil 5C$_{18}$MS column (4.6 mm i.d. × 25 cm), a Shimadzu CTO-10A$_{VP}$ column oven (temperature, 30°C) and a JASCO FP-1520S fluorescence detector. Methanol/water (7 : 3, v/v) was used as a mobile phase at a flow rate of 1 ml min^{-1}. Detection wavelengths were 365 and 460 nm for excitation and emission, respectively.

RESULTS AND DISCUSSION

Solid-Phase Extraction

We decided to use a solid-phase extraction (SPE) disk method to extract 3-NBA and 1-NP from rainwater, since a faster flow rate is possible than with a traditional SPE cartridge method. In this study, we compared three types of SPE disks: 3M Empore Extraction Disks C_{18}, SDB-XD (styrene-divinylbenzene copolymer) and Carbon (activated carbon). The recoveries of 3-NBA and 1-NP were measured using 1000 ml water containing 0.1 ml of 100 ng ml^{-1} 3-NBA or 1-NP methanol solution. The recoveries of 3-NBA and 1-NP from water are shown in Table 1. High recoveries (3-NBA, 89 ± 5%, n = 4; 1-NP, 95 ± 3%, n = 3) were observed with the Empore Disk C_{18}, and these recoveries were much higher than those in the Empore Disk SDB-XD (3-NBA, 62 ± 4%, n = 4; 1-NP, 77 ± 7%, n = 3) or the Empore

Table 1. Recoveries (%) of 3-NBA and 1-NP from Water by SPE Disks

SPE disk	3-NBA	1-NP
Empore Disk C_{18}	89 ± 5	95 ± 3
	($n = 4$)	($n = 3$)
Empore Disk SDB-XD	62 ± 4	77 ± 7
	($n = 4$)	($n = 3$)
Empore Disk Carbon	< 5	< 5
	($n = 2$)	($n = 2$)

Each value represents mean ± S.D.

Disk Carbon (3-NBA, less than 5%, $n = 2$; 1-NP, less than 5%, $n = 2$). Therefore, we used an Empore Disk C_{18} as the SPE Disk to extract 3-NBA and 1-NP from rainwater.

Platinum Black Column Reduction

We previously reported a reduction method for 3-NBA by refluxing with hydrazine and Raney nickel, but this method is tedious and time consuming. Thus, we optimized the reduction conditions (temperature of the reduction column and flow rate of the carrier) for 3-NBA using a reduction column packed with platinum black as a catalyst and methanol as a carrier. Figure 2 shows the relationship between the temperature in the reaction oven and the peak area of 3-aminobenzanthrone (ABA), a reduced product of 3-NBA. When the flow rate of the carrier was set at 0.2 ml min^{-1}, large peak areas were observed at 50 and 80°C, but a significant peak was not observed at 120 or 150°C. When the flow rate was 1 ml min^{-1}, a large peak area was observed at all temperatures (50, 80, 120 and 150°C). Peak area except for 120 and 150°C at a flow rate of 0.2 ml min^{-1} were all the same level. Considering the boiling point of methanol (64°C) and consumption of carrier, the temperature of the reduction column and the flow rate of carrier were set at 80°C and 0.2 ml min^{-1}, respectively. The reduction efficiency at the optimized condition was 92% when 10 ng ml^{-1} 3-NBA solution (0.2 ml) was used.

A typical chromatogram of a standard solution of 3-NBA (10 ng ml^{-1}) under the optimized conditions is shown in Fig. 3. A large peak at a retention time of 12.4 min was identified to 3-ABA by retention time of authentic 3-ABA. Calibration was examined by injecting 0.2 ml of standard solution into the reduction system. A calibration graph showed good linearity ($r > 0.99$) in the range of 1–200 ng ml^{-1}. The detection limit calculated by the

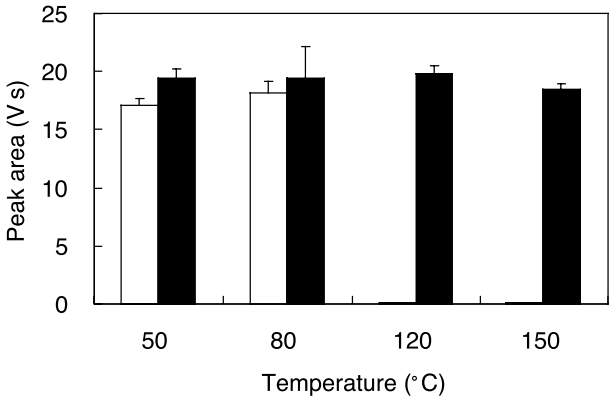

Fig. 2. Relationship between Reduction Column Temperature and Peak Area of 3-Aminobenzanthrone

Amount of 3-nitrobenzanthrone injected, 20 ng. Flow rate, open box: 0.2 ml min^{-1}, solid box: 1 ml min^{-1}.

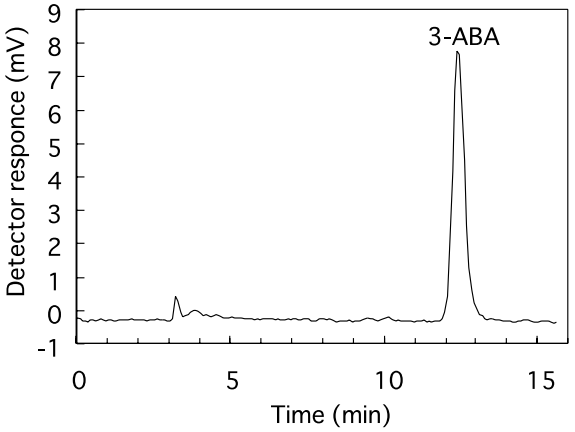

Fig. 3. HPLC Analysis of a Standard Solution of 3-Nitrobenzanthrone (10 ng ml^{-1})

ratio of signal to noise ($S/N = 3$) was 0.2 ng ml^{-1}. The reproducibility of this method was examined for 10 ng ml^{-1}. The relative standard deviation ($n = 5$) was less than 4%.

Determination of 3-Nitrobenzanthrone in Rainwater

We determined 3-NBA in rainwater collected at a residential area of Kyoto, Japan using the above optimized extraction and reduction conditions. A typical chromatogram of the extract from rainwater is shown in Fig. 4. Although large peaks were observed from 3 to 6 min, there was no significant interfering peak near the peak of 3-ABA.

The concentration of 3-NBA in rainwater was calculated from the peak area of 3-ABA. The data were subjected to recovery correction. Recovery for

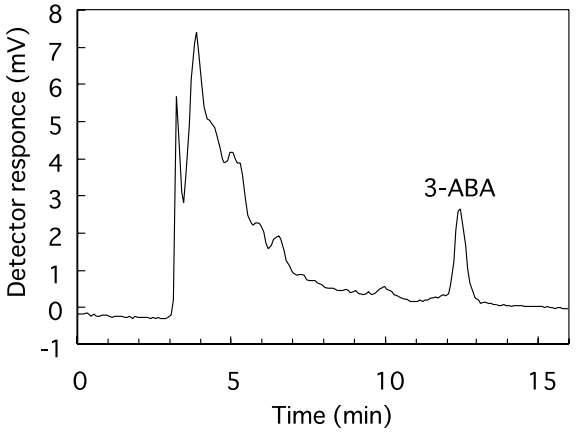

Fig. 4. Typical Chromatogram of Extract from Rainwater
Sampling period, October 2–29, 2001. Detected concentration, 3.0 ng ml^{-1}.

extraction was 90%, which was obtained by recovery test using 5000 ml water containing 0.5 ml of 1 ng ml^{-1} 3-NBA methanol solution. Recovery for clean-up was 96% that was reported previously.[16] The calculated results are listed in Table 2. 3-NBA in rainwater was detected within the range of 0.07–2.6 ng l^{-1}. This is the first detection of 3-NBA in rainwater. The concentration in rainwater was higher from May to July (0.26–2.6 ng l^{-1}) and lower from October to December (0.07–0.2 ng l^{-1}).

The concentration of 1-NP, one of the major nitroarenes in airborne particulate matter, was also determined for comparison with that of 3-NBA. 1-NP determination was carried out by HPLC with fluorescence detection, which was reported by Tejada *et al*.[17] The concentration of 1-NP in rainwater is also listed in Table 2. 1-NP in rainwater was detected within a range of 0.0056–0.19 ng l^{-1}. 1-NP concentration varied about one or two orders of magnitude lower concentration than 3-NBA. Feilberg *et al*. has reported that atmospheric concentration of 3-NBA at Riso in Denmark was lower than that of 1-NP, and that the photolysis rate for 3-NBA was smaller than that for 1-NP.[12] Although 3-NBA has been detected in diesel exhaust particulate matter,[11] the major source of 3-NBA has been still unclear. The difference of composition was observed between rain collected in Kyoto and airborne particulate matter collected in Riso, probably because of not only difference of emission source but also difference of photolysis.

There have been several studies of polycyclic aromatic hydrocarbons (PAHs) in rainwater.[18–20] PAH concentrations in rainwater have been reported

Table 2. Concentrations of 3-NBA and 1-NP in Rainwater

Sampling period (y/m/d)	Volume (l)[a]	3-NBA (ng l^{-1})	1-NP (ng l^{-1})
2001/05/22–31	4.5	2.6	0.19
2001/06/05–30	9.0	0.26	0.0074
2001/07/07–25	4.2	0.30	0.0071
2001/10/02–29	9.1	0.07	0.015
2001/11/08–30	1.5	< 0.2	NA[b]
2001/12/05–14	2.9	< 0.1	0.0056

a) Volume of sample. *b*) Not analyzed.

to range from a few to several hundred ng l^{-1} at sampling points in Poland, Hungary and United States. We recently reported the concentrations of eight mutagenic nitroarenes in rainwater collected at a residential area in Kanazawa, Japan.[9] These nitroarene concentrations in rainwater were within the range of 0.0047–3.7 ng l^{-1}. The 3-NBA concentration in rainwater determined in this study was lower than those of major PAHs such as pyrene and benzo[*a*]pyrene and higher than those of minor nitroarenes such as 1,3-, 1,6- and 1,8-dinitropyrenes and 4-nitropyrene.

Pollutants in the atmosphere can be removed by either dry or wet deposition. 3-NBA has been detected in the air within a range of not detected (N.D.)–11.5 at Tokyo, Japan and N.D.–68.4 pg m^{-3} at Riso, Denmark.[11,12] Recently, we detected 3-NBA in surface soil collected in the Chubu area of Japan within a range of 1.2–1020 pg g^{-1}.[16] In this study, we detected 3-NBA in rainwater, and it is clear that 3-NBA in the atmosphere is transported into rainwater.

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