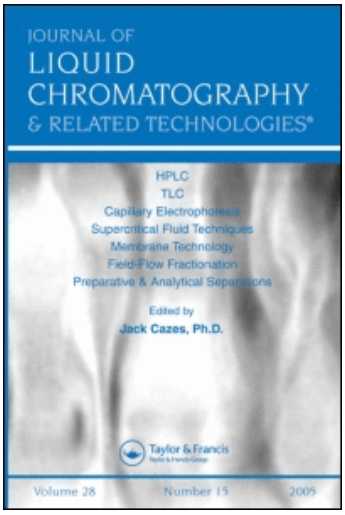

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C. Raha[a]; M. Hart-anstey[a]; E. Bresnick[a]
[a] The Eppley Institute for Research in Cancer University of Nebraska Medical Center, Nebraska

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HIGH-PRESSURE LIQUID CHROMATOGRAPHIC SEPARATION OF 6-ACETOXYBENZO(A)PYRENE FROM IN VITRO INCUBATION OF 6-NITROBENZO(A)PYRENE

C. Raha, M. Hart-Anstey, and E. Bresnick
The Eppley Institute for Research in Cancer
University of Nebraska Medical Center
42nd and Dewey Avenue
Omaha, Nebraska 68105

ABSTRACT

We carried out studies of the microsomal metabolism of 6-nitrobenzo(a)pyrene (NBaP) to understand why the compound is a marginal carcinogen. Microsomal incubation products were separated by reverse phase high-pressure liquid chromatography. A chromatographic peak with a retention time of 24.8 mins was isolated and examined by ultraviolet, mass and nuclear magnetic resonance spectra. The product was characterized as 6-acetoxybenzo(a)pyrene. The source of the acetylating agent could be cytosolic.

INTRODUCTION

Nitrated polycyclic aromatic hydrocarbons (nitro-PAHs) are found in the environment and some are highly potent, direct-acting bacterial mutagens, while others in this class require metabolic activation (1).

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Using the mouse skin model, El-Bayoumy et al. (2) examined some of the nitro-PAHs as tumor initiators and reported NBaP to be a marginal initiator, in comparison to benzo(a)pyrene (BaP). Later Wislocki et al. (3) found that compared to BaP, NBaP was weakly carcinogenic in the newborn mouse liver and lung.

To determine why NBaP is a presumed weak carcinogen, we examined its metabolism in rat liver microsomes and compared our past findings (4,5) and those of others (6) with the results of the present study.

MATERIALS

NBAP was prepared by nitration (7) of BaP (Aldrich Chemical Co., Milwaukee, WI) and purified by chromatography on both alumina and silica gel columns, followed by further separation on silica gel layers (6). The NBaP used in the present experiments contained no BaP, but had 0.16% of the other nitro isomers, i.e., 1- and 3-nitro-BaP, as demonstrated by a reverse phase high-pressure liquid chromatography (RP-HPLC) method to be described later. HPLC-grade solvents were from EM Science Industries, Gibbstown, NJ; Bondelut (3 cc's) C18 bonded phase, from Analytichem International, Harbor City, CA and all biochemicals from Sigma Chemical Co., St. Louis, MO.

METHODS

Animals

Male Sprague-Dawley rats (80-100 g) were obtained from Sasco, Inc., Omaha, NE and induced with three intraperitoneal injections of 25 mg/kg 3-methylcholanthrene (MC) (4).

Preparations of Liver Microsomes

In the past (4) we used microsomes prepared by differential centrifugation of a 20% homogenate in 0.25 M sucrose, pH 7.5. In the present work, we used the calcium precipitation method

of Benson et al. (8). To remove cytosolic contamination, the microsomal pellet was suspended (20 ml per 8 g rat liver) in 0.154 M KCl containing 50 mM Tris, pH 7.5, and resedimented at 100,000 x g for 60 mins. The pellet was used as the source of microsomes. Protein concentration was estimated according to Lowry et al. (9).

Incubation Mixture

Incubation and preliminary isolation of products were carried out according to Fu et al. (6), with minor modifications. In 250 ml 50 mM Tris, pH 7.5, the following were added: 500 mg microsomal protein, 5.2 mg NBaP in 4.4 ml acetone, 3 mM $MgCl_2.6H_2O$, 20 mM glucose-6-phosphate (G-6-P), 0.5 mM NADP and 160 units G-6-P dehydrogenase. The mixture was incubated for 1 hr in air at 37°C in ordinary light and the reaction was terminated by cooling and adding 500 ml acetone and 1,000 ml ethyl acetate. The upper organic layer was dried over 500 g anhydrous sodium sulfate, filtered, and the solvent was removed. Following removal of moisture by azeotropic distillation with benzene and alcohol, a residue was obtained for reverse phase-HPLC work. A second incubation was carried out with 50 mg microsomal protein and proportional amounts of other reagents. Experiments were also carried out under argon atmosphere or in the presence of 1 mM 1-benzylimidazole (BI), as a cytochrome P-450 inhibitor (10). Yellow light was used during the isolation of the products.

Purification of Residue for RP-HPLC Work

The brownish red residue was dissolved in 15 ml methanol, filtered to remove insoluble (perhaps proteinaceous) material and then passed through Bondelut. The eluate was collected, concentrated to 10 ml and again purified twice through Bondelut in 5 ml methanol solution. The third filtrate was concentrated to 2.5 ml and centrifuged. The supernatant was injected into an

HPLC instrument in a concentrated form to minimize the number of injections.

Reverse Phase High-Pressure Liquid Chromatography (RP-HPLC)

The method of Fu et al. (6) was employed, using a Beckman C_{18} Ultrasphere ODS 5 µm 4.6 mm x 25 cm column. A precolumn (Upchurch Scientific, Oak Harbor, WA and Whatman, Clifton, NJ) was used to enhance column longevity. We injected 50-µl samples into a Beckman 332 gradient liquid chromatograph with model 110A pumps, 210A injection valve, 420 programmer, and 164 variable wavelength detector with a 235988 mini-controller. The injection loop was 250 µl and detection was at 254 mm (AUFS 1.0). A Kipp and Zonen BD 40 recorder and a Hewlett-Packard 3392A integrator were also used. The gradient consisted of 75-90% methanol in water gradient over 45 min (6) with a flow rate of 1 ml/min. Peaks were collected manually. Samples were evaporated under reduced pressure at temperatures not exceeding 35°.

Characterization of Products

We used mass, ultraviolet (UV) and nuclear magnetic resonance (NMR) spectra to characterize metabolites.

High resolution and chemical ionization mass spectra were carried out by the Midwest Center for Mass Spectrometry, University of Nebraska, Lincoln.

UV spectra were taken in methanol-methylene chloride (1:1) using a Cary 15 UV-vis spectrophotometer. NMR spectra were determined on a Varian XL-300 spectrometer with $CDCl_3$ (99.96%) as solvent.

RESULTS

In previous work (4,5) we found BaP as a product of the metabolism of NBaP and in the present studies BaP could be seen

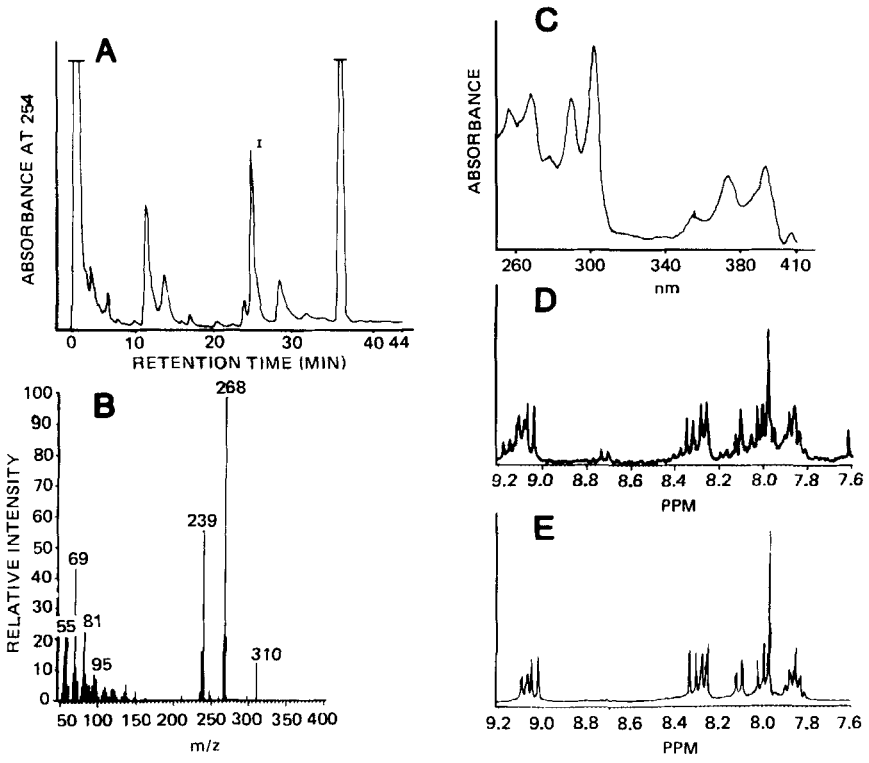

FIGURE 1

with a retention time (R_t) of 41.6 min when Bondelut was not used. To avoid protein contamination of the column when we processed products from 500 mg microsomes, we used Bondelut three times and perhaps because BaP is a non-polar substance it may have been retained by the C_{18} bonded phase of Bondelut.

In this publication, we have confined ourselves to the identification of peak I (Fig. 1A), which had an R_t of 24.84 min. Both incubation experiments produced similar HPLC profiles of products.

Integration (area %) showed peak I to consist of 10.4% total metabolites, i.e., as much as the other major peaks (Fig.

1A). The peak at R_t 36.28 was NBaP. High resolution mass spectra showed the substance in peak I to fit a molecular formula of $C_{22}H_{14}O_2$ to 0.0 ppm. Chemical ionization peak matching (methane reagent gas) gave (MH^+) 311.1065, $C_{22}H_{15}O_2$ to 2 ppm. The mass spectrum (bar graph) of the substance is shown in Fig. 1B. The fragment at m/z 268 corresponds to $C_{22}H_{14}O_2$-C_2H_2O (Ketene), i.e., $C_{20}H_{12}O$. These data suggest that peak I is a mono-acetoxyBaP, a possibility that is further supported by the UV-spectrum (peak I) in Fig. 1C, which is reminiscent of a substituted BaP (11).

Further characterization of the substance (Fig. 1D) was via NMR spectrum. Fig 1E shows the NMR spectrum of an authentic sample of 6-acetoxyBaP. The similarity between the two spectra (1D and 1E) suggests that the substance (peak I in Fig. 1A) isolated from the incubation mixture is 6-acetoxyBaP. A few extraneous peaks seen in Fig. 1D are due to BaP quinones (NMR spectra of BaP-3,6- and -6,12-quinones were compared). The substance in peak I (Fig. 1A) appeared to decompose in air to form a yellow solution.

Formation of this substance (peak I) was inhibited by BI, carbon monoxide or argon atmosphere, suggesting the need for oxygen and cytochrome P-450 activity for its formation.

DISCUSSION

By using electron spin resonance measurements Mason and Holtzman (12) showed that nitro-PAHs can form a nitro anion radical: $RNO_2 + e^- \rightarrow RNO_2^-$, which can participate in two types of reactions: (1) $RNO_2^- \rightarrow R^\bullet$ (carbon-centered radical) + nitrite. Nitrite has been detected (13). The carbon-centered radical may yield BaP, which may produce BaP phenols via metabolism. In our results we note that either BI, a cytochrome P-450 inhibitor (10), or argon atmosphere inhibited the formation of the mono-acetoxyBaP (peak I, Fig. 1A). In the second reaction the nitro-anion radical may transfer the electron to oxygen to produce

superoxide anion: $RNO_2^{\cdot-} + O_2 \rightarrow RNO_2 + O_2^{\cdot-}$, which may lead to other oxygen radicals, including hydroxyl radicals that can produce an aromatic phenol from a nitro-PAH [e.g., nitrobenzene yields phenol (14)]. The identity of a mono-acetoxyBaP from a microsomal incubation reaction suggests acetylation of a BaP phenol formed via one of the above pathways. We washed our microsomal preparations, but apparently cytosolic activity was not completely removed.

Fu et al. (6) reported isolation of ring-hydroxylated NBaP derivatives that are phenols, and the products we report (peak I) are phenol derivatives. Tong and Selkirk (15) suggested that phenols are detoxification products. Accordingly the weak carcinogenicity of NBaP could very well be due to the appearance of phenolic substances as the major metabolites.

However, since under aerobic metabolic conditions nitro-PAHs catalyze the rapid reduction of molecular oxygen to superoxide and superoxide-derived species it is conceivable that NBaP could act as a tumor promoter (16).

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