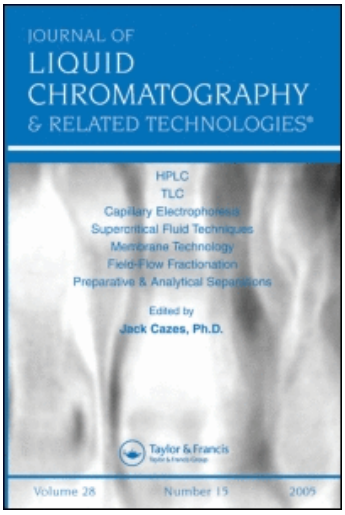

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The Liquid Chromatographic Separation of Metabolites of Benzo[a]pyrene with γ-Cyclodextrin as a Mobile Phase Additive

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THE LIQUID CHROMATOGRAPHIC SEPARATION OF METABOLITES OF BENZO[a]PYRENE WITH γ-CYCLODEXTRIN AS A MOBILE PHASE ADDITIVE

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ABSTRACT

The effects of γ-cyclodextrin as a mobile phase additive on the retention characteristics of fourteen metabolites of benzo[a]pyrene were investigated with reversed-phase liquid chromatography. It was possible to separate dihydrodiols, diones, and monohydroxyl-benzo[a]pyrenes as individual classes of metabolites. Also, with γ-cyclodextrin in the mobile phase, monohydroxyl-benzo[a]pyrene isomers that were very difficult to separate were readily separated. For example, 6-hydroxyl-benzo- [a]pyrene was separated from 12-hydroxyl-benzo[a]pyrene and 9-hydroxyl-benzo[a]pyrene was separated from 2-hydroxyl-benzo- [a]pyrene. The four tetrols were readily separated with shorter retention times using 4.0 mM of γ-cyclodextrin in a methanol-water (55:45) mobile phase. Overall, the results showed that γ-cyclodextrin was more effective in separating the metabolites than was β-cyclodextrin.

INTRODUCTION

Benzo[a]pyrene (B[a]P) is a common environmental pollutant, and it is considered to be a carcinogen (1-4). The microsomal enzyme complex, aryl

hydrocarbon hydroxylase (AHH), and metabolically related enzymes convert B[a]P to epoxides, phenols, dihydrodiols, diones, and water-soluble conjugates (1-3). It has been shown that reversed-phase high-performance liquid chromatography (HPLC) is a sensitive and rapid technique for the isolation and separation of B[a]P metabolites (4-14). As an example, Selkirk et al. (7) analyzed the metabolites of benzo[a]pyrene from human liver microsomes and human lymphocytes using HPLC. In 1979, Tjessum and Stegeman (15) used an alkaline gradient system to improve the separation of monohydroxyl-B[a]P isomers using a highly alkaline eluent consisting of a series of alkylamines in a linear gradient mode. This procedure, however, did not separate other B[a]P metabolites such as, tetrols, diones, and dihydrodiols. Also, the high pH may have damaged the column. Elnenaey and Schoor (6) developed a method to separate twelve isomeric monohydroxyl-B[a]P by using HPLC with fluorescence detection and various sequences of methanol-water gradients. They showed that with this approach many isomers had almost identical retention times (6). Recently, Rozbeh and Hurtubise (5) reported a systematic method for optimizing binary and ternary mobile phases for the separation of a complex mixture of fourteen metabolites of benzo[a]pyrene using HPLC.

The use of cyclodextrins in chromatography is important, and it has been discussed in several books and reviews (16-18) Cyclodextrins (CD) are tours-shaped molecules with α-1,4 linkage of glucopyranose units. The interior of the cyclodextrin cavity is fairly nonpolar due to the glucosidic oxygen and hydrogen atoms in the cavity (18). The most frequently studied cyclodextrins are α-, β-,

and γ-CD, which have different cavity diameters (18). Cyclodextrins molecules can form complexes with several types of compounds. For example, Zukowski et al. (19) investigated the use of mobile phases containing α-CD or β-CD for the separation of disubstituted benzene derivatives with a C_{18} column. Their results indicated that only β-CD imposes a distinct selectivity toward ortho, meta, and para isomers with reversed-phase systems, which resulted in a complete separation of the isomers (19). Also, the effects of different concentrations of β-CD on the retention and selectivity of various aromatic isomers have been investigated (20).

Frequently, γ-CD is used in chromatography as a complexing reagent for large molecules. For example, γ-CD chemically bonded to silica gel is a highly selective stationary phase for the HPLC separation of C_{60} and C_{70} fullerenes (21). Hurtubise and co-worker (22) showed that polycyclic aromatic hydrocarbons, nitrogen heterocycles, and hydroxyl aromatics of different sizes will interact with β-CD to different extents, and this permits the separation of some of these compounds from their isomers. In a previous paper (23), we described the chromatographic separation of fourteen metabolites of benzo[a]pyrene in reversed-phase HPLC with β-CD as a mobile phase additive. Woodberry et al. (24) demonstrated that a bonded γ-CD column with methanol-water mobile phase is capable of separating benzo[a]pyrene-trans-7,8-dihydrodiol from benzo[a]pyrene-trans-9,10-dihydrodiol with better selectivity than β-CD bonded phases. γ-Cyclodextrin has not been used as extensively as a mobile phase modifier because of its expense. However, Roussel and Favrou (25-26) described the chromatographic separation of eight enantiomers using β- or γ-CD as a mobile

phase modifier. They concluded that γ-CD shows better selectivity toward the enantiomers, while weaker complexes were formed with β-CD.

The aim of this paper is to demonstrate the practical significance of γ-CD as a mobile phase additive in the chromatographic separation of a complex mixture of fourteen metabolites of benzo[a]pyrene. Also, the work reported here considers the chromatographic behavior of different pairs of metabolites in methanol-water mobile phases modified by γ-CD. Also, comparisons were made between γ-CD versus β-CD for the retention characteristics of pairs of metabolites, and a complex mixture of fourteen metabolites. Moreover, the effects of the cavity size of β-CD versus γ-CD on the selective separation of some of the metabolites were emphasized.

EXPERIMENTAL

Apparatus

HPLC was performed with a Waters liquid chromatograph equipped with model 6000A pump (Waters Assoc., Milford, MA, U.S.A.), a U6K injector, a dual channel free-standing model 440 UV detector set at 254 nm, and a linear 1200 dual channel, 5.0 V recorder (Linear Instruments Co. Concord, CA). A model FIAtron heating block (Oconomowoc, WI, U.S.A.) constant temperature control system was used to keep the temperature of the column at 25 ± 0.1°C. Separations were carried out with a 5-μm Baker-bond C_{18} (250 mm x 4.6 mm i.d.) from J.T.Baker (Pillipsburg, NJ, U.S.A.).

Reagents

Methanol (MeOH) and water were HPLC grade and were purchased from J.T.Baker Inc. (Pillipsburg, NJ). The γ-CD samples were obtained from American Maize-Products Company (Amaizo, Hammond, IN). The benzo[a]pyrene (B[a]P) metabolites were purchased from the National Cancer Institute (NCI) repository at Midwest Research Institute (MRI, Kansas City, MO).

Procedures

The mobile phases were prepared as follows. Methanol and water were prefiltered through a membrane 0.45 μm filter (Millipore type HA). An accurate amount of γ-CD, which was vacuum dried at 75° C for 8 hr, was dissolved in an appropriate amount of water. Then, a specific quantity of methanol was added to the γ-CD solution. After complete dissolution of the γ-CD at room temperature, an amount of MeOH:H_2O, which was the same composition used to dissolve the γ-CD, was added to the volumetric flask to bring the volume to 1000 ml. Solutions of 0.1 mg/ml for individual metabolites and 0.01 mg/ml for the mixture of fourteen standards were prepared in methanol. To assure stability, the solutions were stored under nitrogen gas at -15°C and in the dark. The amount of the sample injected into the liquid chromatograph was 3-4 μl for individual metabolites and 10 μl for the mixture of fourteen compounds. All chromatograms were obtained at 25°C. The flow rate was 1.0 ml/min. The void volume was determined by injection of a methanol solution of potassium nitrite. The capacity factors were calculated from the expression, $k' = (t_R - t_o)/t_o$, where t_R is the

retention time of the solute and t_o is the retention time of potassium nitrite. Triplicate injections for the metabolites of benzo[a]pyrene were carried out using a mobile phase without and with γ-CD. The values of k′ in Table 1 and Table 2 are the averages from these injections.

RESULTS AND DISCUSSION

Mobile Phases and γ-CD Concentrations

A preliminary investigation with nine different concentrations of methanol-water indicated that by decreasing the methanol content of the mobile phase the k′ values increased for each individual metabolites. Therefore, lower concentrations of methanol resulted in larger elution times for the metabolites, which is undesirable. Thus, two different compositions of binary mobile phases were employed in this investigation to determine the effects of γ-CD on the retention characteristics of the fourteen metabolites. The methanol-water (MeOH:H_2O, V:V) mobile phases used were 75:25 and 80:20 with different concentrations of γ-CD. The maximum analytical concentration of γ-CD in MeOH:H_2O (75:25) was 3.5 mM and for the 80:20 composition it was 2.5 mM.

The Effects of Mobile Phase Additives on the Retention Characteristics of the Metabolites

It is known that the hydrophobic interactions and dispersion forces operating between the bonded alkyl moiety of the stationary phase and the nonpolar part a molecule play an important role in determining the retention

characteristics of the sample in HPLC (27). Since hydrophobic interactions are affected by several factors such as the chain length and the amount of bonded alkyl moieties in the stationary phase, as well as the type and content of the organic modifier in the mobile phase, the addition of cyclodextrin to the mobile phase is expected to cause changes in the capacity factors of the solutes. It is also known that both the molecular size and geometry of benzo[a]pyrene are such that benzo[a]pyrene will not fit entirely into the cavity of a single β-CD molecule (24,28,29). Also, Patony and Warner (29) showed that a benzo[a]pyrene complex exhibits a significant induced ellipticity in the presence of γ-CD, while α-CD or β-CD did not produce a major induced circular dichroism signal. β-CD is comprised of seven glucose units as opposed to the eight glucose units of γ-CD (16-18). Consequently, the interior cavity of γ-CD is larger than the cavity of β-CD (18). Also, two factors that are important for inclusion of a solute into a cyclodextrin cavity are the size and geometry of the compound. γ-CD can accommodate larger molecules than β-CD, and this would lead to size selectivity with the two cyclodextrins. An earlier investigation from this laboratory showed that twelve metabolites from a complex mixture of fourteen compounds can be separated by an optimum binary mobile phase of acetonitrile-water (ACN:H_2O) (65:35) and an optimum ternary mobile phase containing acetonitrile-methanol-water (ACN:MeOH:H_2O) (17:50:33) (5). However, with methanol-water (81.75:18.25) two pairs of isomers, namely, 2-OH-B[a]P and 9-OH-B[a]P, and tetrol I-2 and tetrol II-2 could not be resolved into their individual pairs. With other MeOH:H_2O binary mobile phase compositions, two pairs of monohydroxyl-

TABLE 1

The k'-Values of the Metabolites of Benzo[a]pyrene for Methanol-Water (80:20) with Different Concentrations (mM) of γ-CD

		γ-CD (mM)				
No.	Compound[a]	0.0	1.0	1.5	2.0	2.5
1	Tetrol I-1	0.55	0.49	0.44	0.41	0.38
2	Tetrol II-1	0.65	0.53	0.49	0.48	0.45
3	Tetrol I-2	0.66	0.62	0.55	0.52	0.50
4	Tetrol II-2	0.74	0.76	0.71	0.67	0.62
5	B[a]P-t-9,10-dihydrodiol	0.66	0.62	0.59	0.57	0.51
6	B[a]P-t-7,8-dihydrodiol	2.31	2.17	2.15	2.05	1.95
7	B[a]P-1,6-dione	7.6	7.15	6.45	6.02	5.66
8	B[a]P-3,6-dione	8.95	8.4	7.62	7.2	6.74
9	12-OH-B[a]P	9.96	8.6	8.05	7.45	6.89
10	9-OH-B[a]P	12.7	10.4	9.96	9.46	9.05
11	2-OH-B[a]P	12.7	11.7	11.4	10.5	9.95
12	7-OH-B[a]P	14.5	13.2	12.6	11.8	10.8
13	3-OH-B[a]P	18.8	15.0	14.4	12.7	11.0
14	6-OH-B[a]P	10.8	9.26	8.72	8.31	7.36

[a] 1. Benzo[a]pyrene-r-7,t-8,9,c-10-tetrahydrotetrol (I-1)
2. Benzo[a]pyrene-r-7,t-8,c-9,t-10-tetrahydrotetrol (II-1)
3. Benzo[a]pyrene-r-7,t-8,9,10-tetrahydrotetrol (I-2)
4. Benzo[a]pyrene-r-7,t-8,c-9,10-tetrahydrotetrol (II-2)
5. Benzo[a]pyrene-trans-9,10-dihydrodiol
6. Benzo[a]pyrene-trans-7,8-dihydrodiol
7. Benzo[a]pyrene-1,6-dione
8. Benzo[a]pyrene-3,6-dione
9. 12-Hydroxybenzo[a]pyrene
10. 9-Hydroxybenzo[a]pyrene
11. 2-Hydroxybenzo[a]pyrene
12. 7-Hydroxybenzo[a]pyrene
13. 3-Hydroxybenzo[a]pyrene
14. 6-Hydroxybenzo[a]pyrene

The structures of the compounds are given in Ref. 5.

B[a]P isomers, 12-OH-B[a]P and 6-OH-B[a]P, and 2-OH-B[a]P and 9-OH-B[a]P were the most difficult metabolites to separate (5).

In a follow-up paper, Rozbeh and Hurtubise (23) employed several concentrations of β-CD as a mobile phase additive to improve the separation of the two most difficult to separate pairs of isomers. Substantial improvements were obtained for the separation of a mixture of fourteen metabolites with MeOH:H_2O (65:35), ACN:H_2O (65:35), and ACN:MeOH:H_2O (50:17:33) with β-CD. The isomers, 6-OH-B[a]P and 12-OH-B[a]P, were separated with a relatively high concentration of β-CD in the methanol-water mobile phase (23). However, one pair of metabolites, 2-OH-B[a]P and 9-OH-B[a]P, could not be resolved with β-CD in methanol-water(23). Also, large capacity factors for monohydroxyl-B[a]P and low solubility of β-CD, at lower concentrations of methanol were a problem (23). Therefore, in this work, the effects of different concentrations of γ-CD in the mobile phase on the retention of the metabolites were examined.

Table 1 shows the effects of different concentrations of γ-CD on the capacity factors of fourteen metabolites of benzo[a]pyrene with MeOH:H_2O (80:20). The k′ values of the metabolites for MeOH:H_2O (75:25) with various concentrations of γ-CD are shown in Table 2. Comparison of the data from the separation of benzo[a]pyrene metabolites with β-CD in MeOH:H_2O mobile phases (23) with results from this investigation indicated that the capacity factors of all fourteen metabolites decreased to a greater extent in the presence of γ-CD compared to β-CD. For example, the k′ value of 3-OH-B[a]P changed from 18.8 with no γ-CD in the mobile phase to 11.0 with 2.5 mM γ-CD in MeOH:H_2O

TABLE 2

The k′-Values of the Metabolites of Benzo[a]pyrene for Methanol-Water (75:25) with Different Concentrations (mM) of γ-CD

	γ-CD (mM)					
Solute[a]	0.0	1.0	1.5	2.0	3.0	3.5
Tetrol I-1	0.61	0.58	0.56	0.49	0.43	0.40
Tetrol II-1	0.80	0.66	0.62	0.59	0.58	0.56
Tetrol I-2	0.84	0.73	0.69	0.61	0.60	0.57
Tetrol II-2	1.08	0.94	0.92	0.83	0.77	0.74
B[a]P-t-9,10-dihydrodiol	0.95	0.85	0.75	0.74	0.68	0.58
B[a]P-t-7,8-dihydrodiol	3.94	3.68	3.22	3.06	2.80	2.5
B[a]P-1,6-dione	9.1	8.9	8.6	8.12	7.62	7.3
B[a]P-3,6-dione	11.0	10.7	10.2	9.64	9.5	8.57
12-OH-B[a]P	11.9	10.7	10.2	9.9	9.03	8.90
9-OH-B[a]P	14.7	13.4	13.0	11.9	11.3	10.7
2-OH-B[a]P	15.1	14.7	14.1	13.5	12.9	12.3
7-OH-B[a]P	20.9	19.0	17.9	16.6	15.7	14.8
3-OH-B[a]P	23.2	21.2	20.3	19.6	19.1	18.0
6-OH-B[a]P	11.9	11.4	11.1	11.0	9.74	9.50

[a] See the footnote of Table 1 for the full names of the compounds. The structures of the compounds are given in Ref. 5.

(80:20) (Table 1). In contrast, the k′ value of 3-OH-B[a]P decreased from 24.0 with no β-CD to only 22.0 with 3.5 mM β-CD in the MeOH:H_2O (70:30) (23). These results clearly indicate that the larger decrease in the k′ values of the metabolites caused by the addition of γ-CD in the mobile phase, resulted from the weakening of the hydrophobic interaction between the solutes and stationary phase.

Also, the ability of γ-CD to form a stronger complex with a given metabolite compared to β-CD was apparent. In addition, the higher solubility of γ-CD compare to β-CD permitted complex formation to occur more readily compared to β-CD (17-18). It is logical that β-CD exhibited a smaller effect on the retention times of the metabolites because of its cavity size (~7.8 Å). This would permit only partial inclusion complex formation with the nonpolar part of the B[a]P molecule (30). In contrast, the larger cavity of the γ-CD permits greater interaction with the B[a]P isomers.

Separation of Structural Isomers

The separation of isomers in HPLC is of great importance. In this work, separate mixtures of four tetrols, two dihydrodiols, two diones, 12-OH-B[a]P and 6-OH-B[a]P, 9-OH-B[a]P and 2-OH-B[a]P, and 7-OH-B[a]P and 3-OH-B[a]P were prepared. In previous work, a mixture of four tetrols could be separated with MeOH:H_2O (55:45) with 4.0-5.0 mM of β-CD (23). In this work, a similar investigation for the tetrols with different concentrations of γ-CD in the mobile phase was undertaken. A larger reduction in the k′ values of the four tetrols was obtained with γ-CD compare to β-CD (23). The decrease in the retention times of tetrols with 4.0 mM of γ-CD in MeOH:H_2O (55:45) resulted in the sharpening of the bands and a reasonably good separation of the four stereoisomers. Therefore, it was concluded that MeOH:H_2O (55:45) with 4.0 mM γ-CD was a better mobile phase for the separation of this class of metabolites for the β-CD and γ-CD mobile phases investigated.

TABLE 3

Selectivity Factors (α) for Some Pairs of Benzo[a]pyrene Metabolites in MeOH:H_2O (70:30) Without and With 3.0 mM β-CD

	α-Values[b]	
Pairs of Metabolites[a]	0.0	3.0
B[a]P-t-9,10-dihydrodiol and B[a]P-t-7,8-dihydrodiol	4.38	4.73
B[a]P-1,6-dione and B[a]P-3,6-dione	1.21	1.12
12-OH-B[a]P and 6-OH-B[a]P	1.00	1.05
9-OH-B[a]P and 2-OH-B[a]P	1.01	1.02
7-OH-B[a]P and 3-OH-B[a]P	1.05	1.15

[a] See the footnote of Table 1 for the full names of the metabolites.

[b] α-values were calculated from the equation $\alpha = k_2'/k_1'$.

TABLE 4

Selectivity Factors (α) for Some Pairs of Benzo[a]pyrene Metabolites in MeOH:H_2O (75:25) Without and With 3.0 mM γ-CD

	α-Values[b]	
Pairs of Metabolites[a]	0.0	3.0
B[a]P-t-9,10-dihydrodiol and B[a]P-t-7,8-dihydrodiol	4.15	4.11
B[a]P-1,6-dione and B[a]P-3,6-dione	1.20	1.25
12-OH-B[a]P and 6-OH-B[a]P	1.00	1.08
9-OH-B[a]P and 2-OH-B[a]P	1.02	1.14
7-OH-B[a]P and 3-OH-B[a]P	1.11	1.22

[a] See the footnote of Table 1 for the full names of the metabolites.

[b] α-values were calculated from the equation $\alpha = k_2'/k_1'$.

The data in Tables 3 and 4 compare the selectivity factors (α) for several pairs of metabolites obtained with MeOH:H_2O (70:30) containing 3.0 mM β-CD and MeOH:H_2O (75:25) with 3.0 mM γ-CD. These mobile phases were compared because with MeOH:H_2O (70:30) 3.0 mM of β-CD can be dissolved, which is the same as the concentration of γ-CD in MeOH:H_2O (75:25) (23). With larger concentrations of methanol, the solubility of β-CD decreases (18). For example, with MeOH:H_2O (75:25), only 2.5 mM of β-CD completely dissolved in the mobile phase. The capacity factors were reduced to a larger degree, and the selectivity increased for the two diones with γ-CD in the mobile phase compare to β-CD (compare Table 3 and 4). For example, the α-values for the two diones increased from 1.12 with 3.0 mM of β-CD to 1.25 with 3.0 mM γ-CD in methanol-water mobile phases (Table 3 and Table 4). The two diones can be separated with γ-CD or β-CD in the methanol-water mobile phases, but with γ-CD shorter retention times are obtained. It is shown, that in all but one case, the α-values for all pairs of metabolites increased with γ-CD in methanol-water mobile phases.

A substantial decrease in the capacity factors of monohydroxyl-B[a]P metabolites was obtained with γ-CD versus β-CD in methanol-water mobile phases (Table 1 and Table 2, see reference 23). A mixture of 6-OH-B[a]P and 12-OH-B[a]P showed only one peak with MeOH:H_2O (75:25) (Table 2) (Figure 1). However, the two components were resolved with γ-CD in MeOH:H_2O (Figure 2). For example, the α-value of 12-OH-B[a]P and 6-OH-B[a]P increased from 1.0 without γ-CD to 1.08 with 3.0 mM γ-CD in MeOH:H_2O (75:25) (Table 4). The

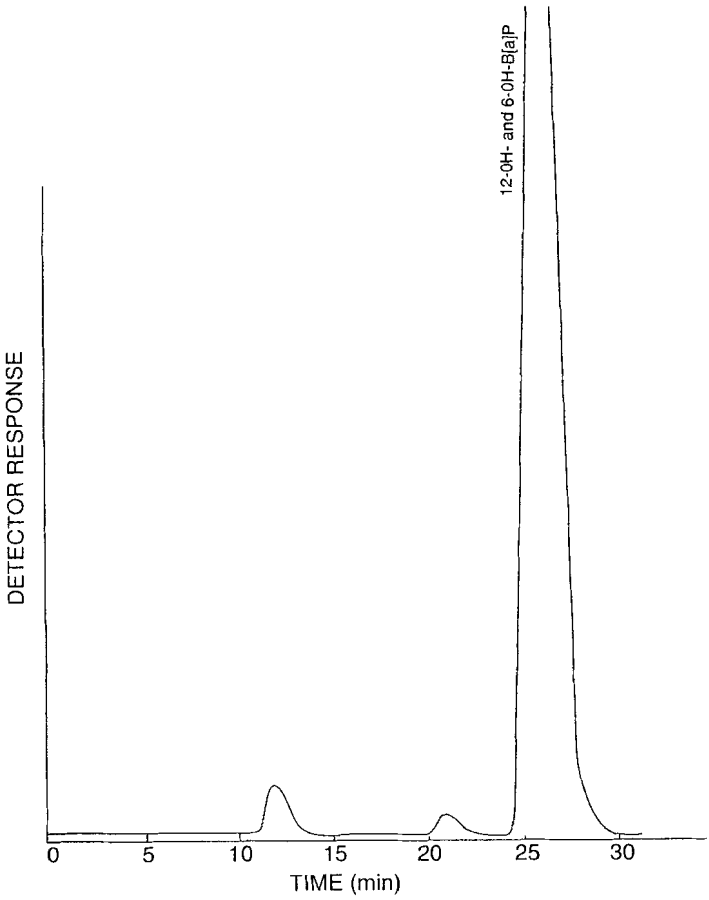

FIGURE 1. Chromatogram of 6-OH-B[a]P and 12-OH-B[a]P obtained with MeOH:H_2O (75:25). The full names of the compounds are given in the footnote of Table 1. The smaller peaks in the chromatogram are due to impurities.

addition of γ-CD significantly reduced the k′ values of these two metabolites and improved the efficiency of separation compared to the mobile phase without cyclodextrin (Table 2). It was reported earlier that a relatively high concentration of β-CD was also capable of separating these two metabolites. For example, the

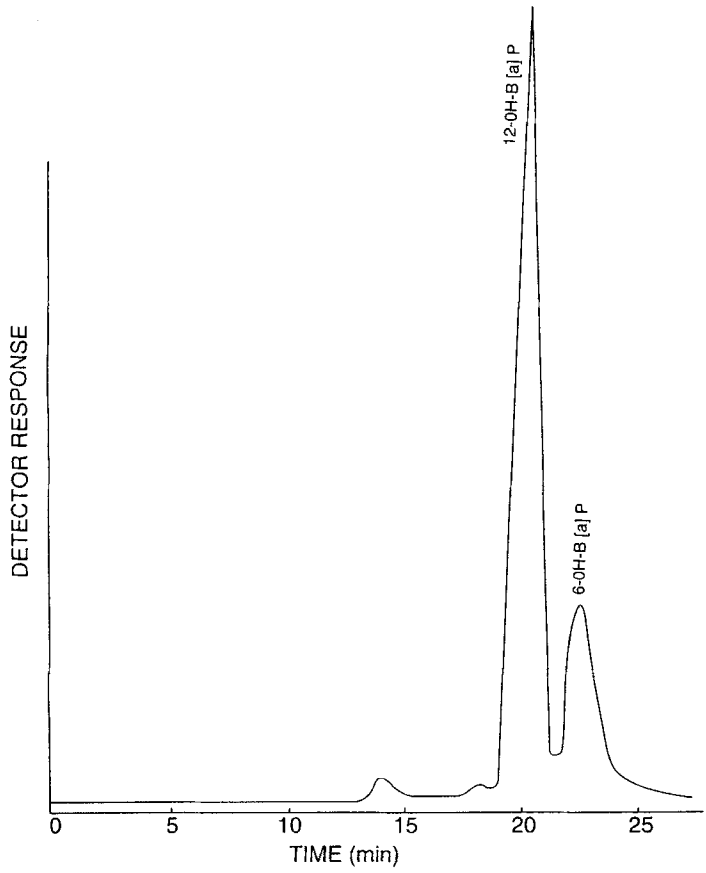

FIGURE 2. Chromatogram of 6-OH-B[a]P and 12-OH-B[a]P obtained in MeOH:H_2O (75:25) with 3.5 mM γ-CD. The full names of the compounds are given in the footnote of Table 1. The smaller peaks in the chromatogram are due to impurities.

capacity factors of 12-OH-B[a]P and 6-OH-B[a]P with 3.5 mM of β-CD in MeOH:H_2O (70:30) were 16.4 and 17.2, respectively (23). However, as Table 2 shows, the capacity factors are much smaller with 3.5 mM γ-CD, which illustrates one of the advantages of using γ-CD for these compounds.

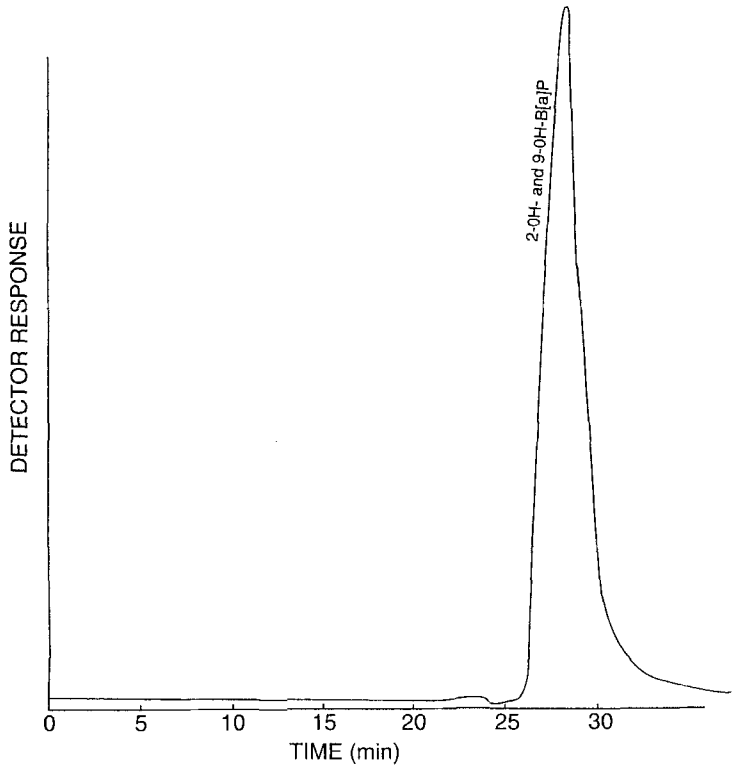

FIGURE 3. Chromatogram of 2-OH-B[a]P and 9-OH-B[a]P obtained with MeOH:H_2O (80:20). The full names of the compounds are given in the footnote of Table 1.

Figures 3 and 4 shows the chromatograms for 2-OH-B[a]P from 9-OH-B[a]P in methanol-water (80:20) with and without γ-CD. In the absence of γ-CD, the chromatographic bands of the two metabolites overlapped severely and no separation was achieved (Figure 3). With β-CD in the mobile phase, no separation was acquired with methanol-water for these two isomers (23). In contrast, with γ-CD in the mobile phase, the two metabolites were easily separated and baseline

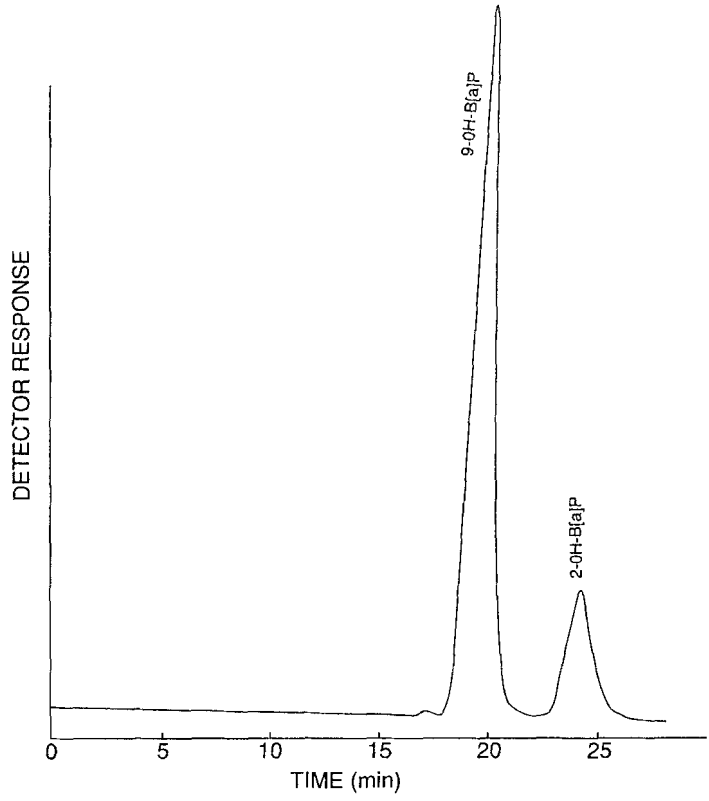

FIGURE 4. Chromatogram of 2-OH-B[a]P and 9-OH-B[a]P obtained in MeOH:H_2O (80:20) with 1.7 mM γ-CD. The full names of the compounds are given in the footnote of Table 1.

resolution was obtained (Figure 4, Table 1). Similar results were obtained in MeOH:H_2O (75:25) with γ-CD in the mobile phase for 2-OH-B[a]P and 9-OH-B[a]P (Table 2). This was due mainly to the greater interaction between γ-CD and 9-OH-B[a]P, which resulted in a larger decrease in the k′ values for this metabolite. The 2-OH-B[a]P and 9-OH-B[a]P isomers were the most difficult

pairs of metabolites to separate with methanol-water mobile phases (5,23). A previous investigation indicated that the optimization procedure with MeOH:H_2O mobile phases resulted in the separation of all pairs of metabolites, except for the 2-OH-B[a]P from 9-OH-B[a]P (5). Also, addition of β-CD as a mobile phase modifier resulted in a very good separation of twelve of the fourteen metabolites, except for the 2-OH-B[a]P from 9-OH-B[a]P (23).

Effects of γ-CD Concentration on the Separation of a Complex Mixture of Metabolites

As the data in Tables 1 and 2 show, the tetrols and B[a]P-t-9,10-dihydrodiol have very small k' values, and it was not possible to separate these compounds completely. However, a mixture of the four tetrols was completely separated with good baseline resolution in MeOH:H_2O (55:45) with 4.0 mM of γ-CD. Thus, for investigating a complex mixture of the metabolites with the mobile phase systems in Table 1 and 2, it was decided to omit the tetrols in this mixture. With γ-CD in methanol-water, the retention times of all the metabolites of B[a]P decreased substantially compare to β-CD (Table 1 and Table 2, see reference 23). Figure 5 shows a chromatogram of a mixture of ten metabolites of benzo[a]pyrene. The chromatogram indicates a successful separation of the ten B[a]P metabolites is possible with MeOH:H_2O using γ-CD as a mobile phase modifier. Three classes of compounds were completely separated in MeOH:H_2O (80:20) with 1.7 mM of γ-CD (Figure 5). These were dihydrodiols, diones, and monohydroxyl-B[a]P. If the tetrols were present in the mixture, they would have

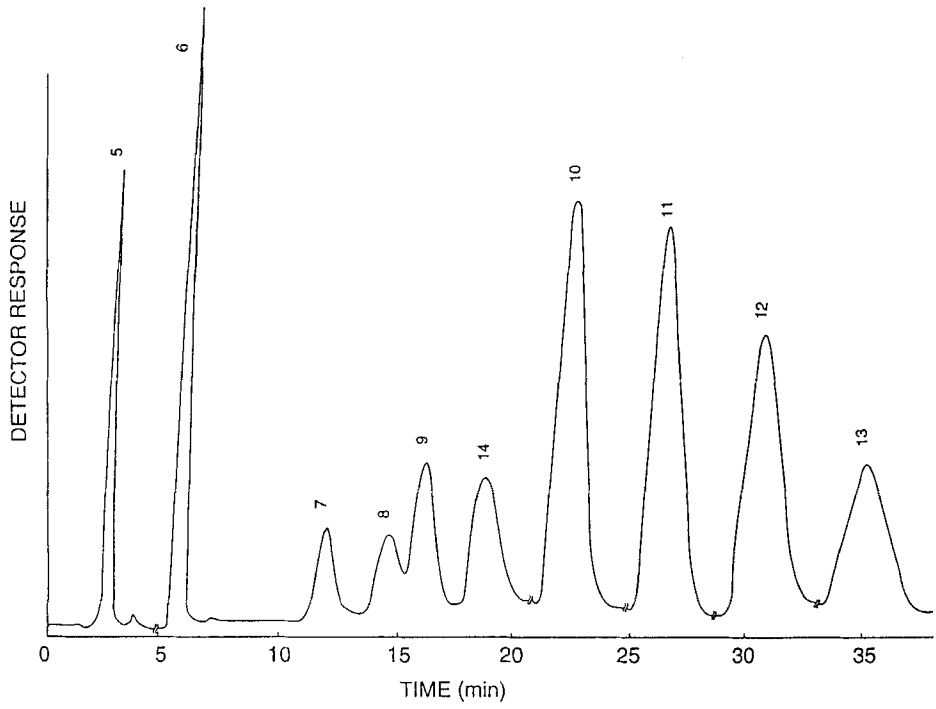

FIGURE 5. Chromatogram of ten metabolites of B[a]P obtained with MeOH:H_2O (80:20) with 1.7 mM γ-CD. The full names of the compounds are given in the footnote of Table 1.

essentially the same retention as B[a]P-t-9,10-dihydrodiol and would have co-chromatographed with tetrol I-2. As discussed earlier, without γ-CD in the mobile phase, there was overlap between 2-OH-B[a]P and 9-OH-B[a]P, and the presence of β-CD in methanol-water system didn't affect the separation of this pair of metabolites (23). Figure 5 shows that the six monohydroxyl-B[a]P metabolites can be readily separated. Comparison of Figure 5 and the chromatogram of fourteen metabolites separated with MeOH:H_2O (65:35) with 4.0 mM of β-CD

from a previous investigation (23), showed that in the region of tetrols and B[a]P-t-9,10-dihydrodiol, β-CD was more effective than γ-CD in separating the tetrols and the dihydrodiol. However, for the separation of dihydrodiols, diones, and monohydroxyl-B[a]P metabolites, MeOH:H_2O (80:20) with γ-CD resulted in a good compound-class separation, band sharpening, shorter retention times, and better separation of the ten metabolites than with β-CD in the mobile phase (23).

CONCLUSIONS

It can be concluded that a good separation of a mixture of three classes of metabolites, namely, dihydrodiols, diones, monohydroxyl-B[a]P metabolites is possible using methanol-water with γ-CD as a mobile phase additive. Mobile phases containing γ-CD showed better selectivity toward the monohydroxyl-B[a]P isomers compare to an optimum binary methanol-water mobile phase (5) or methanol-water with β-CD (23). The capacity factors for the meta- bolites decreased to a larger extend with γ-CD compare to β-CD and a desirable capacity factor range of $1< k'< 15$ was obtained for the metabolites with MeOH:H_2O (80:20) and γ-CD. Also, the selectivity factors for monohydroxyl-B[a]P metabolites increased with γ-CD compared to β-CD in methanol-water mobile phases. Isomers that were difficult to separate, 6-OH- and 12-OH-B[a]P, and 9-OH- and 2-OH-B[a]P, were separated with good resolution with γ-CD in methanol-water. With γ-CD, the retention behavior of the 2-OH-B[a]P and 9-OH-B[a]P was remarkably different than with methanol-water or methanol-water with β-CD. A good separation for the four tetrols can be obtained with 4.0 mM of

γ-CD in MeOH:H_2O (55:45). The elution order of the fourteen metabolites didn't change by addition of γ-CD. Earlier work showed that the elution order of some of the metabolites changed with β-CD in the mobile phase. Also, γ-CD, as a mobile phase additive, was shown to be more effective than was β-CD for the separation of the most difficult to separate pairs of monohydroxyl-B[a]P metabolites.

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