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METABOLISM OF BENZO(a)PYRENE BY FISH LIVER MICROSOMES: LITERATURE REVIEW AND PRELIMINARY STUDIES

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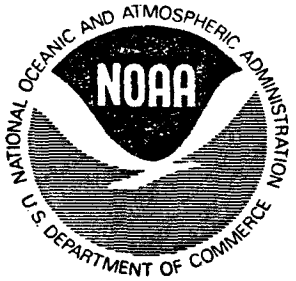

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METABOLISM OF BENZO(a)PYRENE BY FISH LIVER MICROSOMES: LITERATURE REVIEW AND PRELIMINARY STUDIES

Certain environmental contaminants in the marine biota pose a threat to aquatic organisms and, ultimately, to the consumer of fishery products. One group of contaminants which possesses toxic properties, the polynuclear aromatic hydrocarbons (PAHs), enters the marine environment from a wide range of sources, including petroleum spills and natural seepage, fallout from air pollution, industrial waste effluents, storm drain run-off, and creosoted wharfs and pilings. Polynuclear aromatic hydrocarbons have been isolated from a variety of shellfish (Chesler et al., 1978; Dunn and Fee, 1979; Heit et al., 1980; Pancirov and Brown, 1977; Sirota and Uthe, 1981; Sirota et al., 1983), finfish (Brown and Pancirov, 1979; Pancirov and Brown, 1977), and processed seafoods (Dunn and Fee, 1979).

Benzo(a)pyrene (BAP), the most prevalent PAH detected in the atmosphere (Harvey, 1982), is among those PAHs most commonly isolated from seafoods (Dunn and Fee, 1979; Dunn and Stich, 1975, 1976; Pancirov and Brown, 1977). Dunn and Fee (1979) detected BAP levels as high as 2300 ng/g wet weight in the digestive gland and 281 ng/g wet weight in edible tail meat of lobsters held in commercial impoundment. These levels were considerably higher than levels previously reported for any foodstuff and were probably the result of creosoted timbers used in construction of the impoundment tanks.

Considerable information is known about the relative mutagenic and carcinogenic properties among some PAHs and their metabolites, based on mammalian cell and tissue culture (DiPaolo et al., 1969, 1971; Huberman et al., 1971; Mager et al., 1977; Marquardt et al., 1976, 1977, 1978; Wislocki et al., 1976) and in vivo rodent studies (DiPaolo et al., 1969, 1971; Marquardt et al., 1976, 1977, 1979). Unfortunately, less is known about the extent of fishery product

contamination, the specific metabolites produced or accumulated by any given fish species, or the public health consequences as a result of PAH contamination of the marine environment.

Benzo(a)pyrene, like most PAHs, is relatively non-mutagenic and non-carcinogenic in its unmetabolized form, but is transformed by mixed function oxidases (MFO), aryl hydrocarbon hydroxylases, and related enzymes (Gelboin, 1967; Gelboin et al., 1972; Payne, 1977) into potentially mutagenic and carcinogenic compounds. Mixed function oxidase conversion of PAH to metabolites has been extensively studied on mammalian systems (Kinoshita et al., 1973; Selkirk et al., 1975). Certain types of oxidases have also been isolated from fish (Ahokas et al., 1975; Burns, 1976; Payne, 1977; Pohl et al., 1974). Some of the MFO activity is associated with the microsomal hemoprotein, cytochrome P-450 (Kriek and Westra, 1979). Mammalian microsomes have been used extensively to metabolize PAH compounds (Autrup et al., 1975; Gold and Eisenstadt, 1980; Kinoshita et al., 1973; Selkirk et al., 1974). The specific metabolites produced depend largely on the source of the microsomes since biotransformation products appear to be highly species and organ specific (Varanasi et al., 1983). Limited research has been conducted to elucidate the role of fish microsomes in the biotransformation of PAH (Ahokas, 1979; Melius et al., 1979; Schoor and Couch, 1979; Varanasi and Gmur, 1980).

The degree of microsomal activity in the metabolism of PAH compounds is related to the amount of cytochrome P-450 present (Kinoshita et al., 1973). Certain xenobiotics such as barbiturates and PAHs can induce production of P-450 by mammalian hepatic cells (Cooney, 1967; Park, 1975; Remmer, 1968); however, fish are refractive to the barbiturate class of compounds (Addison et al., 1977; Bend et al., 1973; Buhler and Rasmusson, 1968; Statham et al., 1978). An effective inducer of cytochrome P-450 in small rodents and fish is the PAH,

3-methylcholanthrene (Kinoshita et al., 1973; Melius and Elam, 1983; Melius et al., 1979; Selkirk et al., 1971, 1975). In this study, 3-methylcholanthrene (3-MC) induced liver microsomes from sea bass were used to metabolize BAP in vitro.

In this paper we provide an overview of some of the preliminary research on BAP conducted at the Charleston Laboratory including information on the methods used and citations of relevant literature. Topics covered in the report include (i) the induction of cytochrome P-450 in fish liver microsomes, (ii) the conversion of BAP into metabolites by a microsome and coenzyme activation system, (iii) the extraction of BAP metabolites from the activation mixture, (iv) the analysis of extracts for BAP metabolites by high performance liquid chromatography (HPLC), and (v) the evaluation of metabolites for mutagenicity and carcinogenicity with mammalian cell culture, and in vivo animal models.

Most current publications address one or two of these areas, but it is virtually impossible to obtain all the methods required from induction of the microsomes to analysis of compounds for mutagenicity and carcinogenicity in a single text. Other researchers who are conducting petroleum hydrocarbon studies may find the consolidation of these methods useful in their attempts to quantify PAH metabolites in seafoods, determine their distribution throughout the food chain, detect species and organ specificity, and predict the consequences of PAH metabolites to the consumer of fishery products.

Chemicals: Crystallized BAP was purchased from Ultra Scientific, Hope, RI.[1/] The 3-MC was from Eastman Kodak Co., Rochester, NY. Benzo(a)-pyrene and 3-MC metabolites were kindly provided by the National Cancer Institute Chemical Carcinogen Reference Standard Repository of the National Institutes of Health, Bethesda, MD. Standards consisted of the BAP metabolites:

1/ Use of trade names or reference to commercial firms does not imply endorsement by the National Marine Fisheries Service, NOAA.

trans-4,5-dihydrodiol; cis-7,8-dihydrodiol; trans-7,8-dihydrodiol, trans-7,10-dihydrodiol; 1,6-dione; 3,6-dione; 6,12-dione; 4,5-dihydroepoxide; 7,8-tetrahydroepoxide; 1-hydroxy; 3-hydroxy; 5-hydroxy; 6-hydroxy; 7-hydroxy; and 9-hydroxy as well as the 3-MC metabolites: 3-methyl-7-phenylacenaphthene-2,8-dicarboxaldehyde; cis-11,12-diol; 11,12-dione; 11,12-epoxide; 1-hydroxy; 2-hydroxy; 11-hydroxy; and 1 keto. Metabolite standards were dissolved in HPLC-grade dimethyl sulfoxide (Burdick & Jackson Laboratories, Inc., Muskegon, MI), overlayed with nitrogen, and stored at -30°C. All solvents were HPLC-grade: acetone (J. T. Baker Chemical Co., Phillipsburg, NJ, or Fisher Scientific, Fair Lawn, NJ); acetonitrile and hexane (J. T. Baker Chemical Co.); ethyl acetate (Fisher Scientific); and methanol and water (Burdick & Jackson Laboratories, Inc.). Other reagents included tris-HCl (Sigma Chemical Co., St. Louis, MO) and reduced nicotinamide adenine dinucleotide phosphate (NADPH, Calbiochem-Behring, La Jolla, CA). Reagents were tested by HPLC for purity and for possible contamination from handling at various stages in the procedures.

Preparation of fish liver microsomes: Black sea bass (Centropristis striata) weighing 75-225 g (150 g average) were caught 10 miles off the coast of Charleston, SC, and maintained for 4 months in a closed, recirculating and biologically filtered seawater system. Fish were placed in tanks of aged, artificial seawater maintained at 21°C, a salinity of 27°/oo, pH 8.0, and ammonia $\leq$100 ppb. The bass were acclimated without feeding for 3 days after which 3-MC (20 mg 3-MC/kg body mass) in sterile corn oil (10 mg/ml) was administered by a single intraperitoneal injection (Kinoshita et al., 1973) to induce the production of fish liver microsomal enzymes above normal levels (Melius and Elam, 1983). Control fish were inoculated with corn oil only. Treated fish were placed in clean tanks containing aged, artificial seawater for a 3 day induction period, after which they were sacrificed by a blow to the head.

Crude microsomal preparations were produced according to the procedures of Garner et al. (1972) and were designated as the S-9 fractions (Ames et al., 1973). In this procedure, livers were aseptically excised, rinsed with sterile, ice cold 0.15 M KCl, weighed, and homogenized with a Polytron tissue homogenizer (Brinkmann Instruments, Inc., Cantiague, NY) in 3 times the liver weight of cold 0.15 M KCl. The homogenates were centrifuged at 9770 x g for 10 min at 4°C. Supernatants containing the microsomal enzymes were dispensed into amber vials, stored at -70°C, and used within 4 months. Each milliliter of supernatant contained microsomes from 250 mg of wet liver. The amount of protein in the S-9 fractions was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Metabolism of benzo(a)pyrene: The procedure for metabolizing BAP in vitro was essentially that used by Kinoshita et al. (1973) and Selkirk et al. (1974). Flasks containing a total volume of 1.0 ml consisting of 50 uM tris-HCl, pH 7.5; 0.36 uM of NADPH; 3 uM $MgCl_2$; 2.0-2.6 mg of microsomal protein; and 1.0 ug BAP dissolved in 50 ul ethyl acetate were shaken for 30 min at 37°C. The metabolism of BAP was stopped by addition of 1.0 ml of acetone. This procedure was repeated, excluding BAP from the reaction mixture, in order to analyze for endogenous PAH and 3-MC metabolites.

Metabolite extraction: Each reaction mixture was extracted with 2.0 ml of ethyl acetate and centrifuged at 3000 x g for 20 min at 4°C. The supernatant was allowed to separate into two layers. The aqueous lower layer was removed with a Pasteur pipet and reextracted with 1.0 ml of ethyl acetate. The organic layers were combined with 1.0 ml of acetone and filtered through an ethyl acetate-rinsed 0.5 um pore size Millex SR teflon filter (Millipore Corp., Bedford, MA). Initial quality control testing showed that these filters, once rinsed, did not leach interfering contaminants into filtered solvents; that is, no interfering

peaks were observed on chromatograms in the areas where the metabolites were expected to elute. Extracts were evaporated to near dryness with a Buchi Rotovapor-R (Brinkmann Instruments, Westbury, NY) under a vacuum of 650 mm Hg at 40°C. Each sample was brought up to 500 ul with methanol and stored at -10°C in an amber vial with a teflon faced silicone septum.

Prior to injecting the extracts on the HPLC, the last traces of ethyl acetate were substantially removed with a Waters Sep-pak C_{18} cartridge (reverse phase). This procedure involved rinsing the column first with 5 ml of acetonitrile and then 5 ml of water. The thawed sample was applied to the top of the Sep-pak cartridge and washed into the column with 2 ml of water. Then, an additional 5 ml of water was flushed through the column and discarded. The sample was eluted with 10 ml of acetonitrile and the eluate was evaporated to 250 ul for injection on the HPLC. Chromatograms showed that all metabolites, as well as the parent PAH, were eluted from the Sep-pak by this procedure.

Separation of BAP Metabolite Standards: Experiments were conducted to determine HPLC parameters necessary to separate and identify BAP metabolites from stock solutions containing single metabolite standards as well as from mixtures. A Waters liquid chromatography system (Waters Associates, Inc., Milford, MA), fitted with a model 440 UV absorbance detector at 254 nm (attenuation, 0.01 absorbance units full scale) and a model 420 fluorescence detector (excitation filter 254 nm, emission filter 370 nm) was used. Separation was achieved with a Waters Radial-Pak, 10 um, C_{18} cartridge, 8 mm diameter in an RCM-100 Radial Compression Module.

Separation of the metabolites was achieved at a flow rate of 2.0 ml/min and at ambient solvent temperatures. The column was equilibrated with 40% water/60% acetonitrile for 10 min before injection. After injection, the solvent ratio was held at 40% water/60% acetonitrile for 10 min, then changed linearly

over 5 min to 100% acetonitrile and then returned to 40% water/60% acetonitrile over the next 5 min, linearly. Detection of metabolites was by UV absorption at 254 nm. Injection volumes were 30 ul and contained approximately 40 ng of each metabolite.

Although recycling techniques and more sophisticated gradients have been devised to better separate the individual metabolites (Elnenaey and Schoor, 1981; Selkirk et al., 1976), our attention was focused, initially, on general classes of metabolites. The elution sequence of BAP metabolites was diols first, followed by quinones (diones), phenols, and epoxides. Non-metabolized BAP eluted well after the epoxides. Separation of individual diols, quinones, and epoxides was possible with the gradient described above as monitored with the ultraviolet detector (Figure 1), while phenols did not separate as well. It appears that the 1-, 3-, and 5-hydroxy (OH) coeluted, as expected, since individual metabolites had similar retention times. The 6-, 7-, and 9-OH were distinguishable as separate peaks.

Fluorescence was used as confirmatory evidence to pinpoint some of the metabolites. The sensitivity of fluorescence was reduced so that only moderately and highly fluorescent compounds would be detected. This provided a means to further separate and tentatively identify compounds based on fluorescence. In the case of the phenols, 1-, 3-, 5-, and 9-OH were highly fluorescent, 6-OH was nonfluorescent, and 7-OH was only slightly fluorescent. All the diols were highly fluorescent. Epoxides and quinones were either non-fluorescent or only slightly fluorescent.

Analysis of microsomes for endogenous PAH: Data on the occurrence of BAP, 3-MC, and their metabolites in 3-MC induced fish liver microsomes were determined and compared with the occurrence of endogenous PAH in non-induced microsomes. Microsomes were rapidly thawed at 35°C and 1.0 ml

volumes extracted with ethyl acetate/acetone (2:1) according to the procedure described above. HPLC analyses were conducted on the extracts. Results indicated the presence of 3-MC and 2-hydroxy-3-MC in the 3-MC induced extracts; however, neither BAP nor BAP metabolites were detected. Benzo(a)-pyrene, 3-MC, and their metabolites, if present, were at levels below their detection limits in extracts of non-induced microsomes (corn oil controls).

Analysis of BAP metabolites from the activation mixture: Extracts of microsomal activated BAP were assayed by HPLC as described above. Control extracts of the metabolism mixture, excluding the BAP, were also analyzed for base level contaminants. Five presumptive metabolite peaks were observed from the microsomal activated BAP extracts. These peaks were not observed in chromatograms of control extracts. The positions of these peaks are represented in Figure 2. The first peak was observed in the vicinity of the trans-7,10-dihydrodiol peak. The next two did not match any of the standard peaks. These may represent BAP metabolites for which we did not have standards. The next two peaks were tentatively identified as quinones or phenols. The first of these matched the 1,6-quinone, while the other had a retention time similar to both the 3,6-quinone and the 6-OH. Non-metabolized BAP was observed as a late eluting peak.

Overlap in the elution of quinones and phenols makes tentative identification of these metabolites difficult. The use of gas chromatography-mass spectrometry can conclusively identify these two classes of compounds (Takahashi et al., 1979). Refinements in the gradients can also provide better separation within specific classes of compounds and eliminate overlap of the quinone and phenolic peaks (Elnenaey and Schoor, 1981).

Analysis of BAP metabolites for mutagenicity and cytotoxicity: After the PAH metabolites that are formed in fishery products have been identified, it is

possible to determine the relative mutagenicity and carcinogenicity of these compounds in mammalian systems. Studies are underway at the Charleston Laboratory to evaluate the relative mutagenic effects of trace levels of selected BAP metabolites produced by in vitro and in vivo fish systems. These studies center around the use of mammalian cell cultures, primarily, a V79 Chinese hamster lung cell line and C3H mouse prostate cells. The V79 cells were first used for mutagenicity studies by Chu and Malling (1968). Since then, the V79 mutagenicity assay has served as a model for determining the mutagenic potential of PAH compounds (Huberman et al., 1971; Marquardt et al., 1977, 1979). The model uses a change from 8-azaguanine susceptibility to resistance as a marker for chemically induced mutagenesis.

The C3H mouse prostate cell line was established from normal ventral prostates from 6-8 week old C3H mice (Harlan Sprague Dawley Inc., Indianapolis, IN), using the procedures of Chen and Heidelberger (1969A). This model has been used to determine petroleum hydrocarbon induced mutations (Chen and Heidelberger, 1969B; Marquardt et al., 1976, 1977, 1979). Transformed cells that grow to produce colonies can be cloned and injected into mice to determine carcinogenicity (Chen and Heidelberger, 1969B; Marquardt et al., 1976, 1977, 1979). The mice are observed for up to 6 months for tumor production.

In summary, this report provides a review of current literature on PAHs and consolidates analytical procedures. It also describes our efforts to metabolize BAP with fish microsomes and to extract and identify the metabolites. Chromatographic separation of metabolites can be accomplished by UV or fluorescence or both, depending on the metabolite in question. The variability in UV or fluorescence spectra of some metabolites aids in their identification. Separation of general metabolite classes indicates some overlap of quinones,

phenols, and epoxides, which could be eliminated by incorporation of more sophisticated gradients or through recycling (Elnenaey and Schoor, 1981; Selkirk et al., 1976). Analysis of microsomal activated BAP by HPLC produced five presumptive metabolite peaks, i.e., a dihydrodiol, 1,6-quinone, either 3,6-quinone or 6-hydroxy, and two peaks for which standards were not available. These results are consistent with the findings of others (Ahokas et al., 1975; Melius et al., 1979).

Assays to determine possible mutagenic and carcinogenic effects of BAP metabolites on mammalian cells are discussed. Currently, data is being collected on the mutagenic effects of BAP metabolites on mammalian cell cultures and will be published at the completion of this project.

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Figure 1. Separation profile of BAP metabolite standards obtained from a Waters Radial-Pak C_{18} column, flow rate 2.0 ml/min, ambient temperature. The column was equilibrated with 40% water/60% acetonitrile for 10 min before injection. After injection of a standard mix containing 30 ng of each metabolite, the solvent ratio was held at 40% water/60% acetonitrile for 10 min, then, changed linearly over 5 min to 100% acetonitrile and, then, linearly returned to 40% water/60% acetonitrile over the next 5 min. Identity of peaks was based on results obtained by injection of individual standards and groups of standards and by using fluorescence as a qualitative marker for some metabolites. Abbreviations used: (t) = trans; (c) = cis; D = benzo(a)pyrene dihydrodiol; Q = benzo(a)pyrene dione (quinone); OH = benzo(a)pyrene phenol; DHE = benzo(a)pyrene dihydroepoxide; THE = benzo(a)pyrene tetrahydroepoxide.

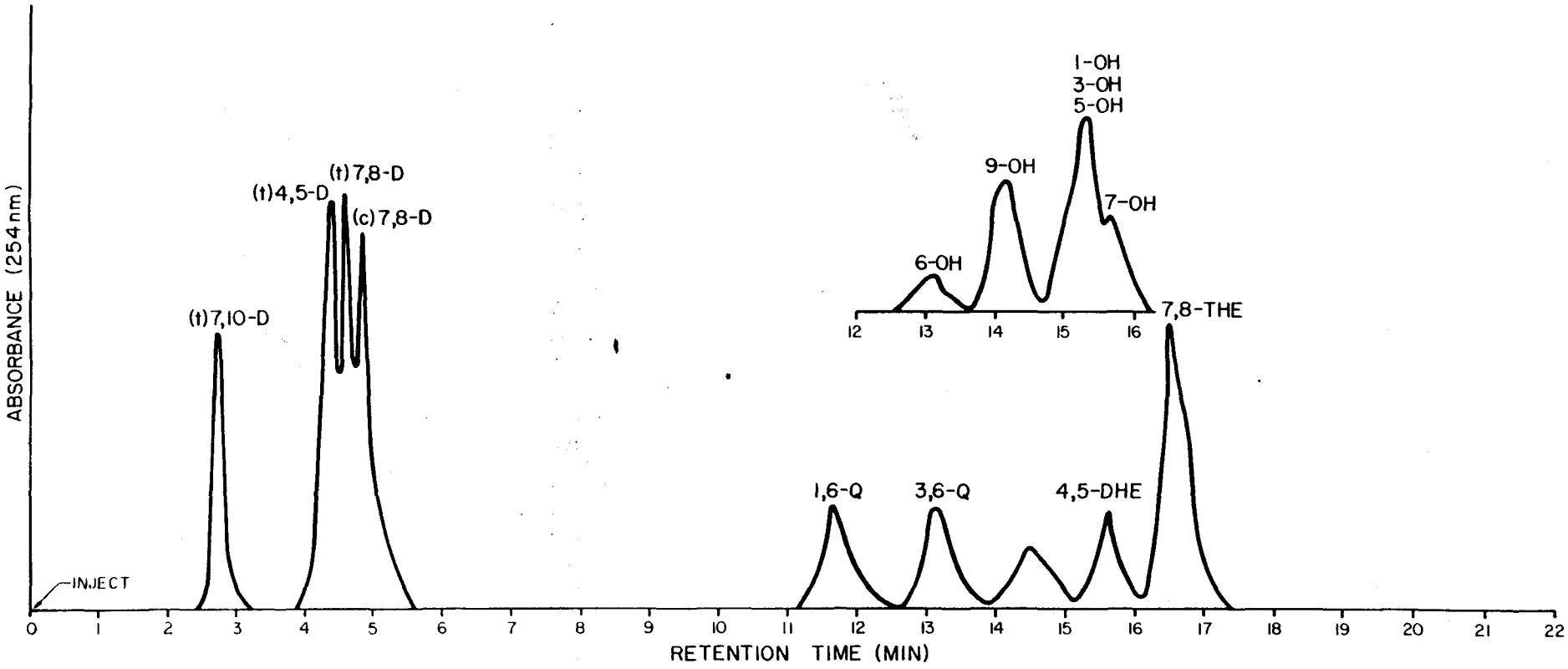
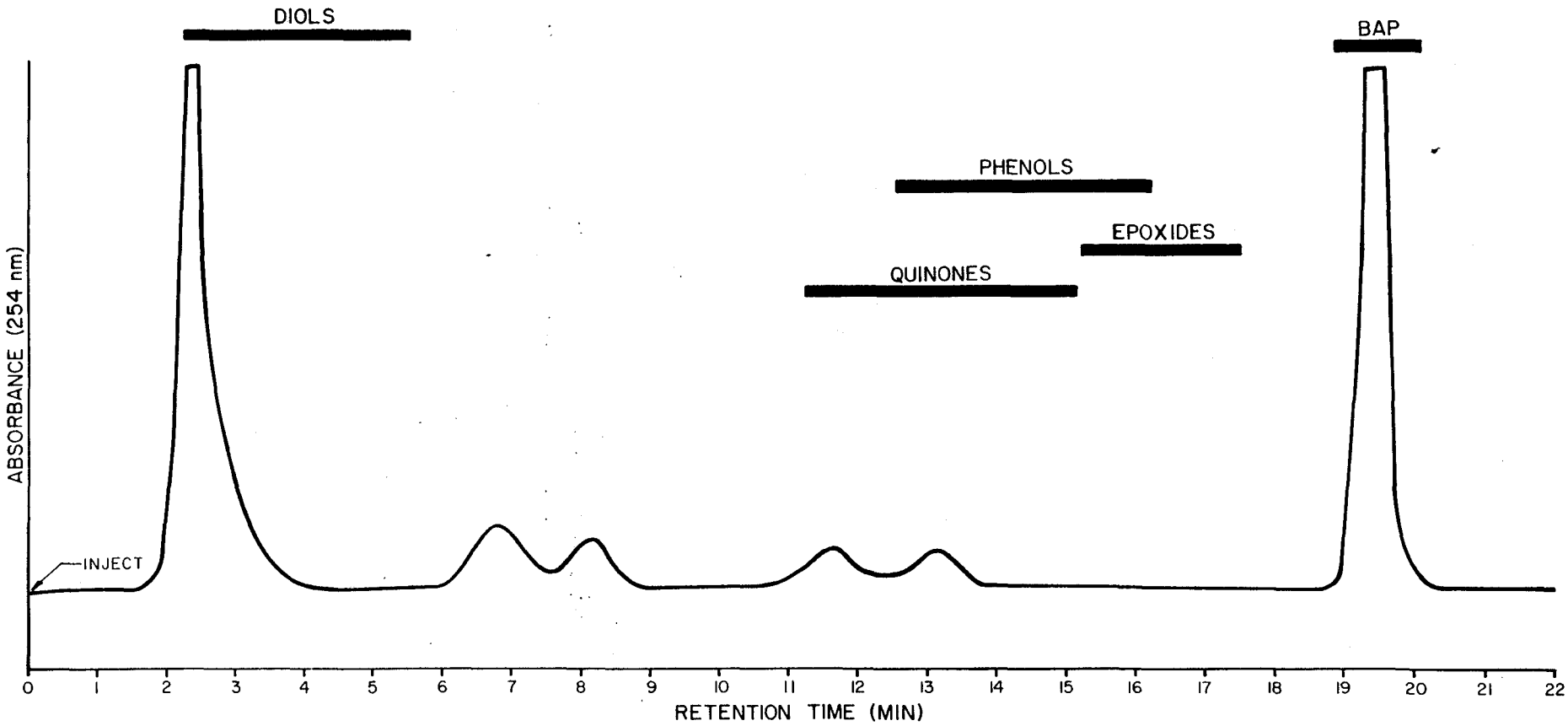

ABSORBANCE (254nm)
(t)7,10-D
(t)4,5-D
(t)7,8-D
(c)7,8-D
INJECT
1,6-Q
3,6-Q
4,5-DHE
7,8-THE
6-OH
9-OH
1-OH
3-OH
5-OH
7-OH
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
RETENTION TIME (MIN)

Figure 2. Separation profile of BAP metabolites produced from a fish liver microsome activation system obtained from a Waters Radial Pak C_{18} column, flow rate 2.0 ml/min, ambient temperature. The column was washed for 10 min with 40% water/60% acetonitrile before injection. After injection, the solvent ratio was held at 40% water/60% acetonitrile for 10 min, then, changed linearly over 5 min to 100% acetonitrile and, then, linearly returned to 40% water/60% acetonitrile over the next 5 min.


DIOLS
BAP
PHENOLS
EPOXIDES
QUINONES
INJECT
ABSORBANCE (254 nm)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
RETENTION TIME (MIN)