Journal of Chromatography, 385 (1987) 293–298
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 997

REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF PHENOLIC DERIVATIVES OF BENZO[*a*]PYRENE, BENZ[*a*]ANTHRACENE, AND CHRYSENE WITH MONOMERIC AND POLYMERIC C_{18} COLUMNS*

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SUMMARY

The separation of monohydroxylated derivatives (phenols) of benzo[*a*]pyrene, benz[*a*]anthracene, and chrysene was studied by reversed-phase high-performance liquid chromatography using a monomeric Zorbax ODS column and a polymeric Vydac C_{18} column. The Vydac C_{18} column resolved the phenols of each hydrocarbon with a wide range of retention times than the Zorbax ODS column. Four K-region phenols of benzo[*a*]pyrene are either not separated or marginally separated on both monomeric and polymeric columns. Other K-region and non-K-region phenols of all three hydrocarbons can be separated by using the monomeric and polymeric columns in combination.

INTRODUCTION

Epoxides are initial products formed in the metabolism of polycyclic aromatic hydrocarbons (PAHs)[1,2]. The epoxidation reactions are catalyzed by microsomal cytochrome P-450 isozymes[1,2]. The metabolically formed epoxides are either enzymatically hydrated by microsomal epoxide hydrolase to *trans*-dihydrodiols or non-enzymatically rearranged to monohydroxylated (phenolic) products[1,2]. In order to elucidate the mechanism of enzyme-catalyzed epoxidation and hydration reactions, it is desirable to separate the phenolic products which are derived either from non-enzymatic rearrangement of the enzymatically formed epoxides or from acid-catalyzed dehydration of *trans*-dihydrodiol metabolites[2–4]. Earlier studies using a monomeric ODS column indicate that phenols of benzo[*a*]pyrene (BaP), benz[*a*]anthracene (BA), and chrysene (CR) (structures and numbering systems are shown in Figs. 1–3)

* The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences. The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals", Institute of Animal Resources, National Research Council, DHEW Pub. No. (NIH) 78-23.

0021-9673/87/$03.50

are poorly separated[3–8]. BaP phenols are not completely separated on a silica gel column by 3–5 passages in recycle chromatography[9].

In this study, we compare the separations of phenolic derivatives of BaP, BA, and CR by reversed-phase high-performance liquid chromatography (HPLC) using a monomeric Zorbax ODS column and a polymeric Vydac C_{18} column. The results indicate that the polymeric Vydac C_{18} column generally provides better separation of the phenolic derivatives. By using two columns in combination, non-K-region phenols of all three PAHs can be separated. The polymeric Vydac C_{18} column was recently used to separate BA *trans*-3,4- and 8,9-dihydrodiols[10] which were not separable on the monomeric Zorbax ODS column[5].

EXPERIMENTAL

Materials

Monohydroxylated derivatives of BaP, BA, and CR were obtained from the Chemical Repository of National Cancer Institute (Bethesda, MD, U.S.A.). A mixture of chrysene phenols was also obtained by incubation of chrysene with liver microsomes from phenobarbital-treated male Sprague–Dawley rats in the presence of a microsomal epoxide hydrolase inhibitor, 3,3,3-trichloropropylene 1,2-oxide, similar to the procedure described in ref. 4.

Chromatography

HPLC was performed using a Waters Assoc. (Milford, MA, U.S.A.) 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 or 280 nm) detector. Samples were injected via a Valco Model N60 loop injector (Houston, TX, U.S.A.). Retention times were recorded with a Hewlett-Packard Model 3390 integrator (Palo Alto, CA, U.S.A.). HPLC-grade solvents were purchased from Mallinckrodt (Paris, KY, U.S.A.).

The retention times of phenolic derivatives were determined at ambient temperature using either a monomeric Zorbax ODS column (250 mm × 4.6 mm I.D.; DuPont, Wilmington, DE, U.S.A.) or a polymeric Vydac C_{18} column (250 mm × 4.6 mm I.D.; The Separations Group, Hesperia, CA, U.S.A.). The elution solvents were methanol–water (3:1, v/v) for the separation of BA and CR phenols and methanol–water (4:1, v/v) for the separation of BaP phenols, respectively. The flow-rate was 1.2 ml/min in every case. After the retention time of each phenol was established, a mixture of two to four isomeric phenols was chromatographed in order to establish the relative elution order and reproducibility of retention times.

RESULTS

The phenolic derivatives of BaP were eluted between 11.1 and 15.2 min from the monomeric ODS column (Fig. 1). Under the chromatographic conditions used in this study, 1-OH-BaP and 10-OH-BaP were eluted together with 3-OH-BaP. However, these three phenols were separated on the polymeric Vydac C_{18} column (Fig. 1). BaP phenols were eluted between 8.8 and 30.7 min from the polymeric Vydac C_{18} column with a much wider spread of retention times than that observed with the

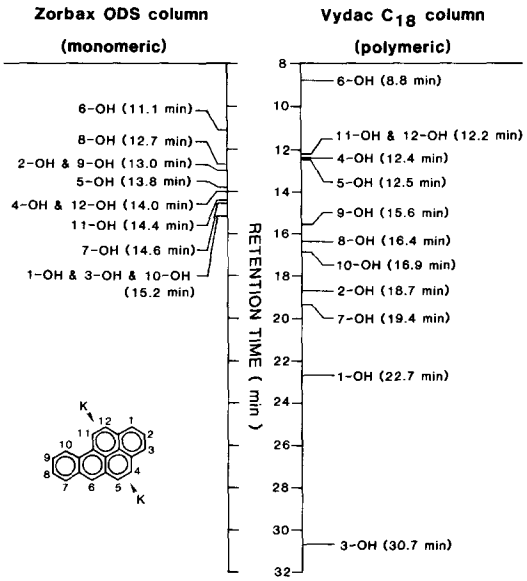

Fig. 1. Retention times of BaP phenols on the monomeric Zorbax ODS and the polymeric Vydac C_{18} columns. The eluent was methanol–water (4:1, v/v) at 1.2 ml/min.

monomeric Zorbax ODS column (Fig. 1). Two pairs of K-region phenols (4- and 5-OH-BaP and 11- and 12-OH-BaP) could not be separated on either of the two columns used. Other BaP phenols could be separated by using two columns in combination (Fig. 1).

Ten of twelve BA phenols were available for this study, and they were eluted

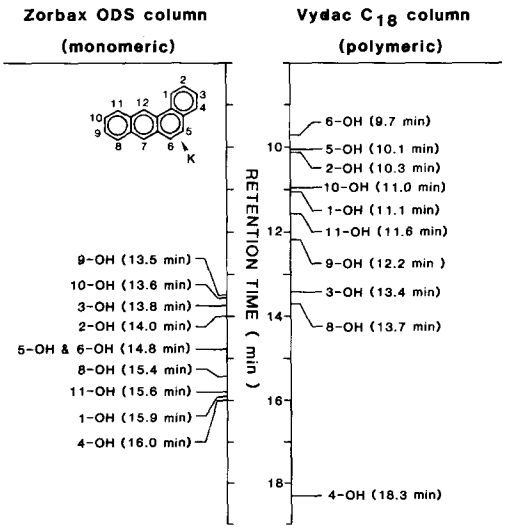

Fig. 2. Retention times of BA phenols on the monomeric Zorbax ODS and the polymeric Vydac C_{18} columns. The eluent was methanol–water (3:1, v/v) at 1.2 ml/min.

within a narrow range (2.5 min) of retention times from the monomeric Zorbax ODS column and more widely (9.0 min) from the polymeric Vydac C_{18} column (Fig. 2). Similar to the K-region phenols of BaP, the K-region phenols of BA (5-OH-BA and 6-OH-BA) were not separated on the Zorbax ODS column and were only marginally separated on the Vydac C_{18} column. All other BA phenols were separated from K-region phenols, and they can be all separated from one another by using two columns in combination.

Chrysene is a symmetric molecule and has a total of six possible phenolic derivatives. 5-Hydroxychrysene was not available for this study. Five of the six possible phenols of chrysene were also more efficiently separated on the Vydac C_{18} column with a wider range of retention time than on the Zorbax ODS column (Fig. 3). Perhaps because they were fewer in number and sufficiently dissimilar among one another, all five chrysene phenols can be separated on a Vydac C_{18} column. Only 2-OH-CH and 3-OH-CR were not separated on the Zorbax ODS column.

A mixture of phenolic metabolites was obtained by incubation of chrysene with liver microsomes from phenobarbital-treated rats in the presence of a microsomal epoxide hydrolase, 3,3,3-trichloropropylene 1,2-oxide, and was isolated by normal-phase HPLC[4]. These phenols were analyzed on the polymeric Vydac C_{18} column (Fig. 4). Due to the presence of the epoxide hydrolase inhibitor, the metabolically formed 1,2-, 3,4-, and 5,6-epoxides were not hydrated to dihydrodiol metabolites and were mostly rearranged non-enzymatically to phenolic products. The results shown in Fig. 4 indicated that the metabolically formed 1,2-epoxide was rearranged to 1-OH-CR (*ca.* 85%) and 2-OH-CR (*ca.* 15%), 3,4-epoxide was rearranged to 3-OH-CR (*ca.* 75%) and 4-OH-CR (*ca.* 25%), and 5,6-epoxide was probably rearranged entirely to 6-OH-CR. Since 5-OH-CR was not available for this study, we could not rule out the possibility that 5-OH-CR, a possible non-enzymatic product

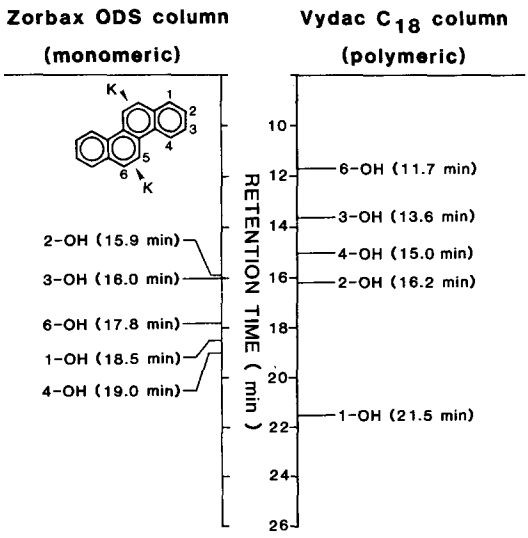

Fig. 3. Retention times of CR phenols on the monomeric Zorbax ODS and the polymeric Vydac C_{18} columns. The eluent was methanol–water (3:1, v/v) at 1.2 ml/min.

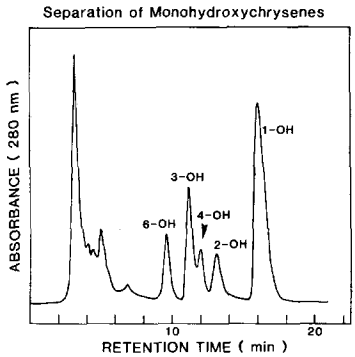

Fig. 4. Reversed-phase HPLC analysis of phenolic products formed in the incubation of chrysene by liver microsomes from phenobarbital-treated male Sprague–Dawley rats in the presence of a microsomal epoxide hydrolase inhibitor (see Materials and methods for details). A polymeric Vydac C_{18} column was used. The eluent was methanol–water (31:9, v/v) at 1.2 ml/min.

formed in the rearrangement of 5,6-epoxide, was contaminated with one of the five authentic phenolic compounds.

DISCUSSION

Wise *et al.*[11] reported that the shape, particularly the length-to-breadth ratio, is an important parameter that determines the elution order of unsubstituted and methyl-substituted PAH in reversed-phase HPLC on either a monomeric or a polymeric C_{18} column. The retention time increases with increasing length-to-breadth ratio. The elution order of a series of methyl-substituted PAH are reliably predicted from the calculated length-to-breadth ratios[11].

When a methyl group is replaced with a hydroxyl group, the relative length-to-breadth ratios of a series of PAH are presumably unchanged. On the assumption that the length-to-breadth ratios are the same for two compounds with either a methyl or a hydroxyl group at the same position of a PAH, the elution orders of phenolic derivatives of BaP, BA, and CR should be similar to those observed for the methylated BaP, BA, and CR, respectively (ref. 11 and Table I). Comparisons of the results in Figs. 1–3 and Table I indicate that the elution orders of phenolic derivatives of BaP, BA, and CR on either the Zorbax ODS column or the Vydac C_{18} column cannot be predicted by length-to-breadth ratios alone. Replacement of a methyl group with a hydroxyl group increases the water solubility of a PAH, thus affecting the partition coefficient of the solute between stationary phase and mobile phase. The elution order of a series of isomeric phenolic derivatives is probably determined by a combination of factors, such as length-to-breadth ratio, water solubility, and lipid/water partition coefficient.

The results of this study can be applied to many areas of study including:

(i) Analysis of the relative amount of various phenolic products formed in the *in vitro* and *in vivo* metabolism of BaP, BA, and CR (*e.g.*, Fig. 4).

(ii) Determination of the relative amount of two phenolic products formed in the non-enzymatic rearrangement of an epoxide[3,4].

TABLE I

RELATIVE RETENTION TIMES OF ISOMERIC PHENOLIC DERIVATIVES OF BENZO[*a*]PYRENE, BENZ[*a*]ANTHRACENE, AND CHRYSENE ON REVERSED-PHASE HPLC PREDICTED BY LENGTH-TO-BREADTH RATIOS*

Benzo[a]pyrene
5-OH < 11-OH < 10-OH < 6-OH < 3-OH ~ 7-OH < 1-OH ~ 9-OH < 2-OH < 8-OH
Benz[a]anthracene
6-OH < 5-OH < 11-OH < 1-OH < 2-OH ~ 7-OH < 12-OH < 8-OH < 10-OH < 4-OH < 9-OH ~ 3-OH
Chrysene
6-OH < 5-OH ~ 4-OH < 3-OH < 1-OH < 2-OH

* If the relative retention times strictly follow the relative length-to-breadth ratios, then it is predicted that the larger the value of length-to-breadth ratio the longer will be the retention time[11].

(iii) Determination of the relative amount of two phenolic products formed in the acid-catalyzed dehydration of either a *cis*- or a *trans*-dihydrodiol derivative of a PAH[3,4].

(iv) Determination of the position of water attack in microsomal epoxide hydrolase-catalyzed hydration of an enantiomeric epoxide[3,4].

(v) Determination of the absolute configuration of a non-K-region epoxide formed in the metabolism of a PAH under molecular oxygen-18[3,4].

ACKNOWLEDGEMENTS

This work was supported by U.S. Public Health Service Grant No. CA29133 and Uniformed Services University of the Health Sciences Protocol No. RO7502.

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