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DIRECT ENANTIOMERIC RESOLUTION OF CYCLIC ALCOHOL DERIVATIVES OF POLYCYCLIC AROMATIC HYDROCARBONS BY CHIRAL STATIONARY PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The enantiomers of some cyclic alcohol derivatives of phenanthrene, benz[*a*]anthracene, benzo[*a*]pyrene, cholanthrene, and 3-methylcholanthrene were resolved by high-performance liquid chromatography using a commercially available preparative column packed with an (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine ionically bonded to γ-aminopropylsilanized silica. Resolution of enantiomers was confirmed by ultraviolet–visible absorption, mass and circular dichroism spectral analyses. This method has been applied to the determination of optical purity of 1-hydroxycholanthrene and 1-hydroxy-3-methylcholanthrene formed in the metabolism of cholanthrene and 3-methylcholanthrene, respectively, by rat liver microsomes.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are stereoselectively metabolized by mammalian drug-metabolizing enzyme systems to optically active intermediates, including epoxides, dihydrodiols, and dihydrodiol-epoxides[1–5]. Enantiomeric epoxides and dihydrodiols as well as diastereomeric dihydrodiol-epoxides of benz[*a*]anthracene (BA) and benzo[*a*]pyrene (BP) vary substantially in their mutagenic and carcinogenic activities[1]. The optical purity of dihydrodiol metabolites of some PAHs can be determined by resolution of diastereomers derivatized with (−)-menthoxyacetyl chloride or (−)-α-methoxy-α-trifluoromethylphenylacetyl chloride[2–4,7–9]. Recently, a chiral stationary phase (CSP) high-performance liquid chromatography (HPLC) method was developed to resolve some dihydrodiol and tetrahydrodiol enantiomers of BA and BP[10] and this method was applied to determine the optical purity and absolute configuration of dihydrodiols formed in the metabolism of several PAHs by hepatic microsomal enzymes[10–12]. PAHs such as cholanthrene (CA)[13] and 3-methylcholanthrene (MCA)[14–16] may undergo enzymatic hydroxylation at the methylene carbons to form metabolites that are biologically more active than the parent compounds. In order to study the detailed mechanism of metabolic activation of carcinogenic PAHs such as CA and MCA, we have applied the CSP-HPLC method pre-

viously developed[10] to resolve the enantiomers of cyclic alcohol derivatives of some PAHs and the results are described in this report. In this study, enantiomers were resolved using a commercially available preparative column packed with an (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine ionically bonded to γ-aminopropylsilanized silica[17].

EXPERIMENTAL

Materials

Racemic 7-hydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (7-OH-H_4BP), 10-hydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (10-OH-H_4BP), 4-hydroxy-1,2,3,4-tetrahydrophenanthrene (4-OH-H_4Ph), 1-hydroxy-1,2,3,4-tetrahydrobenz[*a*]anthracene (1-OH-H_4BA), 4-hydroxy-1,2,3,4-tetrahydrobenz[*a*]anthracene (4-OH-H_4BA), 8-hydroxy-8,9,10,11-tetrahydrobenz[*a*]anthracene (8-OH-H_4BA), 11-hydroxy-8,9,10,11-tetrahydrobenz[*a*]anthracene (11-OH-H_4BA) and 1-hydroxycholanthrene (1-OH-CA) were prepared by reduction of the corresponding keto precursors with $NaBH_4$. Racemic 9-hydroxy-9,10-dihydrophenanthrene (9-OH-H_2Ph) was synthesized by re-

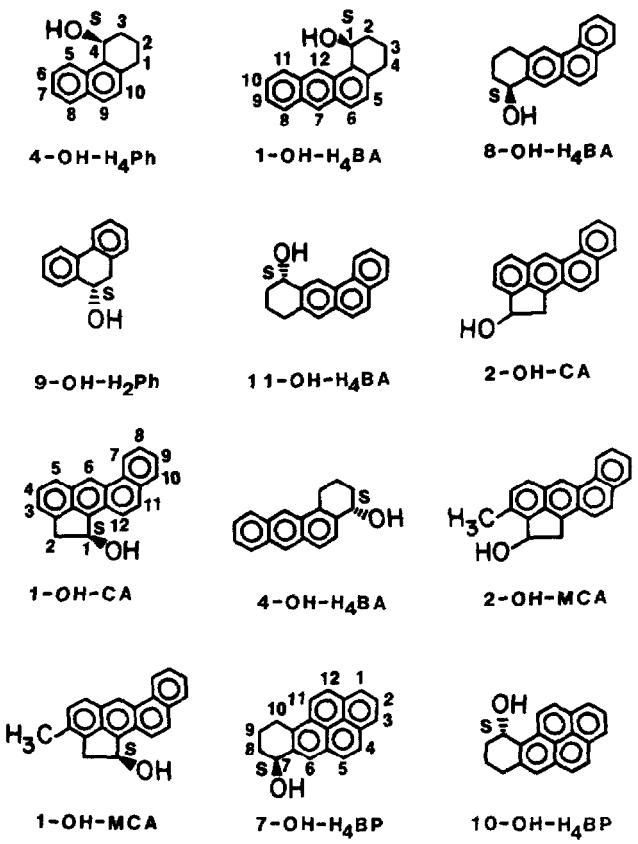

Fig. 1. Structures and designations of cyclic alcohol derivatives used in this study. Except for 2-OH-CA and 2-OH-MCA, the absolute configuration of the enantiomer retained the most on the (*R*)-CSP column as predicted by Pirkle's chiral recognition mechanisms[22] is shown for each compound.

duction of phenanthrene 9,10-epoxide[18] with $NaBH_4$. 8,9-Dihydrobenz-[*a*]anthracene-11(10H)-one, 10,11-dihydrobenz[*a*]anthracene-8(9H)-one[19] and 7,8-dihydrobenzo[*a*]pyrene-10(9H)-one[20] were kindly provided by Dr. Peter Fu of the National Center for Toxicological Research, Jefferson,AR, U.S.A. 9,10-Dihydrobenzo[*a*]pyrene-7(8H)-one, 1,2-dihydrophenanthrene-4(3H)-one and cholanthrene-1-one were purchased from Aldrich (Milwaukee, WI, U.S.A.). Racemic 1-hydroxy-3-methylcholanthrene (1-OH-MCA), and 2-hydroxy-3-methylcholanthrene (2-OH-MCA) were obtained from the Chemical Repository of the National Cancer Institute. 2-Hydroxycholanthrene (2-OH-CA) was obtained biosynthetically as described below.

A mixture of the enzymatically formed 1-OH-CA and 2-OH-CA was obtained by incubation of cholanthrene (CA) with liver microsomes from MCA-treated or phenobarbital-treated rats followed by reversed-phase HPLC isolation as previously described[13]. 1-OH-CA (retention time 13.2 min) and 2-OH-CA (retention time 18.4 min) were separated using a DuPont Zorbax SIL column (25 cm × 6.2 mm I.D.) eluted with tetrahydrofuran–hexane (3:17, v/v) at a flow-rate of 2 ml/min.

A mixture of 1-OH-MCA and 2-OH-MCA was obtained by incubation of MCA with liver microsomes from phenobarbital-treated rats and was purified by reversed-phase HPLC[21]. 1-OH-MCA (retention time 11.9 min) and 2-OH-MCA (retention time 13.9 min) were also separated with the DuPont Zorbax SIL column and the solvent system described above for the separation of 1-OH-CA and 2-OH-CA. Under our experimental conditions, 1-OH-MCA was a very minor metabolite of MCA when liver microsomes of MCA-treated rats were used.

Chromatography

Chemicals were analyzed with a Regis (Regis Chemical, Morton Grove, IL, U.S.A.) Pirkle Type 1-A preparative column (25 cm × 10 mm I.D.), packed with an (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine ionically bonded to spherical particles of 5 μm diameter of γ-aminopropylsilanized silica[17] on a Spectra-Physics liquid chromatograph consisting of a Model SP8700 solvent delivery system, a Model SP8750 injector/organizer, a Model SP8400 variable wavelength UV–vis detector and a Model SP4100 computing integrator. Baseline separation of most compounds used in this study was achieved isocratically with a flow-rate of 4 ml/min using up to 5% (v/v) of solvent A (ethanol–acetonitrile, 2:1, v/v) in hexane at ambient temperature. Solvent was removed from the resolved enantiomers using a speed vac concentrator (Model SVC100H, Savant Instruments, Hicksville, NY, U.S.A.). A minor amount of contaminating CSP leached from the column into the resolved enantiomers was removed by reversed-phase HPLC with a DuPont Zorbax ODS column as described previously[10].

Spectral analysis

UV–visible absorption spectra of samples in methanol were determined using a 1-cm path length quartz cuvette with a Beckman Model 25 spectrophotometer. Optical rotations were measured on a Perkin-Elmer Model 241-MC polarimeter. Mass spectral analysis was performed on a Finnigan Model 4000 gas chromatograph–mass spectrometer–data system by electron impact with a solid probe at 70 eV and 250°C ionizer temperature. Circular dichroism (CD) spectra of samples

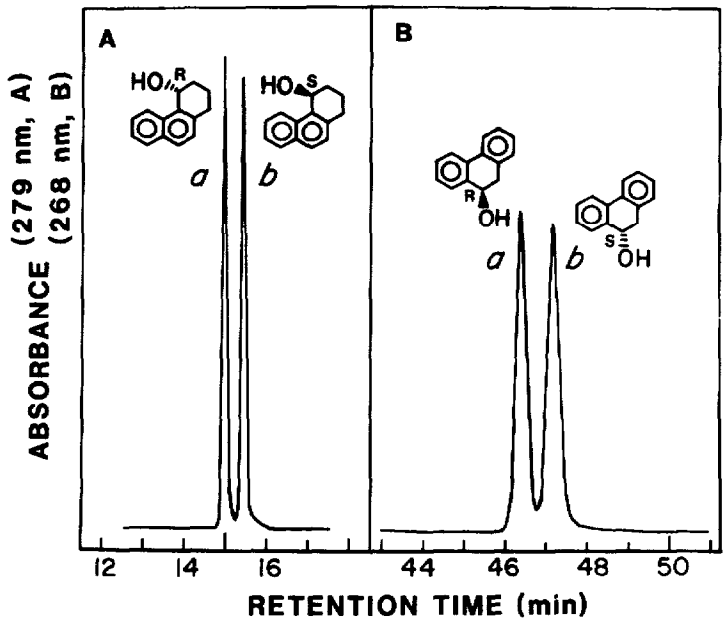

Fig. 2. Resolutions of the enantiomers of 4-OH-H_4Ph (A) with 5% solvent A in hexane and of 9-OH-H_4Ph (B) with 1% solvent A in hexane at a solvent flow-rate of 4 ml/min on a Pirkle 1-A column (25 cm × 10 mm I.D.). The absolute stereochemistries of the enantiomers shown are assigned on the basis of the chiral recognition mechanisms proposed by Pirkle *et al.*[22] and the enantiomers are also designated as *a* and *b* according to the orders of elution.

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. CD spectra were expressed by ellipticity (φ_λ, in millidegrees) for methanol solutions that have an absorbance of 1.0 unit at the wavelength of maximal absorption (λ_{max}) in a quartz cell of 1-cm path length in a UV–vis spectrophotometer. The ellipticity and the molar ellipticity ($[\theta]_\lambda$, in deg · cm^{-1} · $decimol^{-1}$) are related to the molar extinction coefficient (ε_{max}, in $cm^{-1} \cdot M^{-1}$) as follows:

$$[\theta]_\lambda = 0.1\ \varepsilon_{max}\varphi_\lambda$$

CD spectral data obtained from different laboratories are more conveniently compared if they are expressed by φ_λ because the ε_{max} values of many PAH derivatives are either not known or are difficult to determine accurately.

RESULTS AND DISCUSSION

The resolutions of the enantiomers of 4-OH-H_4Ph and of 9-OH-H_2Ph are shown in Fig. 2. The enantiomers of 4-OH-H_4Ph were more easily resolved than those of 9-OH-H_2Ph. The structures of the resolved enantiomers were confirmed by UV absorption and mass spectral analyses in order to assure that decomposition of enantiomers did not occur during the chromatographic process. Decomposition of

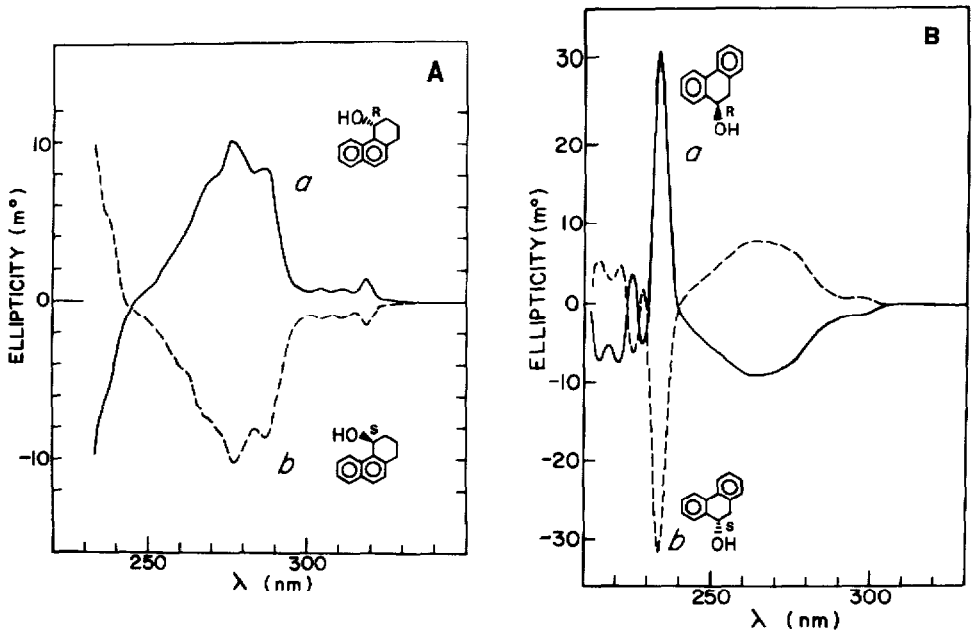

Fig. 3. CD spectra of the resolved enantiomers of (A) 4-OH-H_4Ph (λ_{max} 279 nm) and (B) 9-OH-H_2Ph (λ_{max} 268 nm). The enantiomers were resolved as described in Fig. 2. The CD spectra are expressed by ellipticity (in m^0) for methanol solutions that read 1.0 absorbance unit at the wavelength of maximal absorption. The assignments of absolute stereochemistries of the resolved enantiomers and the designations *a* and *b* are as in Fig. 2.

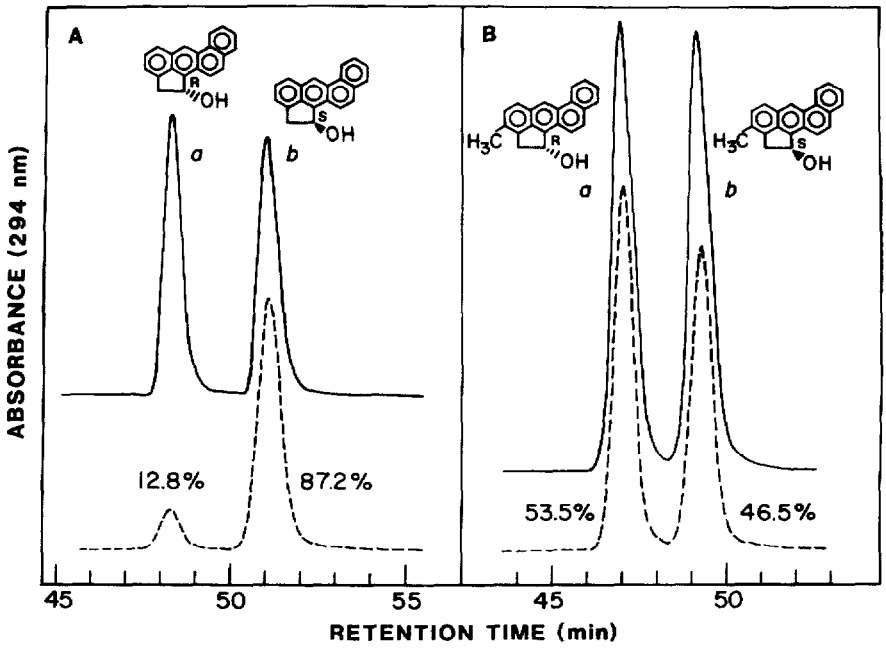

Fig. 4. Resolutions of synthetic 1-OH-CA (solid curve, A), 1-OH-CA formed in metabolism of CA by liver microsomes from phenobarbital-treated rats (dotted curve, A), synthetic 1-OH-MCA (solid curve, B), and 1-OH-MCA formed in metabolism of MCA by liver microsomes from phenobarbital-treated rats (dotted curve, B). The assignments of the absolute stereochemistires of the resolved enantiomers and the designations *a* and *b* are as in Fig. 2.

some dihydrodiol enantiomers of PAHs in the (*R*)-CSP column is known to occur[10]. The CD spectra (Fig. 3) of the optically pure enantiomers obtained by rechromatography of the resolved enantiomers confirmed that enantiomeric pairs were indeed obtained.

Baseline separations of the enantiomers of 1-OH-CA and of 1-OH-MCA were also achieved (Fig. 4). Again the structures of the resolved enantiomers were confirmed by UV and mass spectral analyses. The CD spectra of the optically pure enantiomers are shown in Fig. 5. The spectral data and the CSP-HPLC results of the cyclic alcohol derivatives tested are shown in Table I.

A 1-OH-CA was obtained by reversed-phase and normal-phase isolations (see Materials and Methods) from a metabolite mixture resulting from *in vitro* incubation of CA with liver microsomes from phenobarbital-treated male Sprague-Dawley rats. This 1-OH-CA metabolite was found to contain both the *R* and the *S* enantiomers in a ratio of 12.8:87.2 (enantiomeric purity 74.4% enriched in the S enantiomer, Fig. 4A). Similar analysis of 1-OH-CA metabolite formed in the metabolism of CA by liver microsomes from MCA-treated rats indicated an *R*/*S* enantiomer ratio of

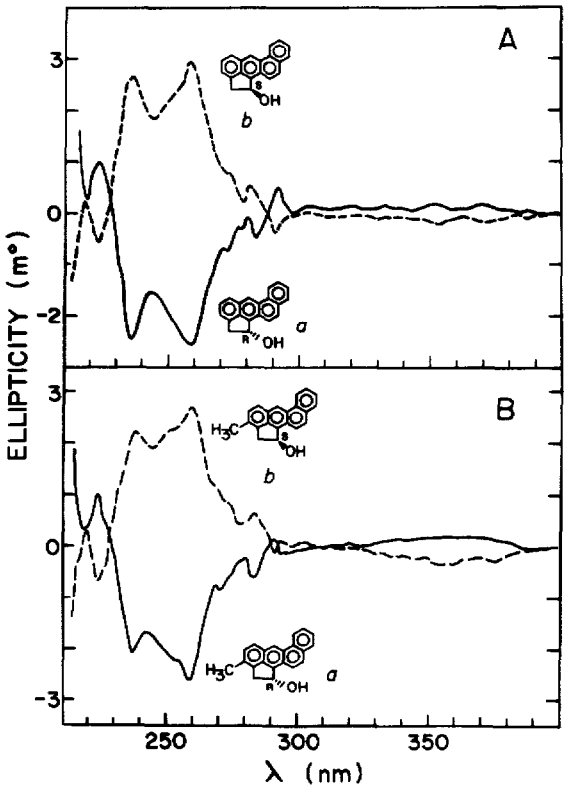

Fig. 5. CD spectra of the resolved enantiomers of (A) 1-OH-CA (λ_{max} 296 nm) and of (B) 1-OH-MCA (λ_{max} 296 nm). The enantiomers were resolved as described in Fig. 4. The CD spectra are expressed by ellipticity (in m^0) for methanol solutions that read 1.0 absorbance unit at the wavelength of maximal absorption. The assignments of absolute stereochemistries of the resolved enantiomers and the designations *a* and *b* are as in Fig. 2.

TABLE I

UV ABSORPTION AND CD SPECTRAL DATA AND CSP-HPLC RESOLUTION OF CYCLIC ALCOHOL DERIVATIVES OF SOME POLYCYCLIC AROMATIC HYDROCARBONS

A Regis Pirkle Type 1-A column (25 cm × 10 mm I.D.) was used which was packed with (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine ionically bonded to γ-aminopropylsilanized silica. N.D. = not determined.

Chemical	λ_{max}* *(nm)*	*Solvent A (%)***	*Retention time (min)****		*R value*§	*Ellipticity (λ)*§§	
			Peak a	*Peak b*		*Peak a*	*Peak b*
(+)4-OH-H_4Ph	279	5	14.9 (*R*)	15.9 (*S*)	2.1	+10.1 (276)	−10.1 (276)
(±)9-OH-H_2Ph	268	1	46.4 (*R*)	48.6 (*S*)	1.2	+30.6 (234)	−31.6 (234)
(±)1-OH-H_4BA	258	5	22.2 (*R*)	23.4 (*S*)	1.6	−3.5 (259)	+3.5 (259)
(±)4-OH-H_4BA	258	2	83.2 (*R*)	85.3 (*S*)	0.7	N.D.§§§	N.D.
(±)8-OH-H_4BA	255	5	30.5 (*R*)	32.4 (*S*)	2.1	−8.8 (255)	+8.8 (255)
(±)11-OH-H_4BA	255	5	30.3 (*R*)	32.3 (*S*)	2.2	−7.0 (255)	+7.0 (255)
(±)7-OH-H_4BP	248	5	71.4 (*R*)	73.8 (*S*)	1.1	+0.6 (277)	−0.6 (277)
(±)10-OH-H_4BP	248	5	54.7 (*R*)	55.8 (*S*)	0.6	N.D.	N.D.
(±)1-OH-CA§§§	294	5	48.1 (*R*)	51.2 (*S*)	2.5	−2.7 (258)	+2.7 (258)
(±)2-OH-CA†	294	5	74.5††	74.5††	0	–	–
(±)1-OH-MCA	296	5	46.9 (*R*)	49.2 (*S*)	1.4	−2.6 (258)	+2.7 (258)
(±)2-OH-MCA†††	296	5	52.3†††	52.3†††	0	–	–

* Wavelength of maximal absorption.

** Percentage of solvent A (ethanol–acetonitrile, 2:1, v/v) in hexane. The flow-rate was 4 ml/min.

*** The order of the enantiomer with the absolute configuration indicated in parenthesis is predicted according to the chiral recognition mechanism proposed by Pirkle *et al.*[22].

§ R (resolution) = $(V_2 - V_1)/[1/2(W_1 + W_1)]$, where V is retention volume and W is peak width at base.

§§ Ellipticity (in millidegrees) at wavelength indicated in the parenthesis for methanol solutions that read 1.0 absorbance unit at the wavelength of maximal absorption indicated in column 2.

§§§ $[\alpha]_D^{25} = -79°$ (2.6 mg/ml in tetrahydrofuran) for peak a and $[\alpha]_D^{25} = +79°$ (1.9 mg/ml in tetrahydrofuran) for peak b.

† A 2-OH-CA formed by metabolism of CA by liver microsomes from MCA-treated male Sprague-Dawley rats was optically active ($\varphi_{258} = +3.9$ m°).

†† These compounds do not have the multiple simultaneous interactions with (*R*)-CSP required to permit separation of the enantiomers[22].

††† A 2-OH-MCA formed by metabolism of MCA by liver microsomes from MCA-treated male Sprague-Dawley rats was optically active ($\varphi_{258} = +4.8$ m°).

6.6:93.4 (enantiomeric purity 86.8%). For comparison, a 1-OH-MCA metabolite was obtained from the metabolism of MCA by liver microsomes from phenobarbital-treated rats. This 1-OH-MCA metabolite had an *R*/*S* ratio of 53.5:46.5 (enantiomeric purity 7% enriched in the *R* enantiomer) as indicated by CSP-HPLC analysis (Fig. 4B). The enantiomers of both 2-OH-CA and 2-OH-MCA were not resolved by the CSP-HPLC procedure described. However, a 2-OH-CA and a 2-OH-MCA obtained from the metabolism of CA and MCA respectively by liver microsomes from MCA-treated rats were found to be optically active (see footnotes of Table I). These results indicate that the cytochrome P-450-containing rat liver microsomal enzyme system has various degrees of stereoselectivity in catalyzing hydroxylation reactions at the 1- and the 2-methylene carbons of both CA and MCA.

Eight of the twelve enantiomeric cyclic alcohol derivatives used in this study were resolved with resolution values greater than 1.0 and two (4-OH-H_4BA and

10-OH-H_4BP) with resolution values less than 1.0 (Table I). Two compounds (2-OH-CA and 2-OH-MCA) were not resolved even when the percentage of solvent A in hexane was reduced to 1% and the flow-rate was lowered. For those resolved, the resolution value was increased when the percentage of solvent A in hexane was reduced. However, the increase in resolution was at the expense of considerably longer retention time and peak broadening.

According to the chiral recognition mechanisms proposed by Pirkle *et al.*[22], interactions with the (*R*)-CSP employed in this study cause the *S* enantiomers of 1-OH-H_4BA and 4-OH-H_4Ph to be more strongly retained than the *R* enantiomers. The absolute configurations of the most retained enantiomers have been assigned on the basis of the chiral recognition mechanisms[22]; these are indicated in Table I.

Inspection of the molecular models indicates that the enantiomers of 2-OH-CA and of 2-OH-MCA can not have the multiple simultaneous interactions with (*R*)-CSP required for stronger retention. Presumably this lack of interactions is the reason why the enantiomers of 2-OH-CA and of 2-OH-MCA were not resolved. The enantiomers of the other ten cyclic alcohols used in this study were all resolved. These ten cyclic alcohol derivatives have structures that allow the required multiple chiral interactions with the (*R*)-CSP and in each case the *S* enantiomer is predicted[22] to be the more retained one.

The absolute configurations of the enantiomeric cyclic alcohol derivatives in this study have not been established by a definitive method and the assignments of the absolute configurations indicated in this study should therefore be regarded as tentative. Current efforts are directed toward further extension of the general usefulness of the CSP-HPLC as a rapid and sensitive method in determining the optical purity and the absolute configuration of oxygenated chiral metabolites of polycyclic aromatic hydrocarbons.

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REFERENCES

1 A. H. Conney, *Cancer Res.*, 42 (1982) 4875, and references therein.
2 S. K. Yang, D. W. McCourt, J. C. Leutz and H. V. Gelboin, *Science*, 196 (1977) 1199.
3 S. K. Yang, D. W. McCourt and H. V. Gelboin, *Biochemistry*, 16 (1977) 3680.
4 D. R. Thakker, W. Levin, H. Yagi, S. Turujman, D. Kapadia, A. H. Conney and D. M. Jerina, *Chem. Biol. Interac.*, 27 (1979) 145.
5 S. K. Yang, *Drug Metab. Disp.*, 10 (1982) 205.
6 S. K. Yang, H. V. Gelboin, J. D. Weber, V. Sankaran, D. L. Fisher and J. F. Engel, *Anal. Biochem.*, 78 (1977) 520.
7 R. G. Harvey and H. Cho, *Anal. Biochem.*, 80 (1977) 540.
8 K. Nakanishi, J. Kasai, H. Cho, R. G. Harvey, A. M. Jeffrey, K. W. Jennette and I. B. Weinstein, *J. Amer. Chem. Soc.*, 99 (1976) 258.
9 D. R. Thakker, H. Yagi, H. Akagi, M. Koreeda, A. Y. H. Lu, W. Levin, A. W. Wood, A. H. Conney and D. M. Jerina, *Chem. Biol. Interac.*, 16 (1977) 281.

10 H. B. Weems and S. K. Yang, *Anal. Biochem.*, 125 (1982) 156.
11 P. P. Fu and S. K. Yang, *Biochem. Biophys. Res. Commun.*, 109 (1982) 927.
12 P. P. Fu and S. K. Yang, *Carcinogenesis*, 4 (1983) 979.
13 X.-C. Li, P. P. Fu, M. W. Chou and S. K. Yang, in M. Cooke and J. D. Anthony (Editors), *Polynuclear Aromatic Hydrocarbons: Formation, Analysis, and Measurement*, Battelle Press, Columbus, OH, 1983, pp. 809–818.
14 A. W. Wood, R. L. Chang, W. Levin, P. E. Thomas, D. Ryan, T. A. Stoming, D. R. Thakker, D. M. Jerina and A. H. Conney, *Cancer Res.*, 38 (1978) 3398.
15 W. Levin, M. K. Buening, A. W. Wood, R. L. Chang, D. R. Thakker, D. M. Jerina and A. H. Conney, *Cancer Res.*, 39 (1979) 3549.
16 C. S. Cooper, P. Vigny, M. Kindts, P. L. Grover and P. Sims, *Carcinogenesis*, 1 (1980) 855, and references therein.
17 W. H. Pirkle, D. W. House and J. M. Finn, *J. Chromatogr.*, 192 (1980) 143.
18 S. Krishnan, D. G. Kuhn and G. A. Hamilton, *J. Amer. Chem. Soc.*, 99 (1977) 8121.
19 R. D. Haworth and C. R. Marvin, *J. Chem. Soc.*, (1933) 1012.
20 P. P. Fu, J. Clark and A. Y. Huang, *J. Chem. Res. (S)*, 5 (1982) 121.
21 T. A. Stoming, W. Bornstein and E. Bresnick, *Biochem. Biophys. Res. Commun.*, 79 (1977) 461.
22 W. H. Pirkle, J. M. Finn, B. C. Hamper, J. Screiner and J. R. Pribish, in E. L. Iliel and S. Otsuka (Editors), *Asymmetric Reactions and Processes in Chemistry*, (ACS Symposium Series No. 185), American Chemical Society, Washington, DC, 1982, pp. 245–260.