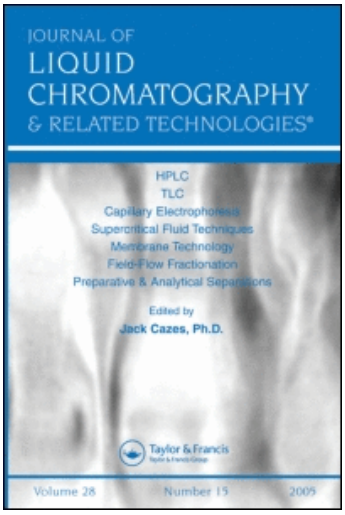

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Reversed-Phase Chromatographic Separation of Benzo[A]Pyrene Metabolites with β-Cyclodextrin as a Mobile Phase Modifier

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REVERSED-PHASE CHROMATOGRAPHIC SEPARATION OF BENZO[a]PYRENE METABOLITES WITH β-CYCLODEXTRIN AS A MOBILE PHASE MODIFIER

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ABSTRACT

β-Cyclodextrin (β-CD) was investigated as a mobile phase modifier for the separation of fourteen metabolites of benzo[a]pyrene. A wide range of β-CD concentrations was employed, and the chromatographic properties of four different classes of metabolites were compared in methanol-water, acetonitrile-water, and methanol-acetonitrile-water mobile phases with and without β-CD. The chromatographic bands for all the metabolites sharpened with β-CD in the mobile phase. The resolution for four classes of metabolites improved with β-CD in the binary and ternary mobile phases. The monohydroxyl-benzo[a]pyrenes used in this study were structurally similar and difficult to separate with methanol-water. However, with β-CD in the mobile phase, overall improvement in the separation of the monohydroxyl-benzo[a]pyrenes was achieved. Substantial improvement was obtained for the separation of 6-hydroxyl-benzo[a]pyrene from 12-hydroxyl-benzo[a]pyrene with β-CD in methanol-water and for 2-hydroxyl-benzo[a]pyrene from 9-hydroxyl-benzo[a]pyrene with β-CD in the acetonitrile-water.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) can be formed by combustion reactions or high-temperature processes involving carbonaceous materials such as

coal, coal tar, pitch, asphalt, and oil (1). The potential carcinogenic effect of PAH is the basic reason for concern about the levels of these compounds in the working environment (2). Some PAH have been shown to cause cancer in animals, and they are suspected to be human carcinogens as well (1-3). Of the PAH, benzo[a]pyrene (B[a]P) has been studied the most (4-6). The profile of the metabolites of B[a]P has been largely worked out by using high-performance liquid chromatography (HPLC) (7-15). For example, Selkirk et al. (9-11) employed HPLC for the isolation and separation of metabolic isomers of B[a]P. Zaleski and co-workers (8) used HPLC to compare the metabolic profiles of B[a]P and B[a]P-t-7,8-dihydrodiol in freshly isolated hepatocyte from mirror carp. However, the separation of some of the structural isomers and closely related B[a]P metabolites has been difficult to achieve by chromatography (14).

The use of CD in liquid chromatography as a mobile phase modifier has been shown to be effective in the separation of several classes of isomers (16,17). Cyclodextrins are a series of macrocyclic oligosaccharides produced by the action of *Bacillus macerans amylase* on starch and contain from 6 to 12 α-1,4 linked D-glucose units (18). The most widely used CD consist of six, seven, and eight glucose monomers arranged in a torus shape and are designated as α-, β-, and γ-CD, respectively (19). The coupling of the glucose moieties gives the CD molecule a rigid, conical molecular structure with a hollow interior. The interior of the cavity of a cyclodextrin molecule is composed of two rings of C-H groups with a ring of glucosidic oxygen in between, allowing the rings to be hydrophobic in nature (20). The internal diameters of the cavities of α-, β-, and γ-CD are

approximately 5.7, 7.8, and 9.5 Å, respectively, and the depths are roughly 7.8 Å (20). The cavities enables the CD to trap guest molecules in their interior, resulting in the formation of inclusion or "host-guest" complexes (16,21-23). The stability of an inclusion complex depends on the size of CD cavity and on intermolecular forces such as hydrogen bonding, van der Waals attraction, and hydrophobic interaction. These macrocyclic carbohydrate molecules can be discriminating in their inclusion complexing tendencies toward different structural, positional, or stereoisomeric molecules.

β-Cyclodextrin has been used as a mobile phase modifier in the separation of structural isomers (16,24-25). For example, Mohseni and Hurtubise (16) investigated the effects of β-CD on the separation of several structural isomers and concluded that the addition of β-CD to the mobile phase in HPLC resulted in dramatic improvements in the separation of structurally similar hydroxyl aromatics. Woodberry et al. (21) investigated the effects of β- and γ-CD bonded-phase columns on the separation of two of the metabolites of benzo[a]pyrene isomers. Their results suggested that HPLC methodology with CD as a stationary phase or as a mobile phase additive could be used to separate B[a]P-t-7,8-dihydrodiol from its 9,10-isomer.

We previously reported a systematic method for determining optimal mobile phases for the separation of a complex mixture of B[a]P metabolites (26). That work was based on the window diagram approach (27-29), and the solubility parameter optimization method (30). A mixture of fourteen B[a]P metabolites was separated by us using optimization procedures developed for binary and ternary mobile phases (26). Also, the retention characteristics of the fourteen different

metabolites of B[a]P were investigated using methanol-water, acetonitrile-water, and methanol-acetonitrile-water mobile phases (26).

The purpose of this work was to assess the effects of β-CD as a mobile phase modifier for separating B[a]P metabolites and the effects of β-CD on the compound-class separation of the metabolites. α-Cyclodextrin was not investigated in this work because its cavity size is too small for the metabolites, and γ-CD was not investigated in this work because of its expense.

EXPERIMENTAL SECTION

Apparatus

The liquid chromatographic system consisted of a Waters Model 6000A pump (Waters Associates, Milford, MA) connected to a U6K injector, a dual-channel ultraviolet detector model 440 detector set at 254 nm and a dual-channel 5.0 V recorder (Linear Instruments Co. Concord, CA). A model FIAtron temperature controller (Oconomowoc, WI) was employed to keep the temperature of the column constant at 25 ± 0.1° C. The column employed was a 5-μm BakerBond C_{18} (250 mm x 4.6 mm i.d.) purchased from J. T. Baker (Pillipsburg, NJ).

Reagents

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). The β-CD samples were obtained from Aldrich (Milwaukee, WI). Methanol and water were HPLC grade and were purchased from J. T. Baker Inc. (Pillipsburg, NJ). Acetonitrile was HPLC grade and was obtained from Fisher Scientific (Fair Lawn, NJ).

Procedures

The organic modifiers and water were prefiltered through a Millipore type HA 0.45 μm filter. An accurately weighed amount of β-CD, which was vacuum dried at 75° C for 8 hr, was dissolved in prefiltered water. Then, the appropriate amount of organic modifier was added to the β-CD solution. After complete dissolution at room temperature, the remaining amount of mobile phase 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 or acetonitrile, depending on the mobile phase composition. To prevent decomposition of the metabolites, the standard solutions were stored under nitrogen gas at -15° C and in the dark. The column void volume was obtained by injecting a methanol or acetonitrile solution of potassium nitrite.

Mobile Phases and β-CD Concentrations

Optimum mobile phases from a previous investigation (26) and other binary mobile phases were employed in this study to determine the effects of β-CD on the retention characteristics of the fourteen metabolites. The methanol:water (MeOH:H_2O, v:v) solvents used for the metabolites were, 55:45 for a mixture of four tetrols, and 65:35, 70:30 and 81.75:18.25 for a mixture of fourteen metabolites. The maximum analytical concentrations of β-CD in MeOH:H_2O mobile phases of 55:45, 65:35, 70:30 and 81.75:18.25 were 5.0, 4.6, 3.5 and 2.0 mM, respectively.

The acetonitrile:water (ACN:H_2O, v:v) composition used for the separation of the fourteen metabolites were 60:40 and 65:35. The largest analytical concentration of β-CD in ACN:H_2O solvents were for 60:40, 3.0 mM, and for 65:35, 2.0 mM. The methanol:acetonitrile :water (MeOH:ACN:H_2O) composition was 17:50:33 (v:v:v). The maximum amount of β-CD that could be dissolved in this ternary mobile phase was 2.0 mM.

RESULTS AND DISCUSSION

Effects of the Percentage of Methanol and β-Cyclodextrin Concentration on the Retention Characteristics of the Metabolites of Benzo[a]pyrene

Previously, we used mobile phase optimization methods to determine an optimum binary mobile phase for the metabolites (26). In this work, several different concentrations of β-CD were investigated with the optimum binary mobile phase, MeOH:H_2O (81.75:18.25). This optimum mobile phase had a high percentage of methanol, therefore the maximum analytical concentration that could be dissolved in this mobile phase was 2.0 mM β-CD. With this concentration, the overall retention times for the mixture of fourteen metabolites were reduced and the bands were sharper. Also, the order of separation of the metabolites was the same as with the mobile phase without β-CD. However, as discussed below, better separation of the metabolites was obtained with β-CD when the percentage methanol was lower than in the optimum binary mobile phase.

Our earlier investigation with different percentages of methanol as an organic modifier indicated that the two most difficult pairs of metabolites to separate were 6-OH-B[a]P and 12-OH-B[a]P, and 2-OH-B[a]P and 9-OH-B[a]P

(26). Also, in this work, lower percentages of methanol and β-CD gave better separation of the metabolites than higher percentages of methanol. Therefore, smaller percentages of methanol with a wide range of β-CD concentrations were emphasized in this work. The capacity factors of the fourteen metabolites of B[a]P with different concentrations of β-CD in MeOH:H_2O (70:30, 65:35 and 55:45) were obtained. The retention properties of the metabolites were compared in MeOH:H_2O with and without β-CD and with other binary and ternary mobile phases as well. Criteria such as, the decrease in the capacity factors, class separation, band sharpening and increased resolution between the most difficult peak pairs of isomers were compared with and without β-CD. It was found that the retention properties of the monohydroxylated B[a]P were affected the most by increasing the β-CD concentration in MeOH:H_2O (70:30 and 65:35). For example, the k′ value of 6-OH-B[a]P changed from 19.4 with no β-CD in the mobile phase to 17.22 with 3.5 mM β-CD in MeOH:H_2O (70:30) (Table 1). The k′ value of 12-OH-B[a]P was reduced from 19.22 without β-CD to 16.4 with the addition of 3.5 mM β-CD in MeOH:H_2O (70:30). With MeOH:H_2O (70:30) and β-CD small changes in k′ values of tetrols were obtained (Table 1). Benzo[a]pyrene-t-7,8-dihydrodiol and the diones showed relatively larger changes in their k′ values with MeOH:H_2O (70:30) and β-CD. Also, because of the small retention times for tetrols and B[a]P-t-9,10-dihydrodiol, there was overlap between these two classes of metabolites when using the MeOH:H_2O (70:30) and β-CD mobile phase. Therefore, a good separation for the four tetrols and B[a]P-t-9,10-dihydrodiol was not obtained in a mixture of fourteen metabolites with this mobile phase. As seen

in Table 1, there was a decrease in retention of all of the metabolites with β-CD in the mobile phase.

The retention characteristics of fourteen metabolites of B[a]P with MeOH:H_2O (65:35) over a wide range of β-CD concentrations were also studied. Table 2 lists the capacity factors of the fourteen metabolites in MeOH:H_2O (65:35) with different amounts of β-CD. The k′ values were rather large for the hydroxyl aromatics with this mobile phase compared to MeOH:H_2O (70:30). However, a relatively large decrease in the k′ values of the monohydroxyl-B[a]P metabolites was obtained with increasing β-CD concentration in MeOH:H_2O (65:35). For example, the k′ values for 3-OH-B[a]P in the absence of β-CD was 42.86, whereas with 4.6 mM β-CD present, it was reduced to 32.11. This was mainly due to the higher concentrations of β-CD that were achieved with MeOH:H_2O (65:35) mobile phase compared to MeOH:H_2O (70:30) (see Table 1 and Table 2).

Separation of a Complex Mixture of the Metabolites

Comparison of the chromatograms in Figures 1a and 1b for a mixture of the fourteen metabolites with MeOH:H_2O (65:35) indicates, band sharpening, a decrease in the retention times, and the separation of 6-OH-B[a]P and 12-OH-B[a]P isomers with 4.0 mM of β-CD as a mobile phase modifier. However, significant improvement in the separation of B[a]P-1,6-dione and B[a]P-3,6-dione was not obtained with β-CD. Three classes of metabolites were separated, namely, monohydroxyl-B[a]P, diones, and dihydrodiols (Figure 1b). Nevertheless, the chromatographic bands overlapped for B[a]P-t-9,10-dihydrodiol and the tetrols with and without β-CD (Figures 1a and 1b).

TABLE 1
The k′-Values of the Metabolites of Benzo[a]pyrene for Methanol-Water (70:30) with Different Concentrations (mM) of β-CD

	β-CD (mM)					
Solute[a]	0.0	1.0	2.0	2.5	3.0	3.5
1	0.81	0.8	0.78	0.78	0.76	0.76
2	0.98	0.94	0.91	0.91	0.85	0.84
3	1.02	1.03	1.04	1.05	1.0	0.85
4	1.36	1.35	1.35	1.35	1.3	1.22
5	1.27	1.25	1.22	1.22	1.17	0.83
6	5.56	5.02	4.26	4.26	4.15	3.93
7	9.51	9.15	8.15	8.11	7.93	7.27
8	11.56	10.91	10.37	10.46	8.9	9.75
9	19.22	19.15	18.11	17.44	17.10	16.40
10	18.76	17.92	16.9	16.5	16.82	16.77
11	19.0	18.3	17.3	16.6	16.4	16.0
12	22.72	21.0	20.6	20.0	19.3	18.3
13	24.02	23.2	22.42	22.42	22.28	22.02
14	19.40	19.1	18.55	18.30	18.01	17.22

[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. 26.

TABLE 2
k'-Values Obtained for Metabolites of Benzo[a]pyrene with Methanol-Water (65:35 v/v) with Different Concentrations (mM) of β-CD

	β-CD (mM)					
Solute[a]	0.0	2.0	3.0	4.0	4.5	4.6
1	1.11	1.1	1.07	0.98	0.96	1.00
2	1.31	1.32	1.31	1.17	1.15	1.15
3	1.57	1.55	1.51	1.35	1.34	1.34
4	2.07	1.98	1.97	1.82	1.8	1.8
5	1.84	1.8	1.8	1.73	1.72	1.71
6	7.27	7.1	6.95	6.51	6.28	6.24
7	13.3	12.96	12.86	12.42	12.33	12.28
8	17.04	16.95	16.55	15.88	15.57	14.91
9	33.04	32.5	30.35	26.42	24.86	24.15
10	31.54	31.05	29.66	25.84	24.51	24.2
11	31.72	31.0	29.75	25.17	24.2	23.89
12	39.8	38.98	37.88	31.93	30.02	30.03
13	42.86	40.05	39.97	33.57	32.37	32.11
14	33.72	32.1	31.84	27.45	25.6	25.4

[a] See the footnote in Table 1 for the names of the compounds.

The structures of the compounds are given in Ref. 26.

Comparison of the results between the two mobile phases, MeOH:H_2O (65:35) and MeOH:H_2O (70:30), indicated similar results for the compound class separation with different concentrations of β-CD, except the k' values were shorter with the MeOH:H_2O (70:30). However, with MeOH:H_2O (65:35) and 4.0 mM of β-CD, the four tetrols and B[a]P-t-9,10-dihydrodiol were partially separated and 6-OH-B[a]P was separated from 12-OH-B[a]P. In contrast, with MeOH:H_2O

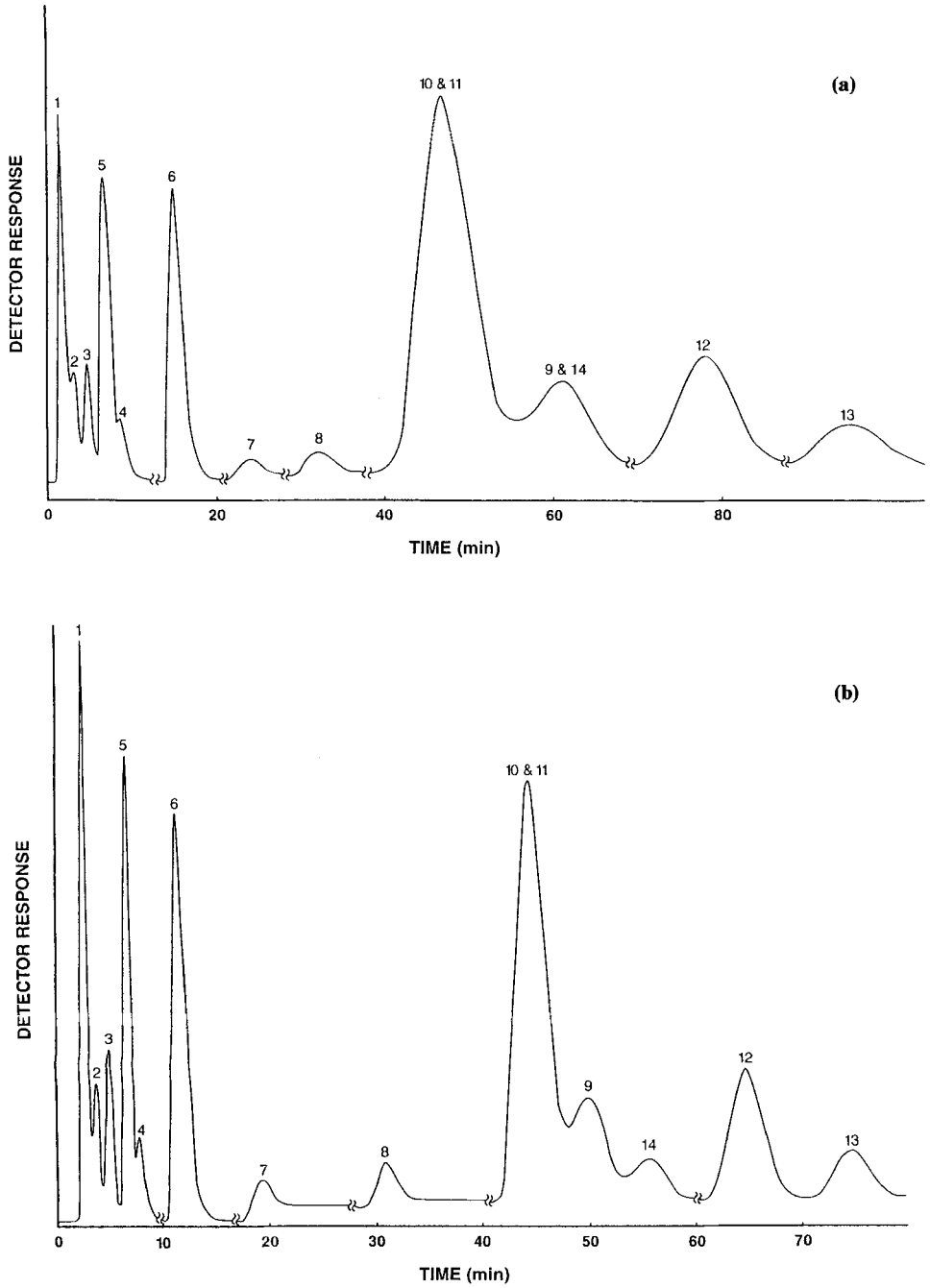

FIGURE 1. Chromatograms of the fourteen metabolites of benzo[a]-pyrene obtained with MeOH:H_2O (65:35) (a), and MeOH:H_2O (65:35) with 4.0 mM β-CD (b) on a 5.0 μm C_{18} column. The names of the compounds are given in the footnote of Table 1.

(70:30) and 3.5 mM β-CD, the four tetrols and B[a]P-t-9,10-dihydrodiol overlapped and 6-OH-B[a]P was partially separated from 12-OH-B[a]P. Also, there was no improvement in the resolution of 2-OH-B[a]P and 9-OH-B[a]P with either mobile phases. An increase in the concentration of β-CD in both MeOH:H_2O (70:30) and (65:35) only affected the retention characteristics of tetrols and dihydrodiols slightly because of the small k′ values for these two classes of metabolites. In spite of the relatively large retention times with MeOH:H_2O (65:35) and 4.0 mM β-CD, a good overall separation of thirteen compounds from the mixture of fourteen metabolites was obtained (Figure 1b). Thus, MeOH:H_2O (65:35) with β-CD was a more useful mobile phase than MeOH:H_2O (70:30) for the separation of a mixture of the metabolites.

Dependence of Capacity Factors of Tetrols on β-CD Concentration

The detection of tetrols in human body fluids and lower animals indicates the presence of B[a]P-DNA adducts. Thus, the study of tetrols is very important in cancer research. To examine the effects of different concentrations of β-CD on the chromatographic properties of the four tetrols, MeOH:H_2O (55:45) mobile phases with and without β-CD were investigated. The k′ values of the tetrols for different analytical concentrations of β-CD (0.0, 2.0, 3.0, 4.0 and 5.0 mM) were obtained. The MeOH:H_2O (55:45) mobile phase was used because the tetrols showed a much greater change in their retention times compared to MeOH:H_2O mobile phases with compositions of 81.75:18.25, 70:30, and 65:35. The k′ values for the monohydroxyl metabolites with MeOH:H_2O (55:45) were very large, and thus this mobile phase was not useful for the separation of the fourteen

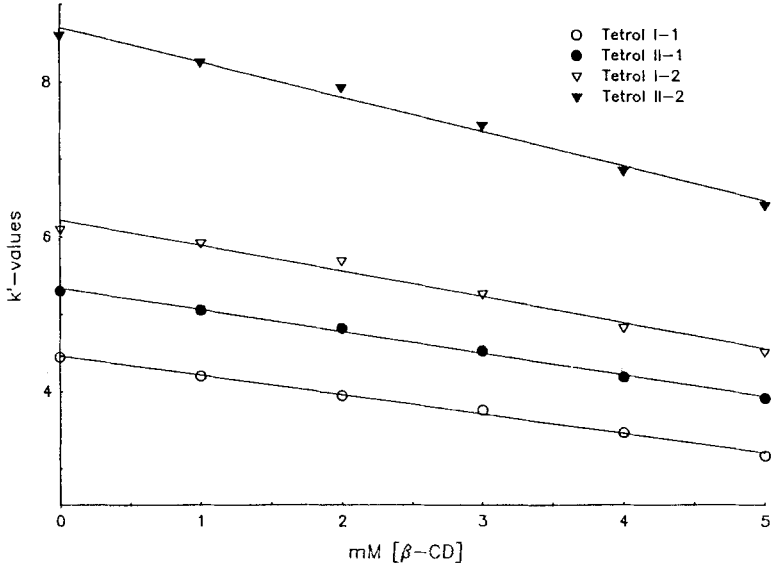

FIGURE 2. Plots of k′ values versus β-CD concentration for the four tetrols in MeOH:H_2O (55:45 v/v) at 25° C.

metabolites. Figure 2 shows the effects of the β-CD concentration on the retention of the four stereoisomers of the tetrols. As indicated in the Figure, a linear relationship was obtained between k′ and β-CD concentration for each of the tetrols. It is important to compare the changes in the capacity factors of the four tetrols with MeOH:H_2O (55:45) in the presence of β-CD. For example, benzo[a]pyrene-r-7,t-8,c-9,10-tetrahydrotetrol (tetrol II-2) showed a larger reduction in its k′ values with an increasing β-CD concentration compare to the other three tetrols (Table 3). The decrease in the capacity factors of tetrols with β-CD in MeOH:H_2O (55:45) resulted in the band sharpening, reduction in band width and a better separation of tetrols, which was due to their ability to form

TABLE 3
k′-Values of Tetrols with Methanol-Water (55:45 v/v) with Different Concentrations (mM) of β-CD

	β-CD (mM)					
Solutes[a]	0.0	1.0	2.0	3.0	4.0	5.0
1 (I-1)	4.45	4.2	3.95	3.76	3.47	3.17
2(II-1)	5.3	5.05	4.82	4.53	4.19	3.92
3 (I-2)	6.1	5.92	5.69	5.26	4.83	4.52
4(II-2)	8.6	8.25	7.93	7.44	6.85	6.41

[a] See the footnote in Table 1 for the names of the tetrols.

The structures of the tetrols are given in ref. 26.

inclusion complexes with β-CD. The best mobile phase investigated for the separation of tetrols was MeOH:H_2O (55:45) with 5.0 mM of β-CD.

Effects of the Composition of Acetonitrile and β-Cyclodextrin Concentration on the Retention of B[a]P Metabolites

In the absence of cyclodextrin, the retention of the fourteen B[a]P metabolites decreased as the acetonitrile (ACN) percentage increased in the mobile phase. At about ACN:H_2O (55:45), the k′ values of the monohydroxyl-B[a]P were approximately 20, and the k′ values became substantially greater at lower percentages of acetonitrile (26). At a percentage of 75% ACN, or greater, a white complex with small concentrations of β-CD formed. Thus, these ACN:H_2O mobile phases could not be used with β-CD. To examine the effects of β-CD and ACN on the retention characteristics of the metabolites, lower percentages of ACN were investigated (60:40 and 65:35).

With ACN:H_2O (60:40) four classes of metabolites, namely, tetrols, dihydrodiols, diones, and monohydroxyl-B[a]P, were separated with or without β-CD as a mobile phase additive. An increase in the concentration of β-CD didn't show a significant affect on the k'-values of tetrols and dihydrodiols. These results are related to the low retention times for these two classes of metabolites. The overall separation of the mixture of fourteen metabolites with ACN:H_2O (60:40) and β-CD was essentially the same as with ACN:H_2O (65:35) and β-CD.

Generally, retention times decreased and the bands were sharper with β-CD in ACN:H_2O (65:35) mobile phase compared to ACN:H_2O (65:35) or MeOH:H_2O (65:35). Also, 2-OH-B[a]P and 9-OH-B[a]P were separated with ACN:H_2O (65:35) and β-CD compared to MeOH:H_2O (65:35) and β-CD (compare Figure 1b and Figure 3). This pair of isomers was one of the most difficult pairs to separate with MeOH:H_2O mobile phases. Evidence for sharpening of the bands is shown in Figure 3 for these two hydroxyl aromatics with β-CD present in ACN:H_2O (65:35) compare to ACN:H_2O (65:35). An earlier investigation with the fourteen metabolites (26) indicated that the ACN:H_2O (65:35) was capable of separating at least thirteen metabolites from a mixture of fourteen metabolites. Similar results were obtained by addition of β-CD to the mobile phase. Figure 3 shows the advantages of using β-CD as a mobile phase additive in ACN:H_2O (65:35). Comparison between this chromatogram and chromatogram obtained with ACN:H_2O (65:35) (26) indicated: a) decrease in the overall retention times, b) sharper bands in the chromatogram (Figure 3), and c) improvement in the compound-class separation.

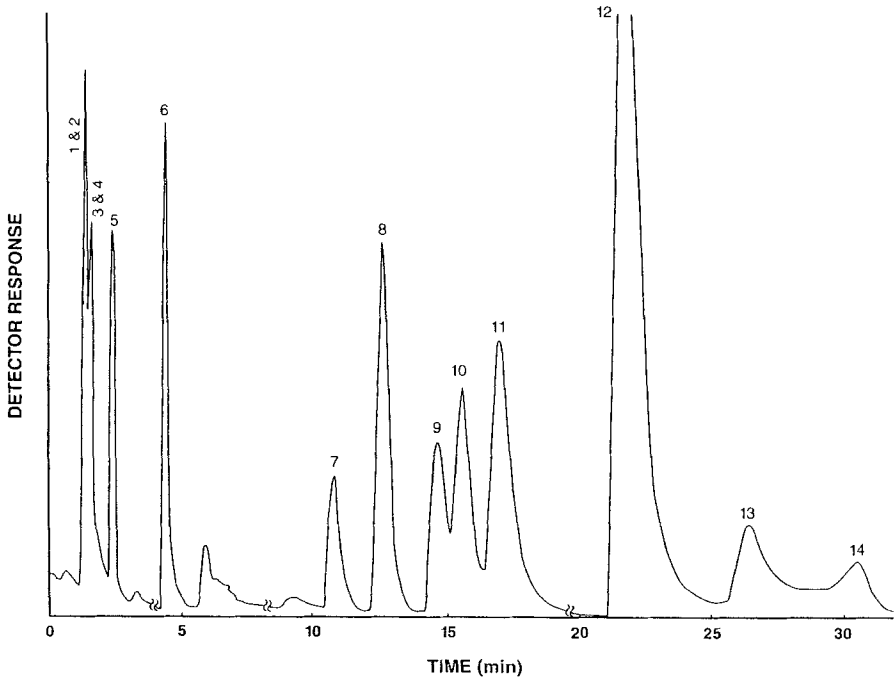

FIGURE 3. Chromatogram of fourteen metabolites of benzo[a]pyrene obtained with ACN:H_2O (65:35) with 1.5 mM β-CD at 25° C. The names of the compounds are given in the footnote of Table 1.

Comparison of MeOH:H_2O and ACN:H_2O

Comparison of Figure 3 and Figure 1b, shows that 2-OH-B[a]P and 9-OH-B[a]P can be separated with ACN:H_2O (65:35) and β-CD. However, with MeOH:H_2O (65:35) and 4.0 mM of β-CD the four tetrols were separated somewhat better than with ACN:H_2O (65:35) and β-CD (compare Figures 1b and Figure 3). In addition, Figure 1b shows the effectiveness of β-CD in the separation of structural isomers, 6-OH-B[a]P and 12-OH-B[a]P. These two isomers could not be separated with just methanol-water (Figure 1a). Also, MeOH:H_2O (65:35) with 4.0 mM of β-CD is capable of separating at least twelve

of the metabolites (Figure 1b). In contrast, ten of the metabolites were separated with ACN:H_2O (65:35) and 1.5 mM of β-CD (Figure 3). Larger k′ values for the six monohydroxyl-B[a]P metabolites were obtained with MeOH:H_2O (65:35) and β-CD (Table 2) compared to ACN:H_2O (65:35) (Figure 3). Generally, ACN:H_2O (65:35) with 1.5 mM β-CD was a better mobile phase for the separation of this class of metabolites. However, to obtain a better separation with a good baseline resolution of the fourteen metabolites MeOH:H_2O (65:35) with 4.0 mM of β-CD is preferable.

Effects of a Ternary Mobile Phases and β-CD Concentration on the Capacity Factors of the Metabolites

The retention characteristics of the fourteen metabolites of benzo[a]pyrene with different analytical concentrations of β-CD (0.0, 1.0, 1.5, 1.7 and 2.0 mM) and MeOH:ACN:H_2O (17:50:33 v/v/v) were obtained, because it was shown earlier that this ternary mobile phase was effective in separating the fourteen metabolites (26). With this mobile phase, the k′ values for the tetrols and dihydrodiols didn't change significantly with increasing β-CD concentration. For example, the k′ value of benzo[a]pyrene-trans-7,8-dihydrodiol decreased from 2.26 with no β-CD to 2.01 with 2.0 mM β-CD. The small changes in the k′ values of the dihydrodiols indicated a weak guest-host interaction between these metabolites and β-CD. The diones had relatively large k′ values with β-CD in the ternary mobile phase. An increase in the concentration of β-CD to 2.0 mM reduced the k′ values of both diones about 1.5-fold. The k′ values for the monohydroxylated compounds also decreased with an increase in the β-CD concentration. For

example, the k′ values of 12-OH-B[a]P and 9-OH-B[a]P were about 9.8 and 10, respectively, with the ternary mobile phase (26). With 2.0 mM β-CD, the k′-values for these two metabolites decreased to 6.4 and 6.9, respectively, resulting in band sharpening and shorter retention times and separation of these two compounds from each other. Thus, an increase in the concentration of β-CD to 2.0 mM resulted in the reasonable elution times $1<k'<15$ and band sharpening for the metabolites of benzo[a]pyrene.

CONCLUSIONS

The addition of β-CD to the mobile phases in this investigation resulted in a decrease in the capacity factors, band sharpening, and compound-class separation. Without β-CD in methanol-water mobile phases, two pairs of monohydroxyl isomers (6-OH-B[a]P and 12-OH-B[a]P, and 2-OH-B[a]P and 9-OH-B[a]P) could not be separated. However, with β-CD in MeOH:H_2O (65:35), 6-OH-B[a]P and 12-OH-B[a]P were separated. MeOH:H_2O (55:45) with 5.0 mM β-CD was a better mobile phase than either the ACN:H_2O or the ternary mobile phases with β-CD for the separation of the four tetrols. Using ACN:H_2O (65:35) and 2.0 mM β-CD, the monohydroxyl metabolites were separated with a shorter retention time compared to the MeOH:H_2O (65:35) with 4.0 mM β-CD. Also, 2-OH-B[a]P was separated from 9-OH-B[a]P with ACN:H_2O (65:35) and 2.0 mM of β-CD. These two metabolites were the most difficult pairs to separate with different concentrations of β-CD in the MeOH:H_2O mobile phases. Four classes of metabolites namely, tetrols, dihydrodiols, diones, and monohydroxyl-B[a]P were separated with ACN:H_2O (65:35) and β-CD. Addition of β-CD to the ternary

mobile phase MeOH:ACN:H_2O (17:50:33) improved the separation of 9-OH-B[a]P and 12-OH-B[a]P, because of a stronger interaction between β-CD and 12-OH-B[a]P (26). The elution order of the fourteen different metabolites of B[a]P didn't change by addition of β-CD to the binary or ternary mobile phases. However, the overall separation of the metabolites improved significantly by addition of β-CD to methanol-water compared to acetonitrile-water or ternary with β-CD (Figure 1b and Figure 3), especially in the region of the tetrols and B[a]P-t-9,10-dihydrodiol. The disadvantage of MeOH:H_2O (65:35) with β-CD was the relatively large k' values for the monohydroxyl compounds.

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