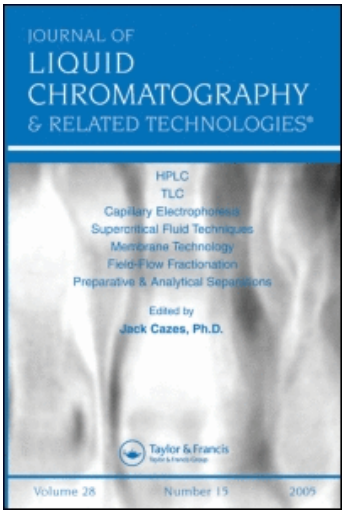

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OPTIMUM SEPARATION AND COMPOUND CLASS SEPARATION OF THE METABOLITES OF BENZO[a]PYRENE-DNA ADDUCTS WITH REVERSED-PHASE LIQUID CHROMATOGRAPHY

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ABSTRACT

Mobile phases were optimized for the reversed-phase liquid chromatographic separation of a complex mixtures of fourteen metabolites of benzo[a]pyrene (B[a]P). The metabolites constituted groups of isomers that were difficult to separate. The groups of isomers were tetrols, dihydrodiols, diones, and monohydroxyl-benzo[a]pyrenes. The window diagram optimization approach was used to initially optimize the binary mobile phases. Based on the data obtained from the optimum binary mobile phases, a solubility parameter optimization method was employed to obtain an optimum ternary mobile phase. Both the binary and ternary mobile phases were very effective in separating the metabolites. However, complete baseline resolution of the complex mixture of the metabolites was not achieved under the conditions investigated. Nevertheless, it was possible to obtain a separation of all fourteen of the metabolites with some overlap of the chromatographic bands. Also, compound class separation was obtained with the classes separating in the order of tetrols, diones, dihydrodiols, and monohydroxyl-benzo[a]pyrenes.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are not highly reactive chemically, but exert their carcinogenic activity after metabolism by humans, or lower animals, through metabolites which are sufficiently reactive to modify cellular macromolecules such as nucleic acids (DNA, RNA) and proteins. Identification of metabolites from some of the PAHs has been reported, with the metabolites from benzo[a]pyrene (B[a]P) being studied the most extensively (1). Information on metabolic activation of B[a]P has been summarized recently (2-4). Benzo[a]pyrene is a ubiquitous environmental contaminant found in the atmosphere, waterways and oceans, soil, marine life and in the food chain. Since the report of B[a]P as a potent carcinogen, numerous studies on the carcinogenesis of B[a]P have been performed (1-5). In laboratory experiments, the biotransformation products of B[a]P are usually analyzed by means of HPLC (6-14). The separation of some B[a]P metabolites using HPLC was initiated by the work of Selkirk et al. (11). However, the approach they developed did not completely resolve an isomeric mixture of four tetrols, dihydrodiols, diones, and monohydroxylated metabolites. Croy et al. (15) refined the earlier work of Selkirk et al. (11) by chromatographically recycling various isomers. They found that 1-OH-B[a]P and 7-OH-B[a]P co-chromatographed with 3-OH-B[a]P. However, their methodology did not isolate of 2-OH-B[a]P. Elnenaey and Schoor (14) developed a method to separate twelve isomeric monohydroxylated metabolites by using HPLC with fluorescence detection and various methanol-water gradients. Their study showed that several metabolic isomers have almost identical retention times. Wang and O'Laughlin (16) focused on the development of a sensitive method using laser-induced fluorescence detection in conjunction with HPLC for the separation and detection of tetrols that were formed by fish that metabolized B[a]P.

Reported here are HPLC methods for the rapid separation of fourteen B[a]P metabolites into four main compound-class types. The compound-class types are tetrols, dihydrodiols, diones, and monohydroxylated benzo[a]pyrene

compounds. Also, mobile-phase optimization methods for obtaining the optimal binary and ternary mobile phases for the separation of complex mixtures of the metabolites are reported. These methods are based on the work of Cooper and Hurtubise (17,18). They discussed the use of the window-diagram optimization approach and a mobile-phase optimization method for ternary mobile phases based on solubility parameter concepts for the separation of complex hydroxyl aromatic mixtures. The window diagram approach has been applied rather extensively to HPLC (19-24). In addition, Schoenmakers and coworkers (25-27) developed a method for estimating optimum compositions of ternary mobile phases for separating complex mixtures based on solubility parameters. Collectively, most of the metabolites used in this work have not been investigated previously by HPLC. For example, the separation of tetrols from diones, dihydrodiols and monohydroxyl-benzo[a]pyrenes has not been studied in detail. Moreover, little or no HPLC chromatographic data are available for many of the compounds investigated in this work.

EXPERIMENTAL

Apparatus

The liquid-chromatograph used was a Waters unit equipped with a model 6000A pump (Waters Assoc., Milford, MA, U.S.A.), a U6K injector, a dual channel free-standing ultraviolet 440 model 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. The chromatographic column employed was a 5-μm Baker-bond C_{18} (250mm x 4.6mm i.d.) obtained from J.T.Baker (Phillipsburg, NJ, U.S.A.).

Reagents

HPLC grade methanol and water were obtained from J.T.Baker Inc. (Phillipsburg, NJ, U.S.A.). Acetonitrile was HPLC grade and was purchased from

Fisher Scientific (Fair Lawn, NJ, U.S.A.). The organic modifiers and water were prefiltered through a Millipore type HA 0.45 μm filter. The B[a]P metabolites are listed in Table 1 and were obtained from the National Cancer Institute (NCI) repository at Midwest Research Institute (MRI, Kansas City, MO). All chemicals were used without further purification.

Procedures

Solutions of 0.1 mg/mL for an individual metabolite and 0.01 mg/mL for the mixture of standards were prepared in methanol or acetonitrile depending on the mobile phase composition. The retention volumes of each metabolite were determined by injecting 3.0-4.0 μl of the standard solution into the chromatographic system. To assure stability, the solutions were stored under nitrogen gas at -15° C and in the dark. However, under these conditions, 6-hydroxybenzo[a]pyrene was unstable in solution, and it decomposed after four days. Therefore, it was prepared freshly each 4-5 days.

The capacity factors (k') were calculated from the equation, $k'=(\bar{V}_R-\bar{V}_m)/\bar{V}_m$, where $\bar{V}_R$ is the retention volume (mL) and $\bar{V}_m$ is the column void volume (mL). The void volume for the C_{18} column was obtained by injection of a methanol solution of potassium nitrite.

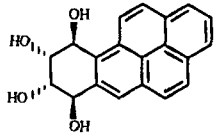
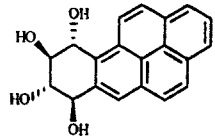
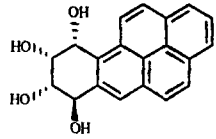
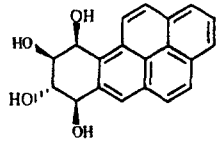
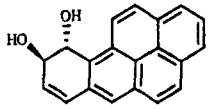
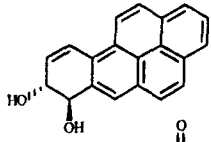
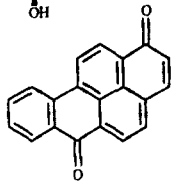
RESULTS AND DISCUSSION

General Considerations

The variable most frequently employed in optimizing liquid-chromatographic separations is the composition of the mobile phase. A number of mobile-phase optimization strategies have been developed over the years to obtain the optimal mobile phases for the separation of mixtures of compounds. In this work, the window diagram approach (23,28,30) and the solubility parameter optimization method developed by Schoenmakers et al. (27) and Drouen et al. (26) were combined to develop a relatively easy way of optimizing the mobile phases for the separation of the B[a]P metabolites. The window-diagram method was employed

TABLE 1

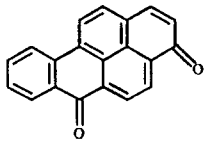
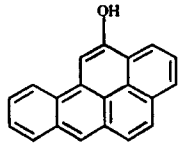
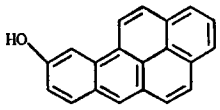
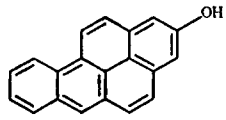
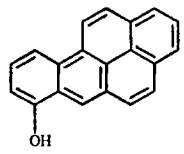
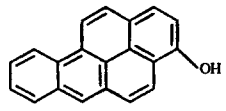
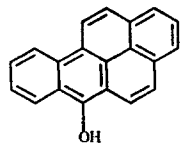
Names and Structures of the Model Compounds

Compound	Structure
1. Benzo[a]pyrene-r-7,t-8,9,c-10-tetrahydrotetrol (Tetrol I-1)	
2. Benzo[a]pyrene-r-7,t-8,c-9,t-10-tetrahydrotetrol (Tetrol II-1)	
3. Bezo[a]pyrene-r-7,t-8,9,10-tetrahydrotetrol (Tetrol I-2)	
4. Benzo[a]pyrene-r-7,t-8,c-9,10-tetrahydrotetrol (Tetrol II-2)	
5. Benzo[a]pyrene-trans-9,10-dihydrodiol	
6. Bezo[a]pyrene-trans-7,8-dihydrodiol	
7. Benzo[a]pyrene-1,6-dione	

(continued)

TABLE 1 (Continued)

Names and Structures of the Model Compounds

Compound	Structure
8. Benzo[a]pyrene-3,6-dione	
9. 12-Hydroxybenzo[a]pyrene (12-OH-B[a]P)	
10. 9 -Hydroxybenzo[a]pyrene (9-OH-B[a]P)	
11. 2-Hydroxybenzo[a]pyrene (2-OH-B[a]P)	
12. 7-Hydroxybenzo[a]pyrene (7-OH-B[a]P)	
13. 3-Hydroxybenzo[a]pyrene (3-OH-B[a]P)	
14. 6-Hydroxybenzo[a]pyrene (6-OH-B[a]P)	

to determine the optimum binary mobile phases. Then, the optimum binary mobile phases were used with the solubility parameter optimization method to acquire the optimum ternary mobile phases. Below brief discussions of the window diagram optimization method and the solubility parameter optimization method are given.

The window diagram method is a graphical method for describing chromatographic retention data. The optimum chromatographic conditions are obtained by maximizing the selectivity of the most difficult to resolve chromatographic peak pairs as a function of various experimental variables (23,28,30). In this work, the separation factor, S, was used as the optimization criterion for the window diagram (29). The separation factor is defined by eqn. 1, where t_i is the retention time of component i, and t_j is the retention time of component j.

$$S = \frac{t_i - t_j}{t_i + t_j} \qquad (1)$$

The advantage of using eqn. 1 is that it not necessary to measure t_o (void time). Schoenmakers et al. (27) and Drouen et al. (26) developed a systematic optimization criterion from their work based on solubility parameter theory. The optimization criterion from their work used in this research was the product of resolution factors, πR_s, which is defined by eqn. 2, where k_i and k_{i+1} are the capacity factors for each pair of adjacent peaks in a chromatogram.

$$\Pi R_s = \Pi_{i=1}^{n-1} \frac{k_{i+1} - k_i}{k_{i+1} + k_i + 2} \qquad (2)$$

The criterion expressed by eqn. 2 gives a relative number designated to select an optimum ternary mobile phase composition, but not necessarily a satisfactory chromatogram. Cooper and Hurtubise (18) reported a detailed comparison between the solubility parameter and window optimization methods for the reversed-phase chromatographic separation of hydroxyl aromatics. They showed that by comparing eqn. 1 and eqn. 2 the only difference between S and

πR_s is that πR_s corresponds to the product of separation factors, or S values, for each adjacent peak pair in a chromatogram. This relationship provides a convenient means of directly comparing the optimization in the window diagram and the solubility parameter optimization method. With both optimization methods, two conditions must be met. First, one must be able to recognize a given solute in different chromatograms. Secondly, one must be able to predict variations of capacity factors with mobile phase composition.

Window Diagram Optimization for the Metabolites of B[a]P

In this work, isomers related to metabolic pathways for B[a]P were selected for separation. Generally, classes of isomers including tetrols, dihydrodiols, diones and monohydroxy-benzo[a]pyrenes can be found in urine, blood sera and lung adenomas samples of cancer patients (5,31). Fourteen different metabolites of B[a]P were investigated with a C_{18} column at 25° C with methanol:water (MeOH:H_2O) and acetonitrile:water (ACN:H_2O) mobile phases.

Initial data for the window diagram optimization procedure were obtained by acquiring k' values for the metabolites with two different isocratic mobile phases which gave a wide range of k' values. Anywhere from 9 to 14 different binary mobile phases were used to construct the window diagrams. The window diagrams were plotted based on separation factors versus mobile phase composition for seven pairs of compounds (eqn. 1). The optimum mobile phase composition for MeOH:H_2O was found to be 81.75:18.25, which gave good resolution of a fourteen component mixture (Figure 1). It is evident from Figure 1 that this binary mobile phase is capable of separating the monohydroxyl isomers, except for the 2-OH-B[a]P and 9-OH-B[a]P and B[a]P-t-9,10-dihydrodiol from the tetrols. Also, 12-OH-B[a]P appears between the two diones. It should be mentioned that a mixture of just the four tetrols can be separated with MeOH:H_2O (55:45). This result is supported by Rojas et al. (8).

In order to examine the selectivity of different mobile phases, other organic modifiers including acetonitrile and tetrahydrofuran were studied. Based on the experimental k' values obtained with different acetonitrile compositions, a window

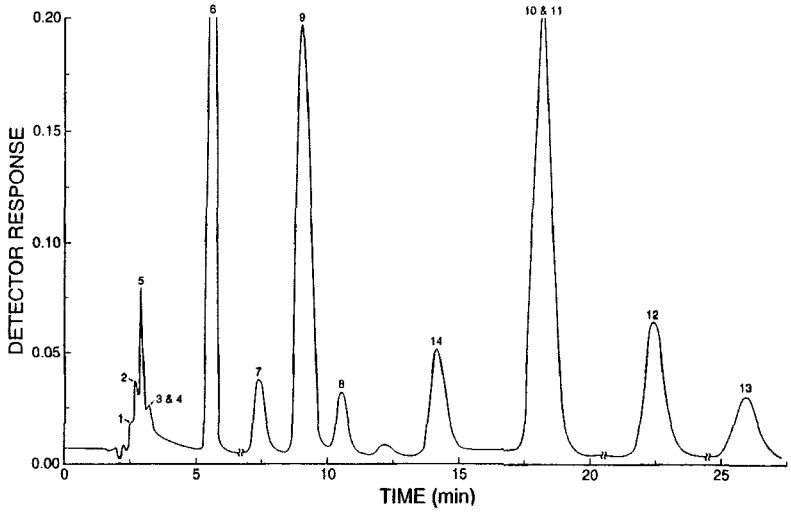


FIGURE 1. Chromatogram of fourteen metabolites of benzo[a]pyrene obtained with the optimum binary mobile phase MeOH:H_2O (81.75:18.25). The names and structures of the compounds are given in Table 1.

diagram was constructed for the ACN:H_2O mobile phase compositions. The optimum mobile phase obtained was ACN:H_2O (65:35). Figure 2 shows chromatogram for the fourteen different metabolites of benzo[a]pyrene separated with ACN:H_2O (65:35). Also, with ACN:H_2O (65:35), the compounds were separated in to four classes, namely, tetrols, dihydrodiols, diones and monohydroxy-B[a]P compounds (Figure 2). Separation of diones and monohydroxy-B[a]P has been confirmed by others using HPLC (10-12,32). The separation order for the monohydroxyl aromatic compounds in the mixture of fourteen metabolites with increasing retention time was in the following order: 12-OH-, 9-OH-, 2-OH-, 7-OH-, 3-OH- and 6-OH-B[a]P (Figure 2). Results for five of the hydroxylated B[a]P (12-OH-, 9-OH-, 2-OH-, 7-OH-, 3-OH-B[a]P), from this investigation, are identical to the results of Elnenaey and Schoor(14). Elnenaey and Schoor (14) used a gradient mobile phase system to separate 12

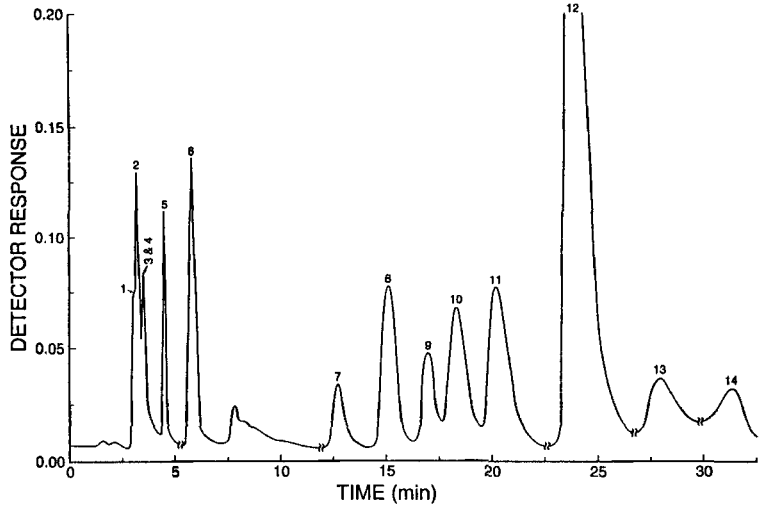


FIGURE 2. Chromatogram of fourteen metabolites of benzo[a]pyrene obtained with the optimum binary mobile phase ACN:H_2O (65:35). The names and structures of the compounds are given in Table 1.

isomeric monohydroxyl-B[a]P and concluded that the 6-OH-B[a]P may co-chromatographed with 9-OH-B[a]P. However, the results from our work indicated that 6-OH B[a]P is unstable, and it has to be used with in 2-3 days of preparation (33). Also, investigation of THF:H_2O (51.5:48.5) which has the same polarity (34) as ACN:H_2O (65:35), indicated several disadvantages of this mobile phase compared to the other binary mobile phases investigated. These include the decomposition of dihydrodiols and 6-OH-B[a]P, and short very retention times for overall separation of the fourteen metabolites.

In general, the optimum mobile phase, ACN:H_2O (65:35), resulted in elution of the complex mixture with the desired resolution between the most difficult to separate pairs, 2-OH-B[a]P and 9-OH-B[a]P. Comparison of Figure 1 and Figure 2 reveals that a much better separation including compound class separation and separation of six monohydroxyl isomers in a complex mixture of fourteen metabolites was achieved using the binary ACN:H_2O (65:35). However,

better baseline resolution was achieved with MeOH:H_2O (81.75:18.25) in the region of monohydroxylated metabolites (Figure 1). Comparison of the respective chromatograms for Figure 1 and Figure 2 shows that ACN:H_2O (65:35) resulted in an increase in the capacity factors (k') for 6-OH-B[a]P and 12-OH-B[a]P. This is most likely due to selective interactions of these two metabolites with acetonitrile in the mobile phase. The increase in the elution times for 6-OH-B[a]P and 12-OH-B[a]P resulted in a better class separation with ACN:H_2O (65:35) compared to MeOH:H_2O (81.75:18.25). Nevertheless, the k' values for the diones increased with ACN:H_2O and caused these compounds to elute near the hydroxyl aromatics (compare Figure 1 and Figure 2). However, ACN:H_2O (65:35) was a better mobile phase than MeOH:H_2O (81.75:18.25) for both compound class separation and for the separation of the fourteen metabolites.

Table 2 shows capacity factors for the fourteen metabolites of B[a]P obtained from the window diagram optimization method for the two different binary mobile phases investigated. The data show that most of these components are eluted in the respective mixtures within a k' range of approximately 1 and 15.61

Solubility Parameter Optimization For the Metabolites of B[a]P

With the binary mobile phases, band broadening was somewhat of a problem suggesting that a ternary mobile phase might provide lower k' values and sharper bands for the metabolites. Also, it was important to improve the separation of some of the pairs of the metabolites and increase resolution between the classes of metabolites such as diones and monohydroxyl-B[a]P metabolites. Therefore, the mobile phase optimization method based on solubility parameters which was developed by Schoenmakers and coworkers (26,27) was applied to the complex mixture of metabolites. Also, because of the results of Cooper and Hurtubise (17,18) for the separation of a mixture of twenty one monohydroxyl aromatic mixtures using a ternary mobile phase from the solubility parameter approach, it was concluded that this approach would be applicable for optimizing the separation of the metabolites on a C_{18} column.

TABLE 2

The k'-Values Obtained for the Metabolites of Benzo[a]pyrene with the Optimum Binary and Ternary Mobile Phases Using the Window Diagram and Solubility Parameter Methods

No.	Compound	k'-values		
		MeOH:ACN :H_2O (17:50:33)	MeOH:H_2O (81.75:18.25)	ACN:H_2O (65:35)
1	Tetrol I-1	0.31	0.53	0.43
2	Tetrol II-1	0.40	0.58	0.50
3	Tetrol I-2	0.47	0.79	0.53
4	Tetrol II-2	0.47	0.82	0.90
5	B[a]P-t-9,10-dihydrodiol	0.74	0.65	0.90
6	B[a]P-t-7,8-dihydrodiol	2.26	1.81	1.76
7	B[a]P-1,6-dione	6.49	2.39	5.51
8	B[a]P-3,6-dione	7.21	4.21	6.08
9	12-OH-B[a]P	9.76	3.56	7.00
10	9-OH-B[a]P	10.0	7.32	7.21
11	2-OH-B[a]P	11.2	7.37	7.64
12	7-OH-B[a]P	13.6	9.23	9.6
13	3-OH-B[a]P	14.8	10.8	10.8
14	6-OH-B[a]P	15.0	5.77	12.4

The binary mobile phase ACN:H_2O (65:35) obtained with the window diagram approach was used to calculate a composition of MeOH:H_2O that had the same polarity as ACN:H_2O. These two binary mobile phases were employed as initial mobile phases to obtain the optimum ternary mobile phase with the solubility parameter approach. After obtaining the initial binary mobile phases the procedure used for optimization of ternary mobile phases was as follows (17,18,34): a) A mobile phase selection diagram was constructed based on ln k' values obtained from ACN:H_2O (65:35) and MeOH:H_2O (83.2:16.8) versus the

composition of the ternary mobile phases of MeOH:ACN:H_2O (27); b) Using eqn. 2, the change in the criterion, πR_s, was calculated from the estimated ln k' values obtained from the mobile-phase selection diagram; c) The experimental values of ln k' were obtained from the first maximum πR_s value; d) The chromatographic data obtained from the first ternary mobile phase was used to construct a new mobile phase selection diagram, and steps c and d were repeated until no further improvement in the separation of metabolites occurred.

Using the solubility parameter approach discussed above, the optimum ternary mobile phase mixture obtained was MeOH:ACN:H_2O (17:50:33). Figure 3 shows the chromatogram obtained with the optimum ternary mobile phase. The chromatogram showed some improvements over the chromatogram obtained with MeOH:H_2O (81.75:18.25) in Figure 1. First, there was no overlap between the tetrols as a group and B[a]P-t-9,10-dihydrodiol. Second, the diones were separated from the hydroxyl aromatics, because of the migration of 12-OH-B[a]P with this optimum ternary (Figure 3). Also, class separation was in order of increasing retention time for tetrols, dihydrodiols, diones and monohydroxyl-B[a]P metabolites, without any overlap of compound classes (Figure 3). In addition, the improved separation of 2-OH-B[a]P and 9-OH-B[a]P was obtained compared to MeOH:H_2O (81.75:18.25) (Figure 1 and Figure 3). Comparison between the chromatograms in Figure 2 and in Figure 3 showed little improvement in the separation in the region of tetrols and dihydrodiols. However, the chromatogram in Figure 3 shows some definite improvements over the binary mobile phases in Figure 1 and Figure 2. The bands are sharper and the compound classes were well separated (Figure 3). Nevertheless, as shown in Figure 3, there was poor separation between 12-OH-B[a]P and 9-OH-B[a]P (Figure 3). In an attempt to further improve the resolution between 12-OH-B[a]P and 9-OH-B[a]P and the other metabolites, the experimental data from Figure 3 were used to obtain another ternary mobile phase using the solubility parameter approach. The composition of that mobile phase was MeOH:ACN:H_2O (11.8:55.8:32.4). However, the resulting chromatogram was clearly poorer than its predecessor, and there was a

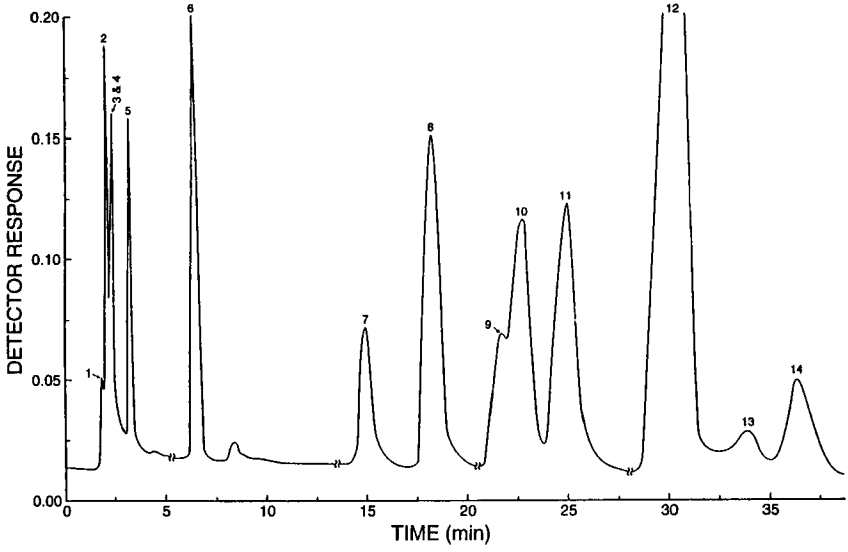


FIGURE 3. Chromatogram of fourteen metabolites of benzo[a]pyrene obtained with the optimum ternary mobile phase MeOH:ACN:H_2O (17:50:33). The names and structures of the compounds are given in Table 1.

decrease in the value of the πR_s criterion. Table 2 shows the k' values obtained for the optimum ternary mobile phase.

CONCLUSIONS

Undoubtedly, the ACN:H_2O (65:35) from the window-diagram optimization method gave a better overall separation for the hydroxyl-B[a]P compounds and also gave good compound-class separation. It was shown that the optimization scheme used for the ternary mobile phase resulted in a somewhat better overall resolution for at least 12 of the compounds and a better class separation for the mixture of metabolites (Figure 3) because each class of compound was separated by a greater range than with ACN:H_2O (65:35). However, ACN:H_2O (65:35) was

essentially as effective as the ternary mobile phase and gave a better separation of 9-OH-B[a]P and 12-OH-B[a]P. A desirable k' value range (1<k'<15) was obtained with both optimum binary mobile phases and the ternary MeOH:ACN:H_2O (17:50:33) (Table 2). The most difficult to separate adjacent peak pairs 2-OH-B[a]P and 9-OH-B[a]P, 6-OH-B[a]P and 12-OH-B[a]P and 7-OH-B[a]P and 3-OH-B[a]P can be resolved with both the optimum ACN:H_2O mobile phase and the ternary mobile phase. However, our results showed that complete baseline resolution with one specific optimum mobile phase was not possible for such a complex mixture of isomers under the conditions described.

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