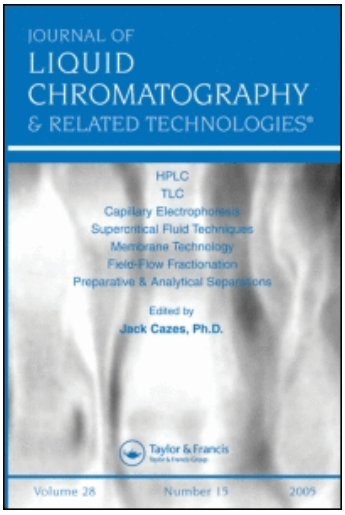

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Liquid Chromatographic Separation of Five *Trans*-dihydrodiols of Benz[*A*] anthracene

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LIQUID CHROMATOGRAPHIC SEPARATION OF FIVE *TRANS*-DIHYDRODIOLS OF BENZ[*A*]ANTHRACENE

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ABSTRACT

Among the five possible *trans*-dihydrodiol derivatives of benz[*a*]anthracene (BA), *trans*-3,4- and *trans*-8,9-dihydrodiols could not be separated by either a normal-phase or a reversed-phase HPLC method unless they were first converted to diacetates. We describe in this report a complete separation of five BA *trans*-dihydrodiols using a Vydac C_{18} column and a column packed with a chiral stationary phase (*R*)-*N*-(3,5-dinitrobenzoyl)leucine covalently bonded to γ-aminopropylsilanized silica. Enantiomeric compositions of BA 3,4-, 5,6-, 8,9-, and 10,11- *trans*-dihydrodiols formed in the metabolisms of BA by liver microsomes from untreated, phenobarbital-treated, and 3-methylcholanthrene-treated rats were determined by chiral stationary phase HPLC or by comparing their circular dichroism spectral data with those of optically pure reference compounds.

INTRODUCTION

The weakly carcinogenic benz[*a*]anthracene (BA, Fig. 1) is an environmental pollutant and its weak carcinogenicity is due to

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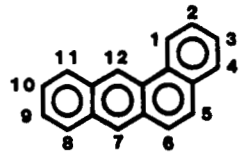

FIGURE 1. Structure and numbering system of benz[*a*]anthracene (BA).

metabolic conversion by mammalian drug-metabolizing enzyme systems to *trans*-3,4-dihydrodiol-1,2-epoxide (1). The quantification of the metabolically formed and proximate carcinogenic BA *trans*-3,4-dihydrodiol in various enzyme systems cannot readily be accomplished owing to its cochromatography with BA *trans*-8,9- and *trans*-10,11-dihydrodiols in a reversed-phase HPLC system using a Zorbax ODS (C_{18}) column (2-4). BA *trans*-10,11-dihydrodiol can be separated from BA 3,4- and 8,9- *trans*-dihydrodiols by normal-phase HPLC using a silica gel column (2,4-7). Thus BA *trans*-3,4-dihydrodiol cannot be separated from BA *trans*-8,9-dihydrodiol in both reversed-phase and normal-phase HPLC systems (2-7). However, BA *trans*-3,4-dihydrodiol and BA *trans*-8,9-dihydrodiol can be separated as their diacetates by reversed-phase HPLC (2). Therefore it was necessary to use two or three chromatographic systems and a derivatization procedure to completely separate and analyze the amounts of various dihydrodiols formed in the metabolism of BA (2-7). This analytical procedure is apparently undesirable when the amounts of BA *trans*-3,4-dihydrodiol formed in many enzyme systems need to be quantified and a large number of samples needs to be analyzed.

Trans-dihydrodiols are formed in the metabolism of polycyclic aromatic hydrocarbons by two consecutive reactions; an epoxidation reaction catalyzed by microsomal cytochrome P-450 isozymes and a hydration reaction catalyzed by microsomal epoxide hydrolase (8,9). In order to facilitate the determinations of the amounts and the enantiomeric compositions of various BA *trans*-dihydrodiols formed in the metabolism of BA in various enzyme systems, we set out to search for either a single HPLC system or a combination of not more than two chromatographic systems that separates all five BA *trans*-dihydrodiols. This paper describes a combination of two chromatographic systems which separates the five possible *trans*-dihydrodiols of BA without the need of a derivatization procedure.

MATERIALS

Racemic *trans*-dihydrodiols of BA were obtained from the Chemical Repository of National Cancer Institute. HPLC grade solvents were purchased from Mallinckrodt, Inc. (Paris, KY). Optically pure enantiomers of BA *trans*-3,4-dihydrodiol (6) and BA *trans*-5,6-dihydrodiol (10) were prepared by resolutions of their diastereomeric di(-)menthoxyacetates.

Male Sprague-Dawley rats weighing 80-100 g were treated i.p. with phenobarbital (75 mg/kg body weight, injected in 0.5 mL of water) once daily on each of three consecutive days, or with 3-methylcholanthrene (25 mg/kg body weight, injected in 0.5 mL corn oil) once daily for each of four consecutive days. The rats were sacrificed the next day after the last injection of the drug and liver microsomes were prepared as described (11). Microsomal protein was determined by the method of Lowry *et al.* (12), with bovine serum albumin as the standard.

METHODS

Preparation of Biosynthetic BA trans-Dihydrodiols: A 100-mL reaction mixture contained 100 mg protein equivalent of rat liver microsomes, 5 mmol Tris-HCl (pH 7.5), 0.3 mmol of $MgCl_2$, 10 units of glucose-6-phosphate dehydrogenase (type XII, Sigma Chemical Co., St. Louis, MO), 10 mg of $NADP^+$, and 48 mg of glucose-6-phosphate. The reaction mixture was pre-incubated at 37°C for 2 min in a water shaker bath. BA (8 μmol in 4 mL of acetone) was

then added and incubated for 30 min. BA and its metabolites were extracted by sequential additions of acetone (100 mL) and ethyl acetate (200 mL). The resulting organic phase was dehydrated with anhydrous $MgSO_4$, filtered, and evaporated to dryness under reduced pressure. The residues were dissolved in methanol and the metabolites were separated by reversed-phase HPLC as described below.

Chromatography: HPLC was performed using a Waters Associates (Milford, MA) liquid chromatograph consisting of a Model 6000A solvent delivery system, a Model M45 solvent delivery system, a Model 660 solvent programmer, and a Model 440 absorbance (254 nm) detector. Samples were injected via a Valco model N60 loop injector (Valco Instruments, Houston, TX). Retention times and ratios of enantiomers, determined by areas under the peaks, were recorded with a Hewlett-Packard Model 3390A integrator.

Reversed-phase HPLC: Dihydrodiol metabolites of BA were isolated by reversed-phase HPLC using a Vydac C_{18} column (4.6 mm i.d. x 25 cm; The Sep/a/ra/tions Group, Hesperia, CA) eluted with methanol/water (3:2, v/v) at a flow rate of 1.2 mL/min. As soon as BA *trans*-3,4-dihydrodiol is eluted, the column was washed with methanol until BA phenols and the unreacted BA were eluted.

Normal-phase HPLC: BA 8,9- and 10,11- *trans*-dihydrodiols were separated using an HPLC column (4.6 mm i.d. x 25 cm, Regis Chemical Co., Morton Grove, IL) packed with spherical particles of 5 micrometer diameter of γ-aminopropylsilanized silica to which (*S*)-*N*-(3,5-dinitrobenzoyl)leucine was covalently bonded. The column was eluted with 10% (v/v) of ethanol/acetonitrile (2:1, v/v) in hexane at a flow rate of 2 mL/min. The enantiomers of BA 3,4-, 8,9- and 10,11- *trans*-dihydrodiols were not resolved under the chromatographic conditions described. BA *trans*-3,4-dihydrodiol cochromatographed with BA *trans*-8,9-dihydrodiol.

Chiral Stationary Phase HPLC: The enantiomers of BA 8,9- and 10,11- *trans*-dihydrodiols were separated with an HPLC column (4.6 mm i.d. x 25 cm, Regis Chemical Co., Morton Grove, IL) packed with spherical particles of 5 micrometer diameter of γ-aminopropylsilanized silica to which (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine was ionically bonded. The enantiomers were eluted with 10% (v/v) of ethanol/acetonitrile (2:1, v/v) in hexane at a flow rate of 2 mL/min (13,14).

Purified dihydrodiols were dried and redissolved in methanol for circular dichroism (CD) spectral measurements. The optical purities of BA 3,4- and 5,6- *trans*-dihydrodiol were determined by comparing their CD spectral data with those of optically pure BA *trans*-(3*R*,4*R*)-dihydrodiol and BA *trans*-(5*R*,6*R*)-dihydrodiol.

Spectral Analysis: Ultraviolet-visible absorption spectra of dihydrodiols in methanol were determined using a 1-cm path length quartz cuvette with a Varian model 118C spectrophotometer. CD

spectra of dihydrodiols in methanol were measured in a cell of 1-cm path length at room temperature using a Jasco model 500A spectropolarimeter equipped with a model DP-500 data processor. The concentration of the sample is indicated by A_λ/mL (number of absorbance unit at wavelength λ per ml of methanol). CD spectra are expressed by ellipticity (in millidegrees) for methanol solutions that have an absorbance of 1.0 unit at wavelength λ (15).

The CD spectral properties of optically pure enantiomers are: BA *trans*-(3*R*,4*R*)-dihydrodiol, Φ_{261}/A_{261} = +3.07 mdeg and Φ_{403}/A_{261} = -0.64 mdeg; BA *trans*-(5*R*,6*R*)-dihydrodiol, Φ_{264}/A_{266} = -6.5 mdeg. CD spectra of enantiomeric *trans*-dihydrodiols of BA were reported earlier (6).

RESULTS AND DISCUSSION

The five possible *trans*-dihydrodiols of BA are separated into four chromatographic peaks by using the Vydac C_{18} column (Fig. 2A). BA *trans*-3,4-dihydrodiol is selectively separated from BA 8,9- and 10,11- *trans*-dihydrodiols. This separation was not achieved by using a Zorbax ODS (C_{18}) column (2-4). Furthermore, BA *trans*-5,6-dihydrodiol is more efficiently separated from BA 8,9- and 10,11- *trans*-dihydrodiols; a considerably improved separation over that using a Zorbax ODS column (2-4). The cochromatography of BA 3,4-, 8,9-, and 10,11- *trans*-dihydrodiols on the Zorbax ODS column was ascribed to the quasidiequatorial conformations of the hydroxyl groups (2-4). Apparently the selectivity provided by the stationary phase of the Vydac C_{18} column enabled the separation of the quasi-diequatorial BA *trans*-3,4-dihydrodiol from the quasi-diequatorial BA 8,9- and 10,11- *trans*-dihydrodiols.

BA 8,9- and 10,11- *trans*-dihydrodiols can be separated by using a silica gel column (2,5-7). These two dihydrodiols are more efficiently separated on a γ-aminopropylsilanized silica column with covalently bonded (*S*)-*N*-(3,5-dinitrobenzoyl)leucine (Fig.

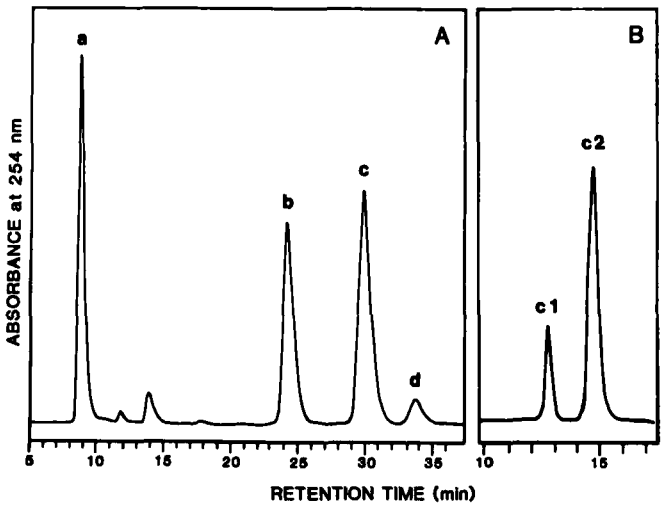

FIGURE 2. (A) Reversed-phase HPLC separation of BA *trans*-dihydrodiols using a Vydac C_{18} column. Solvent; methanol/water (3:2, v/v) at 1.2 ml/min. The identities of the chromatographic peaks are: *a*, BA *trans*-1,2-dihydrodiol; *b*, BA *trans*-5,6-dihydrodiol; *c*, BA 8,9- and 10,11- *trans*-dihydrodiols; *d*, BA *trans*-3,4-dihydrodiol.
(B) Normal-phase HPLC separation of BA *trans*-10,11-dihydrodiol (peak *c1*) and BA *trans*-8,9-dihydrodiol (peak *c2*) using a column packed with γ-aminopropylsilanized silica with covalently bonded (*R*)-*N*-(3,5-dinitrobenzoyl)leucine. Solvent; 10% ethanol/acetonitrile (2:1, v/v) in hexane at 2 mL/min.

2B). BA *trans*-3,4-dihydrodiol cochromatographed with BA *trans*-8,9-dihydrodiol on both the silica gel column (2,5-7) and the γ-aminopropylsilanized silica column with covalently bonded (*S*)-*N*-(3,5-dinitrobenzoyl)leucine (Fig. 2B). Five *trans*-dihydrodiols of BA can thus be completely separated by first using a Vydac C_{18} column (Fig. 2A) and followed by the use of either a Zorbax SIL (silica gel) column (Fig. 2B) or, preferably, a column packed with γ-aminopropylsilanized silica to which a stationary phase (*S*)-*N*-

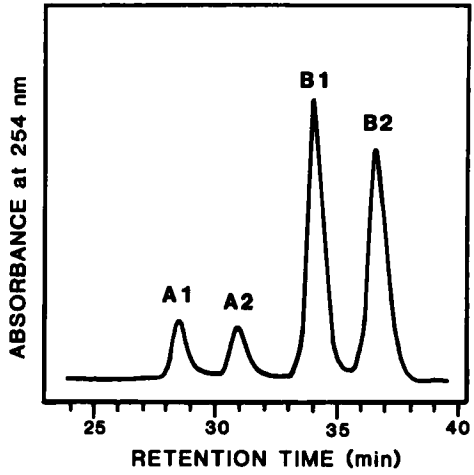

FIGURE 3. Chiral stationary phase HPLC separation of the enantiomers of BA *trans*-10,11-dihydrodiol (peaks A1 and A2) and of BA *trans*-8,9-dihydrodiol (peaks B1 and B2) using a column packed with γ-aminopropylsilanized silica with ionically bonded (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine. The *R,R*-enantiomer of both dihydrodiols is more strongly retained by the chiral stationary phase (13,14). Solvent; 10% ethanol/acetonitrile (2:1, v/v) in hexane at 2 mL/min.

(3,5-dinitrobenzoyl)leucine is covalently bonded (Fig. 2B). Different modes of interactions between the stationary phase and the dihydrodiols, rather than the chirality of the stationary phase, are most likely responsible for the separation of BA 8,9- and 10,11- *trans*-dihydrodiols.

BA *trans*-1,2-dihydrodiol is a very minor metabolite of BA (2,7). BA 3,4-, 5,6-, 8,9-, and 10,11- *trans*-dihydrodiols formed by incubation of BA with liver microsomes from untreated, phenobarbital-treated, and 3-methylcholanthrene-treated male Sprague-Dawley rats, respectively, were purified by the two chromatogra-

TABLE 1

Enantiomeric Compositions of the *Trans*-dihydrodiols Formed in the Metabolism of BA by Liver Microsomes From Rats of the Sprague Dawley Strain.

Enzyme Inducer	Dihydrodiol Enantiomer (%)			
	3*R*,4*R*	5*R*,6*R*	8*R*,9*R*	10*R*,11*R*
None	83	77	94	>99
Phenobarbital	91 (69)*	81 (68)	90 (89)	95 (83)
3-Methylcholanthrene	90	84 (81)	>99 (98)	>99 (98)

*Data in parentheses are from ref. 10 using rats of Long-Evans strain.

phic systems shown in Fig. 2. The enantiomeric compositions of the enzymatically formed BA 3,4- and 5,6- *trans*-dihydrodiols were determined by comparing their CD spectral data with those of optically pure BA *trans*-(3*R*,4*R*)-dihydrodiol and BA *trans*-(5*R*,6*R*)-dihydrodiol, respectively. The enantiomeric compositions of the enzymatically formed BA 8,9- and 10,11- *trans*-dihydrodiols, purified as described in Fig. 2, were each determined by chiral stationary phase HPLC using a column packed with ionically bonded (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine (13,14). Alternatively, the enantiomeric compositions of the BA 8,9- and 10,11- *trans*-dihydrodiols, purified as described in Fig. 2A, can be simultaneously determined by chiral stationary phase HPLC (Fig. 3). The enantiomeric compositions of the metabolically formed BA *trans*-dihydrodiols using three different rat liver microsomal preparations are shown in Table 1.

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