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High-Pressure Liquid Chromatographic Separation of 10 Benzo(a)pyrene Phenols and the Identification of 1-Phenol and 7-Phenol as New Metabolites

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SUMMARY

The separation of ten isomeric benzo(a)pyrene phenols has been accomplished by the use of high-pressure liquid chromatography utilizing a newly developed recycling technique and new column and solvent systems.

Using this new system and comparing the metabolites obtained with authentic standards, we have isolated 1-hydroxybenzo(a)pyrene and 7-hydroxybenzo(a)pyrene and identified them as metabolites formed by rat liver microsomes. In previously reported chromatographic systems, the new metabolites migrated with another metabolite, 3-hydroxybenzo(a)pyrene.

INTRODUCTION

BP[1] is a potent carcinogen in experimental animals and a common environmental pollutant (5). This class of polycyclic aromatic hydrocarbon carcinogens is metabolized by the microsomal cytochrome P-450 containing mixed-function oxygenase, aryl hydrocarbon hydroxylase (10). This enzyme system has been found and is inducible in most animal tissues and is also present in human liver (16), skin (17), placenta (18, 24), macrophages (4), and peripheral blood lymphocytes and monocytes (1, 3, 25). This enzyme system and metabolically related enzymes convert polycyclic aromatic hydrocarbons into several types of oxygenated derivatives which include epoxides, dihydrodiols, phenols, diol-epoxides, quinones, and water-soluble conjugates (20–23). Many of these are detoxification products, but some may be either activated carcinogenic intermediates or precursors thereof (9, 10, 14).

In order to understand the mechanism of carcinogen action, it is necessary to separate and identify metabolites so that their reactive intermediate precursors, formation kinetics, and precursor-product relationships can be determined. In an initial report (19) we demonstrated the extraordinary efficacy of HPLC for the separation of BP metabolites. In subsequent reports from our laboratory (20, 21) and others (13), essentially the same system as originally described was used for studies in BP metabolism. In earlier studies with thin-layer and column chromatography, we isolated and identified the 9-OH-BP as a product of BP metabolism (15). Later studies with HPLC showed the separation of at least 7 distinct compounds (19, 21) and 1 labile epoxide intermediate (13, 20). Phenols of BP may arise by the rearrangement of intermediate epoxides which can also be converted to dihydrodiols by the action of epoxide hydratase. At least 3 dihydrodiols are formed in the metabolism of BP: 7,8-dihydrodiol, 9,10-dihydrodiol, and 4,5-dihydrodiol. Inhibition of the hydratase enzyme results in the complete abolition of diol formation with a concomitant increase of 9-hydroxy formation (21). Diols are also not formed when hydratase-free monooxygenase enzymes are used for oxygenation of BP (13). Formation of phenols by 2 different forms of highly purified cytochrome P-450 results in the formation of phenol mixtures having different fluorescence spectra (26). Furthermore, the ratio of the 2 phenol peaks formed is different for the cytochrome P-450 fraction. Each peak contains phenols with spectra similar to but not identical to 3-OH-BP and 9-OH-BP.

These results and the report that 11 of the 12 possible BP phenols migrate in 2 bands in a similar system (13) led us to believe that other phenols may be present in the 2 phenol metabolite peaks that we previously reported to be separated by HPLC. This report develops the HPLC technique still further in demonstrating that 10 synthetic phenols can be chromatographically separated. This has been accomplished by the use of nonaqueous solvents with a different column and the repeated recycling of unresolved peaks. At least 4 phenols, 1-hydroxy, 3-hydroxy, 7-hydroxy, and 9-hydroxy are formed metabolically. The 1- and 7-hydroxy metabolites are new metabolites that have not been previously separated from the previously identified 3-OH-BP and 9-OH-BP (15, 19).

Chemicals. The following BP phenols were prepared on National Cancer Institute Contract N01-CP-3387 by Dr. J. F. Engel,[2] according to the following procedures: 3-OH-BP by Cook *et al.* (6), 6-OH-BP by Fieser and Hershberg (7), 7-OH-BP by Fieser *et al.* (8), 8-OH-BP and 9-OH-BP by Sims (22), 10-OH-BP by J. F. Engel.[2] The 1-OH-BP (11) and 4-OH-BP, 5-OH-BP, and 12-OH-BP were prepared on National Cancer Institute Contract N01-CP-33385 by Dr. R. Harvey, University of Chicago, Chicago, Ill.[3]

[1] The abbreviations used are: BP, 1-OH-BP, 3-OH-BP, 4-OH-BP, 5-OH-BP, 6-OH-BP, 7-OH-BP, 8-OH-BP, 9-OH-BP, 10-OH-BP, 12-OH-BP, benzo(a)pyrene and its various hydroxy metabolites; HPLC, high-pressure chromatography.

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[2] J. F. Engel, submitted for publication.

[3] R. G. Harvey, submitted for publication.

Male Sprague-Dawley rats weighing 125 to 150 g were given i.p. injections of 5 mg 3-methylcholanthrene in 0.5 ml corn oil 40 hr prior to sacrifice. Liver microsomes were prepared as previously described (15).

Metabolites were formed by incubating rat liver microsomes with BP in the following manner. Each flask contained, in a total volume of 1.0 ml: 0.25 mg microsomal protein; 0.36 μmole NADPH; 3 μmoles $MgCl_2$; 50 μmoles Tris-chloride buffer, pH 7.5; and 100 nmoles of BP dissolved in 0.04 ml methanol.

The flasks were incubated for 10 min at 37° under red illumination, and the reaction was stopped by the addition of 1.0 ml acetone. The mixture was then extracted with 2.0 ml ethyl acetate. Five ethyl acetate extracts were pooled, dried over 1.0 g anhydrous magnesium sulfate, and evaporated under vacuum to dryness, and the metabolites were dissolved in 0.1 ml methanol.

Metabolite separation and isolation of the phenol region was accomplished on a Chromatronix Model 3500 liquid chromatograph fitted with a DuPont 1-m Zipax-ODS-Permaphase column using a methanol:water gradient with methanol at an initial concentration of 30% and a final concentration of 70% (19, 21).

The phenol region was rechromatographed on a DuPont Model 830 high-pressure liquid chromatograph fitted with 2 Zorbax-sil columns (25 cm) connected to a 6-port valve assembly and arranged to direct the flow toward either of 2 columns in series or with flow through an 8-μl UV flow cell between columns (12).

The solvent mixture was hexane:dioxane (9:1) containing 40 μl formic acid per 200 ml solvent. The column was isocratically run at ambient temperature, and the pressure was 1800 psi.

The peaks were collected and the retention times and UV spectra were compared to authentic standards.

Operation of the Recycle System. Chart 1 shows a schematic representation of the recycle system. The system is a modification of the one developed by Henry (14) and incorporates the same alternate pumping design with a 6-port, 2-position valve (Model CV-G-HPAX; Valco, Inc., Houston, Texas).

When the valve is set to Position 1, compounds injected into the system pass first through Ports 1 and 2 and into Column 1. After eluting from Column 1, they pass through the UV cell and back through Ports 5 to 3 and enter Column 2. At this point the valve is changed to Position 2, so the eluting compounds from Column 2 pass through Ports 4 to 2 and reenter Column 1. As the compounds elute from Column 1 and pass through the UV flow cell, they may either be collected at Port 6 after passing through 3 columns or recycled again through another 2 columns by changing the valve back to Position 1 and allowing the compounds to enter Column 2 via Ports 5 to 3. Once the compounds are transferred onto Column 2, which is complete after the final peak has passed through the UV cell, the valve is changed to Position 2. The compounds can be collected after 5 columns at Port 6 or the valve can be changed once again to Position 1 to transfer the compounds once again onto Column 2 repeating the recycle procedure.

Compounds that separate adequately without recycling can be injected into the system with the valve in Position 2 and collected after passing through 2 columns.

RESULTS AND DISCUSSION

Chart 2 shows the elution profile of 10 synthetic BP phenols, separated after 1, 3, and 5 passages through the column. After a single pass through 1 column, 6 peaks are observed; 4 of these peaks each contain 2 phenols and 2 peaks each contain 1 phenol. After the 1st passage the 5-phenol is cleanly resolved as a single peak and the 7-phenol is partially resolved. The 4-phenol and 6-phenol, 10-phenol and 12-phenol, 1-phenol and 3-phenol, and 8-phenol and 9-phenol migrate in the indicated pairs in 4 different peaks. A structural basis for the separation seems to relate to the relative position of the hydroxyl group since each of the latter 4 peaks contain pairs of phenols in which the hydroxyl groups are either adjacent or 2 carbon atoms apart. After passage through 3 columns, 5 peaks were clearly resolved. The 7-phenol, 8-phenol, and 9-phenol were completely separated as single peaks; the 12-phenol and 10-phenol mi-

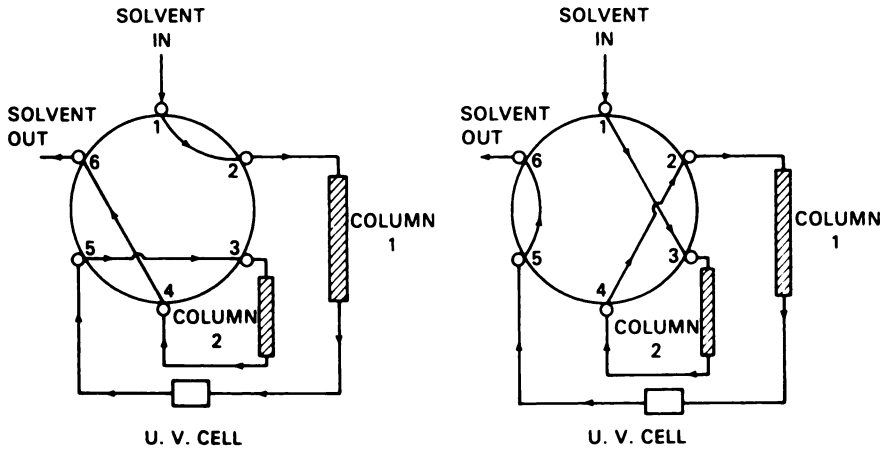

Chart 1. Valve assembly for HPLC recycling.

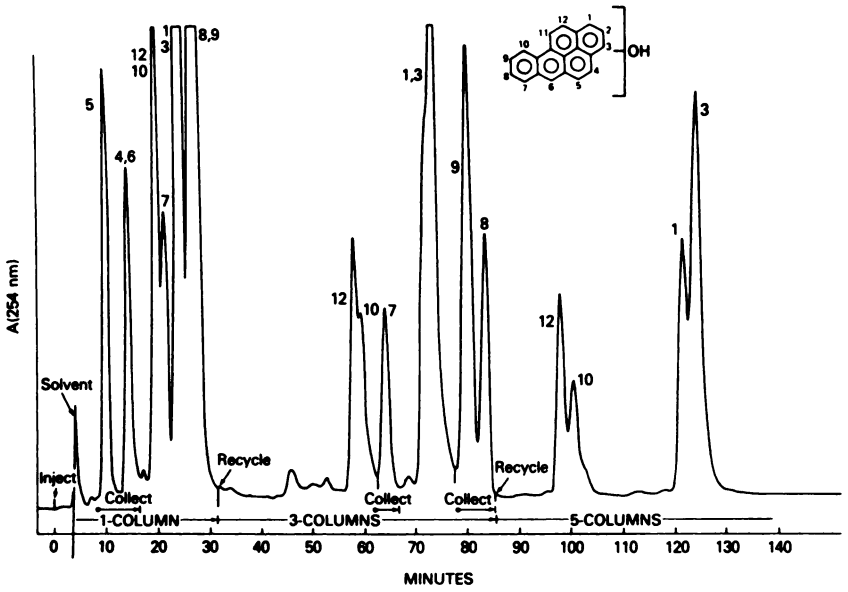

Chart 2. HPLC separation of BP Phenols. Separation was performed on a DuPont Model 830 high-pressure liquid chromatograph fitted with 2 Zorbax-sil columns (25 cm) connected to a 6-port valve assembly and arranged to direct the flow through either of 2 columns in series or through a 8-ml UV flow cell between the columns (12). The flow rate was 0.2 ml/min. The solvent mixture was hexane:dioxane (9:1) containing 40 ml formic acid per 200 ml solvent. The column was isocratically run at ambient temperature and the pressure was 1800 psi. Shown are the phenol patterns after the compounds passed through 1 column, 3 columns, and 5 columns.

grated in 1 peak with the 10-phenol as a sharp shoulder on the 12-phenol and the 1-phenol a slight shoulder on the 3-phenol peak. The single 5-phenol peak and the peak containing the 4-phenol and 6-phenol were collected after a single pass through the column, since they elute first and pass through the UV cell and out of the system before the last peaks (8-phenol and 9-phenol) appear. This prevented us from recycling them further in the same system.

The 2 remaining doublets, the 12-phenol and 10-phenol and the 1-phenol and 3-phenol, were passed through 2 additional columns producing 4 separate peaks, the 12-phenol, 10-phenol, 1-phenol, and 3-phenol, respectively. Additional recycling resulted in some broadening of the peak bases.

The surface activity of the silica packing in these columns was a critical factor in obtaining efficient, reproducible separations. Chart 3 shows the separation of the 10 BP phenols using the same solvent system before the surface activity was adjusted as described in "Materials and Methods." The retention times of all compounds are shorter. After passing through 1 column, the 5-OH-BP and 6-OH-BP isomers elute first in a single peak followed by 3 fused peaks containing 12-OH-BP, 10-OH-BP, 7-OH-BP and 4-OH-BP; 3-OH-BP and 1-OH-BP; and 8-OH-BP and 9-OH-BP, respectively. These 3 peaks were recycled (passed through 2 more columns) resulting in the separation of their 8 components into 6 peaks. The 10-OH-BP and 4-OH-BP and 3-OH-BP and 1-OH-BP cochromatograph. In contrast to the more activated system, further recycling of peaks containing more than 1 component, equivalent to passing through 8 columns, did not result in further separation of their components. A further difference is noted with respect to the position of the 4-OH-BP and 5-OH-BP isomers in Charts 2 and 3. The 5-OH-BP and 6-OH-BP and the 10-OH-BP and 4-OH-BP isomers cochromatograph in the deactivated system with short retention times. Activation of the column results in longer retention times, better resolution, and a shifting of the relative positions of the 4-OH-BP and 5-OH-BP isomers in the chromatogram. The 5-OH-BP now elutes first followed by the 4-OH-BP and 6-OH-BP which elute together.

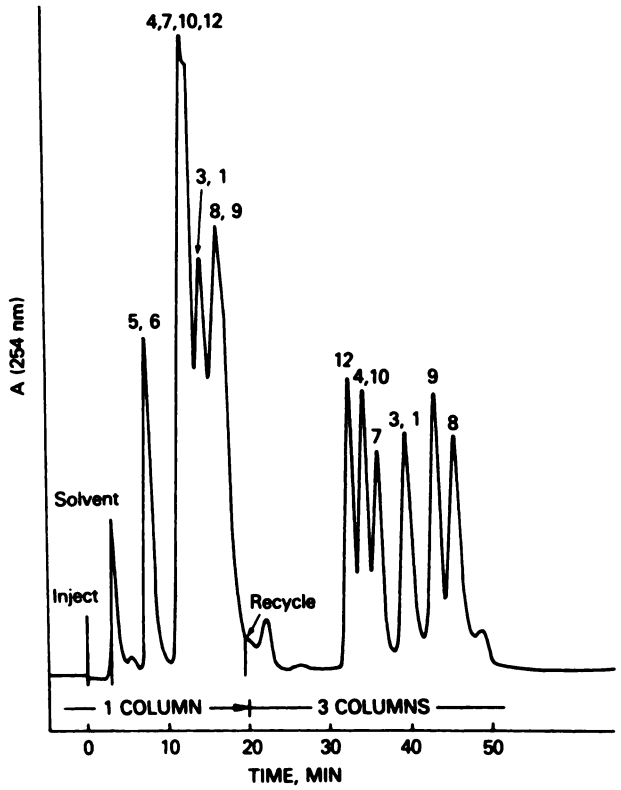

Chart 3. Separation of BP phenols before activation of columns.

Greater activation of the column resulted in less stability of the 1-OH-BP, 3-OH-BP, 6-OH-BP, and 12-OH-BP isomers. The addition of formic acid stabilized all of the latter except the 6-OH-BP. The most labile, 6-OH-BP, when passed through the column eluted with 2 small peaks (5%) identified as the 1,6- and 3,6-benzo*(a)*pyrene quinones.

A gradual deactivation of the column, presumably through water absorption, takes place during prolonged use with the solvent system described. The activity and

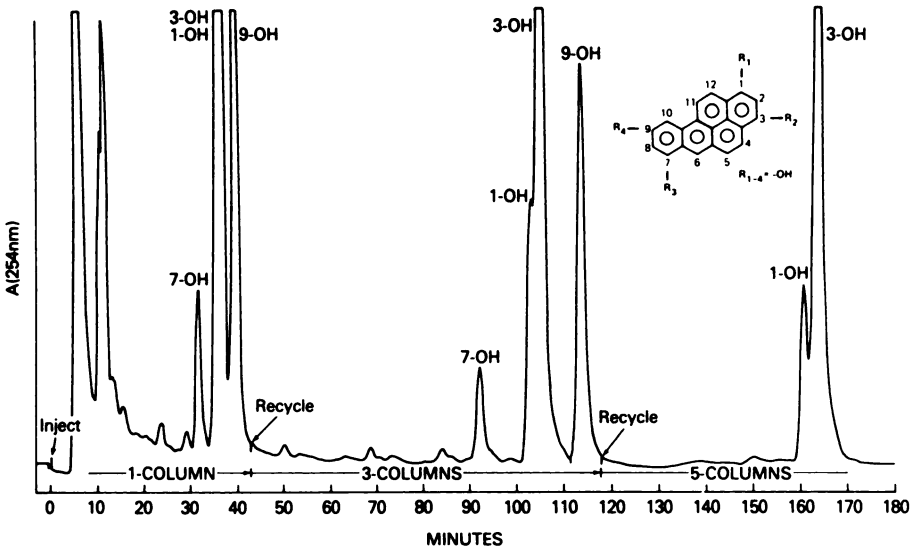

Chart 4. HPLC separation of BP phenol metabolites. The phenol region was preisolated from the metabolite pool as previously described (19, 21). The phenols were then passed repetitively through 2 Zorbax-sil columns at a flow rate of 0.15 ml/min. No other phenols were detected in the 9-hydroxy peak and it was collected after 3 columns.

separation efficiency may be restored by repeating the methanol:isopropyl ether washing procedure. The method reported here combined with the reverse phase method (19, 21) which can separate the 4-OH-BP and 6-OH-BP isomers and allows complete resolution and identification of the 10 phenol isomers of BP that were available to us for this study. Application of these techniques for the analysis of the metabolic products of BP should further elucidate the pathways of BP metabolism. We used this recycle technique to examine BP phenol formation.

Chart 4 shows the separation in an identical system of phenolic metabolites formed when BP was incubated with liver microsomes from methylcholanthrene-induced rats. The 1st 2 unidentified peaks are not metabolites but rather material absorbing at 254 nm which when collected show no UV spectrum related to BP. These peaks are derived from microsomal extracts. Three metabolite peaks are observed after passage through a single column. After passage through the entire 5-column recycling system, 4 distinct peaks are resolved.

Each of the metabolites were isolated and characterized by cochromatography and UV spectra and compared to the 10 synthetic phenols.

These correspond to the 3-phenol and 9-phenol previously identified as metabolites and 2 newly identified metabolites, the 1-phenol and the 7-phenol. The absorption maxima of the latter 2 metabolites and authentic 1-hydroxy and 7-hydroxy metabolites are shown in Table 1. The absorption maxima of the authentic compounds and the compounds formed by rat liver microsomes were identical.

In an early report, Berenblum and Schoental (2) isolated and partially characterized a compound from rabbit which they identified as 1-OH-BP. However, an authentic 1-OH-BP standard was not available at that time, and their characterization cannot be directly compared to this work.

The isolation of the 1-phenol raises the question of the importance of this site for BP carcinogenesis, since metabolism at this position has been indicated only by the identification of the 1,6-quinone which may be a nonenzymatic product derived from activation and oxygenation at position 6. Neither epoxides nor dihydrodiol metabolites at position 1 have been previously reported. The identification of the 7-phenol as a metabolite is consistent with the view that a labile 7,8-epoxide intermediate is formed during BP oxidation. This intermediate can be converted to the 7,8-dihydrodiol, and our present study suggests it can be converted to the 7-phenol.

Table 1
Absorption maxima of metabolites[a] identified as 1-phenol and 7-phenol

	Absorption maxima (nm)
1-OH-BP	257, 267, 287, 298, 278, 387, 460, 415
7-OH-BP	260, 268, 291, 303, 340, 358, 376, 398

[a] The metabolites and authentic compounds give identical maxima shown above. The spectra were determined in 10% dioxane in hexane.

Development of a new analytical technique like HPLC-recycling, which can discriminate between the subtle molecular differences in 10 isomeric phenols of BP will greatly aid in dissecting the metabolic pathways of polycyclic metabolism and in the isolation of the proximate chemical carcinogens of this class of compounds.

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