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Note

Analysis of 3-hydroxybenzo[*a*]pyrene by high-performance liquid chromatography using a post-column method

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Benzo[*a*]pyrene (BaP) is a common pollutant in our environment and a potent carcinogen in experimental animals. Polycyclic aromatic hydrocarbons such as BaP are metabolized by a cytochrome P-450-dependent monooxygenase [1,2], the aryl hydrocarbon hydroxylase (AHH). The AHH and metabolically related enzymes convert polycyclic aromatic hydrocarbons into several types of oxygenated derivative, including epoxides, dihydrodiols, phenols, quinones, diolepoxides and water-soluble conjugates [3]. Many of these are detoxicative products, but some may be either activated carcinogenic intermediates or precursors. Moreover, the AHH activities in the liver and lungs of mice are species-dependent [4] It seems important to investigate the analytical method of AHH for the study of BaP carcinogenicity.

BaP is mainly metabolized to 3-hydroxybenzo[*a*]pyrene (3-OH-BaP) [5] and it is used for AHH assay. Different analytical methods for 3-OH-BaP have been reported [6–12]. Fluorometric measurements [7,10,11] after liquid–liquid extraction with an alkaline solution have been widely used, but not only were the analytical procedures complicated and time-consuming, but other phenols were extracted and measured together. High-performance liquid chromatography (HPLC) with UV detection using gradient elution has been used to effect excellent separation of many metabolites of BaP, but has disadvantages with respect to sensitivity and analysis time [6,8,9,12]. Thus, those methods are not practicable for use in routine AHH assay because there is difficulty in the assay of the small amounts of metabolites formed from relatively small amounts of enzyme and in handling many samples. Therefore, we have tried to develop a more sensitive and less time-consuming method for the determination of 3-OH-BaP by HPLC.

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The present report describes an HPLC technique with fluorescence detection using a post-column method and the application of the method to determination of 3-OH-BaP for AHH assay.

EXPERIMENTAL

Chemicals

BaP was obtained from Aldrich (Milwaukee, WI, U.S.A.) and purified by alumina column chromatography followed by recrystallization from benzene and methanol. 3-OH-BaP and 9-OH-BaP were provided by Dr. Hans Falk of the National Institute for Environmental Health Sciences (Research Triangle Park, NC, U.S.A.) and by Dr. J. Liouel Leong of the Department of Pathology, University of Minnesota (Minneapolis, MN, U.S.A.). NADPH was obtained from Oriental Yeast (Tokyo, Japan). All other chemicals were of analytical-reagent grade.

Apparatus

The chromatographic analyses were performed using a high-performance liquid chromatograph (Hirachi 638) equipped with a fluorescence detector (Hitachi 650-10LC) and a minipulse pump (Gilson, Villiers-le-Bel, France).

AHH assay

Rat livers were removed and homogenized in five volumes of ice-cold 50 m*M* Tris–HCl buffer (pH 7.5) containing 1.15% KCl using a Potter–Elvehjem PTFE homogenizer. The homogenates were centrifuged for 20 min at 9000 *g*, and then the supernatants (S-9) were stored at −80°C until the enzymic assays were performed. AHH activity in the tissue preparation was measured using a modification of the procedure of Nebert and Gelboin [10]. The reaction for AHH assay was carried out for 20 min at 37°C in 1 ml of solution containing 0.1 ml of homogenate (0.3–1.0 mg protein) and 0.9 ml of reaction mixture containing 50 μmol of Tris–HCl buffer (pH 7.5), 0.36 μmol of NADPH and 3 μmol of $MgCl_2$. Just prior to incubation, 10 μl of acetone containing 80 nmol of BaP were added to the mixture. The reaction was stopped by adding 1 ml of cold acetone. BaP and its metabolites were extracted twice with 3 ml of ethyl acetate. The 3 ml of the organic phase were evaporated to dryness under a nitrogen stream, and the residue was dissolved in 0.5 ml of acetonitrile. An aliquot of 50 μl of the resultant sample was injected into the chromatograph. 3-OH-BaP was determined by fluorometric measurement in an alkaline solution after separation by HPLC. The AHH activity was expressed as pmol of 3--OH-BaP/min/mg protein. The protein content of homogenates was determined by the method of Lowry *et al.* [13] using bovine serum albumin as a standard.

Liquid chromatographic conditions

The schematic representation of HPLC using a post-column method is shown in Fig. 1. LiChrosorb RP 18, 5-μm beads (Merck, Darmstadt, F.R.G.) was packed into an analytical stainless-steel column (250 mm × 4 mm I.D.) and a precolumn (10 mm × 4 mm I.D.) by the slurry solvent packing method. The mobile phase, 70% aqueous acetonitrile, was degassed by sonication for 10 min before use. The separation of phenols by HPLC was carried out at a flow-rate of 1.0 ml/min. The aqueous solution of 0.5% sodium hydroxide was supplied at a flow-rate of 0.5 ml/min with a minipulse pump between the analytical column and the detector. The measurement of 3-OH-BaP was carried out by the fluorescence method at an excitation wavelength of 394 nm and an emission wavelength of 520 nm. Some chromatographic parameters, such as retention time and peak area, were printed out using a computing integrator with a liquid chromatograph.

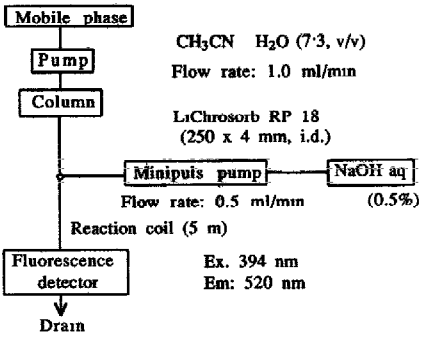

Fig. 1. Schematic representation of HPLC using the post-column method

RESULTS AND DISCUSSION

Reaction conditions

After separation of phenols using the analytical column, the pH of the eluent was raised by the addition of NaOH solution, which was supplied at a flow-rate of 0.5 ml/min with a minipulse pump. The reaction conditions were examined by the flow injection method. The length of the reaction coil was fixed at 5 m. Fig. 2 shows the effect of the concentration of the NaOH solution on the fluorescent intensity of 3-OH-BaP. The concentration of the NaOH solution was varied from 0.005 to 5%. Maximal and constant peak areas were obtained at 0.01–1.0% NaOH solution. Fig. 3 shows the excitation and emission spectra of 3-OH-BaP in 0.15% NaOH solution. Both spectra were displaced to longer wavelengths in the alkaline solution. Therefore, the post-column reaction conditions selected for the determination of 3-OH-BaP were 0.5% NaOH solution and a 5-m reaction coil.

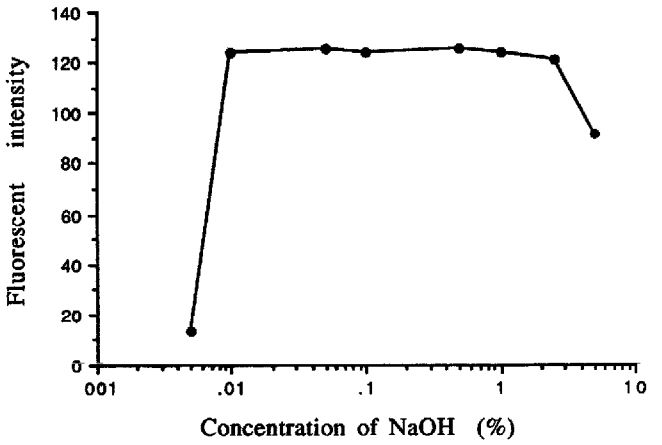

Fig. 2. Effect of the concentration of sodium hydroxide solution on the fluorescent intensity of 3-hydroxybenzo[*a*]pyrene HPLC conditions are shown in Fig. 1.

Liquid chromatography

As 3-OH-BaP and 9-OH-BaP are the main metabolites of BaP, we tried to find the optimal conditions of HPLC for their separation. Acetonitrile–water as a mobile phase gave a good separation, which was improvedly decreasing the percentage of acetonitrile in the mobile phase. However, this prolonged the time

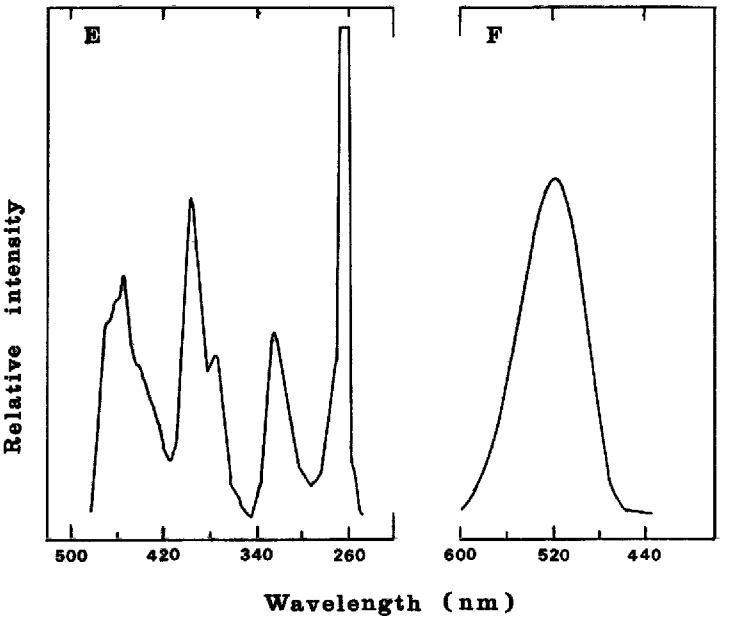

Fig. 3. Fluorescence and excitation spectra of 3-hydroxybenzo[*a*]pyrene in 0.15% sodium hydroxide solution. (E) excitation (emission at 520 nm); (F) fluorescence (excitation at 320 nm).

required for the analysis. We used the 70% aqueous acetonitrile as a mobile phase and found that 3-OH-BaP was completely separated from 9-OH-BaP. Fig. 4 shows the chromatograms of authentic standards and an extract of a reaction mixture obtained from rat liver for AHH assay. The retention times of 3-OH-BaP and 9-OH-BaP were 12.0 and 10.2 min, respectively. The analysis times for metabolites of BaP were less than 40 min.

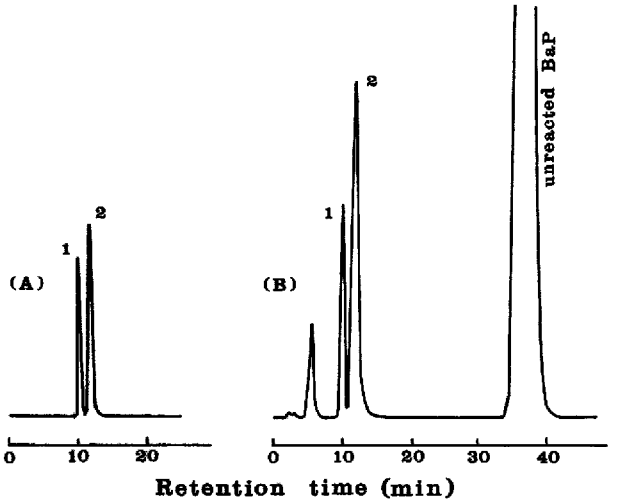

Fig. 4. Chromatograms of (A) a standard (3-OH-BaP, 5 5 ng; 9-OH-BaP, 4 7 ng) and (B) a sample obtained from rat liver for AHH assay HPLC conditions are shown in Fig 1. Peaks 1 = 9-OH-BaP, 2 = 3-OH-BaP.

Detection limit

The calibration curve for the peak area against the amount of 3-OH-BaP was linear ($r = 0.998$) up to 150 ng. The detection limit at a signal-to-noise ratio of 2 was *ca.* 0.2 ng for 3-OH-BaP. As the relationship between the amount of protein in an S-9 and the amount of 3-OH-BaP formed by the reaction was linear up to 1 mg of protein, the detection limit for AHH activity was *ca* 2 pmol/min/mg of protein.

Recovery

In order to apply the present method to the AHH assay, three known amounts (80, 400, 800 ng) of 3-OH-BaP were added to 3 ml of extracts of reaction mixture for AHH assay, and the recoveries were tested. As shown in Table I, the recoveries of each amount of the added 3-OH-BaP, in an experiment using five different reaction mixtures, were 94.9, 97.2 and 97.3%, respectively.

TABLE I

RECOVERY OF 3-HYDROXYBENZO[*a*]PYRENE ADDED TO REACTION MIXTURES FOR AHH ASSAY BY THE PRESENT METHOD

Sample	Original (ng)	Recovery (%)		
		80 ng	400 ng	800 ng
A	74	96.1	98.5	97 7
B	93	91.9	97.2	95 4
C	85	94.5	95.5	97 1
D	81	97.5	97.7	97.8
E	79	94.3	96.9	98 1
Mean		94 9	97.2	97 3

Precision

Ten determinations of 3-OH-BaP were performed on two different reaction mixtures for AHH assay of low (A) and high (B) concentration of 3-OH-BaP. The values (mean ± S.D.) of A and B were 37.0 ± 1.8 and 107.8 ± 4.6 ng/ml, respectively. The reproducibility of this method was satisfactory; the coefficients of variation (C.V.) for A and B were 4.8 and 4.3%, respectively.

CONCLUSION

It is well known that liquid–liquid extraction methods measure the fluorescence of many metabolites of BaP, which are extractable from organic phase by alkali. Thus, the metabolites of BaP measured by such methods are essentially a mixture of phenols quantitatively extracted into the alkali. As 3-OH-BaP and 9-OH-BaP are the main metabolties of BaP, we tried to separate these two phenols using HPLC. Furthermore, in order to detect 3-OH-BaP in an alkaline solution by a fluorescence detector, a post-column alteration of the eluent pH method was used. Consequently, the present method is more sensitive and less time-consuming than HPLC–UV methods. Although the sensitivity of 3-OH-BaP to the present method is less than that to native fluorescence measurement, the present method has advantages with respect to selectivity and analysis time. The native fluorescence measurement sensitively detected a large number of metabolites of BaP, such as phenols, diols, quinones, triols and unchanged BaP, whereas the present method detected only phenols and unchanged BaP in an alkaline solution. As the excitation and emission wavelengths were selected at 394 and 520 nm, respectively, the present method is also less sensitive to unchanged BaP.

We conclude that the present method is specific, sensitive, simple and useful for the determination of 3-OH-BaP for AHH assay.

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