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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF RING-OXIDIZED METABOLITES OF NITRO-POLYCYCLIC AROMATIC HYDROCARBONS*

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SUMMARY

Nitro-polycyclic aromatic hydrocarbons (nitro-PAHs) are widespread genotoxic environmental pollutants, which require metabolic activation to exert their biological activities. Metabolism of nitro-PAHs generates ring-oxidized metabolites including epoxides, phenols, dihydrodiols and tetrahydrotetrols. Separation of the oxidized metabolites and related compounds of a series of isomeric nitro compounds derived from anthracene, benz[*a*]anthracene, benzo[*a*]pyrene and benzo[*e*]pyrene was studied by high-performance liquid chromatography (HPLC) of different types of columns (monomeric and polymeric; reversed-phase and normal-phase). In the reversed-phase HPLC system, the general elution order of these compounds is: parent nitro-PAHs > phenolic derivatives > epoxides > dihydrodiols > tetrahydrotetrols. Among the geometric isomers, *trans*-dihydrodiols with both hydroxyl groups at the quasiaxial positions were eluted earlier than those with the hydroxyl groups at the quasiequatorial positions. Orientation of the nitro substituent has also been found to be an important structural feature for determining the relative retention order. Among the geometric isomers of nitro-PAHs and *trans*-dihydrodiols, the isomers with their nitro groups perpendicular or nearly perpendicular to the aromatic rings were eluted faster than the analogues with their nitro groups parallel or nearly parallel to the aromatic rings. Normal-phase HPLC gave opposite retention order, but with different separability among some of the compounds. Therefore, combination of both reversed- and normal-phase HPLC provides efficient separation of the ring-oxidized derivatives of nitro-PAHs. Results are also presented to compare the separation efficiency among different types of columns used. The results suggest that the polarity of solutes is the principal factor for determining their HPLC retention time.

INTRODUCTION

Nitro-polycyclic aromatic hydrocarbons (nitro-PAHs) are genotoxic environmental pollutants formed by the incomplete combustion of organic material[1–4]. The

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biological effects of these compounds are thought to arise from metabolic activation to reactive electrophiles by ring oxidation and/or reduction of the nitro functional group[1–4]. Recently, it has been found that some structural features of nitro-PAHs can affect metabolism, DNA-binding, mutagenic and tumorigenic potencies, and chemical properties of these types of compounds[5]. It is known that when a compound is eluted from a high-performance liquid chromatographic (HPLC) column, polarity and molecular size of the molecule are important factors in determining the HPLC retention time[6,7]. Polarity is largely related to the type, number, and location of the functional group(s) in a molecule. To date, the relationship between the structures of the ring-oxidized derivatives of nitro-PAHs and their HPLC retention times has not been reported. Consequently, a study of HPLC retention times of ring-oxidized derivatives of nitro-PAHs on different types of HPLC columns will help toward a better understanding of their structural features, and in turn, may be useful for correlating the structures and mutagenicity/tumorigenicity of these compounds.

In this report, a series of ring-oxidized derivatives of nitro-PAHs were used as substrates in an examination of the separating ability of several HPLC columns of different types (monomeric and polymeric; reversed-phase and normal-phase). The structures and abbreviations of the compounds used in this study are shown in Figs. 1 and 2. The analytical HPLC columns employed included: Zorbax ODS column (250 × 4.6 mm I.D.), Zorbax SIL column (250 × 4.6 mm I.D.) (DuPont Medical Products, Wilmington, DE, U.S.A.), Vydac ODS column (250 × 4.6 mm I.D.) (The Separations Group, Hesperia, CA, U.S.A.), Waters μBondapak C_{18} column (300 × 3.9 mm I.D.) (Milford, MA, U.S.A.), Deltabond phenyl column (250 × 4.6 mm I.D.), and Deltabond C_8 column (250 × 4.6 mm I.D.) (Keystone Scientific, State College, PA, U.S.A.).

EXPERIMENTAL

Materials

1-Nitro-7,8,9,10-tetrahydrobenzo[*a*]pyrene (7,8,9,10-H_4-1-NBaP), 7,8,9,10-H_4-3-NBaP, 7,8,9,10-H_4-6-NBaP, 7-hydroxy-6-nitro-7,8,9,10-tetrahydrobenzo[*a*]pyrene (7-OH-7,8,9,10-H_4-6-NBaP), 6-nitro-9,10-dihydrobenzo[*a*]pyren-7(8H)-one (7-keto-7,8,9,10-H_4-6-NBaP), *cis*-5,6-dihydroxy-7-nitro-5,6-dihydrobenz[*a*]anthracene (7-NBA *c*-5,6-dihydrodiol), *cis*-5,6-diacetoxy-7-nitro-5,6-dihydrobenz[*a*]anthracene (7-NBA *c*-5,6-diacetate), *cis*-4,5-diacetoxy-6-nitro-4,5-dihydrobenzo[*a*]pyrene (6-NBaP *c*-4,5-diacetate), 11-hydroxy-7-nitrobenz[*a*]anthracene (11-OH-7-NBA), 7-hydroxy-6-nitrobenzo[*a*]pyrene (7-OH-6-NBaP) and 11-acetoxy-7-nitrobenz[*a*]anthracene (11-OAc-7-NBA) were synthesized as previously described[8–10]. 7-Keto-7,8,9,10-H_4-1-NBaP, 7-keto-7,8,9,10-H_4-3-NBaP, 7-OH-7,8,9,10-H_4-1-NBaP and 7-OH-7,8,9,10-H_4-3-NBaP were obtained as minor products from the nitration of 9,10-dihydrobenzo[*a*]pyren-7(8H)-one, followed by reduction. *cis*-11,12-Dihydroxy-6-nitro-11,12-dihydrochrysene (6-NC *c*-11,12-dihydrodiol) was synthesized according to the procedure of El-Bayoumy and Hecht[11]. *cis*-4,5-Dihydroxy-1-nitro-4,5-dihydrobenzo[*a*]pyrene (1-NBaP *c*-4,5-dihydrodiol) and *cis*-4,5-dihydroxy-3-nitro-4,5-dihydrobenzo[*e*]pyrene (3-NBeP *c*-4,5-dihydrodiol) were similarly prepared.

cis-11,12-Diacetoxy-6-nitro-11,12-dihydrochrysene (6-NC *c*-11,12-diacetate) and *cis*-4,5-diacetoxy-3-nitro-4,5-dihydrobenzo[*e*]pyrene (3-NBeP *c*-4,5-diacetate)

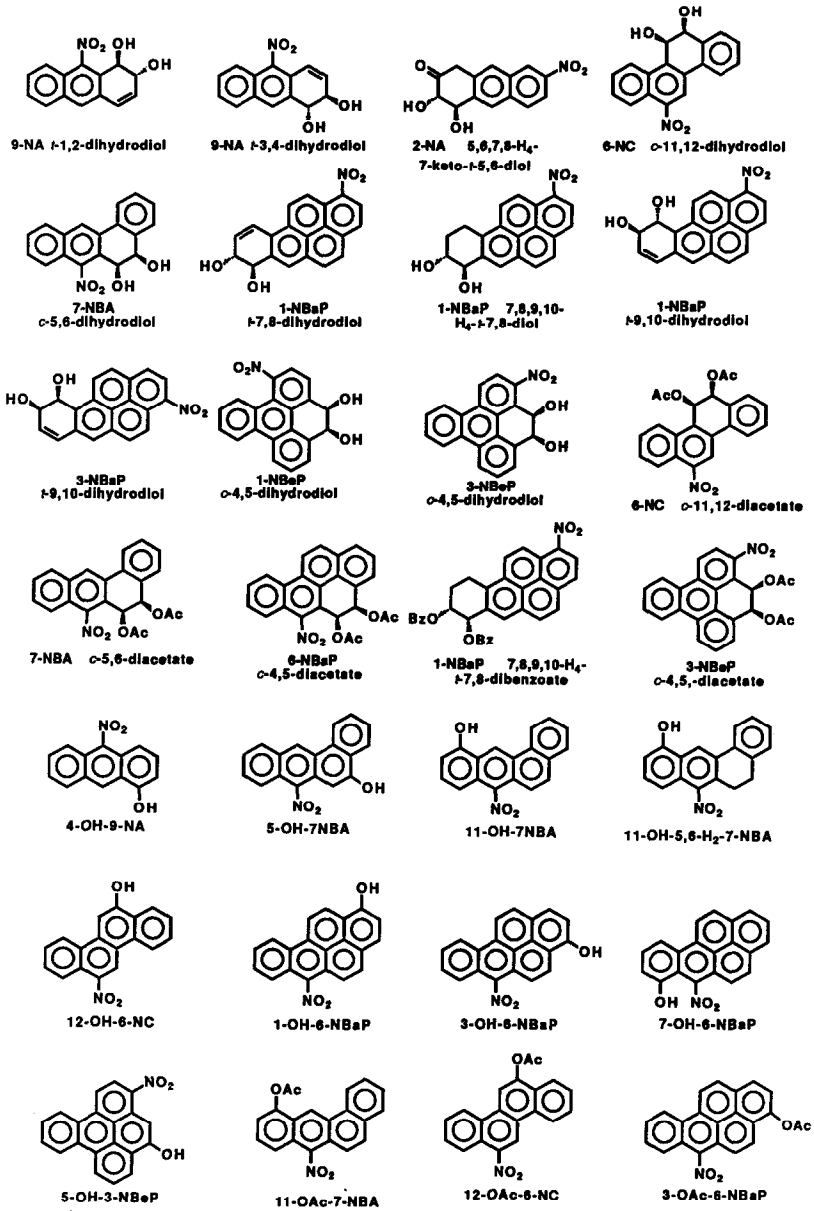

Fig. 1. Structures and abbreviations of nitro-PAH dihydrodiols, hydroxylated nitro-PAHs and their derivatives used in this study.

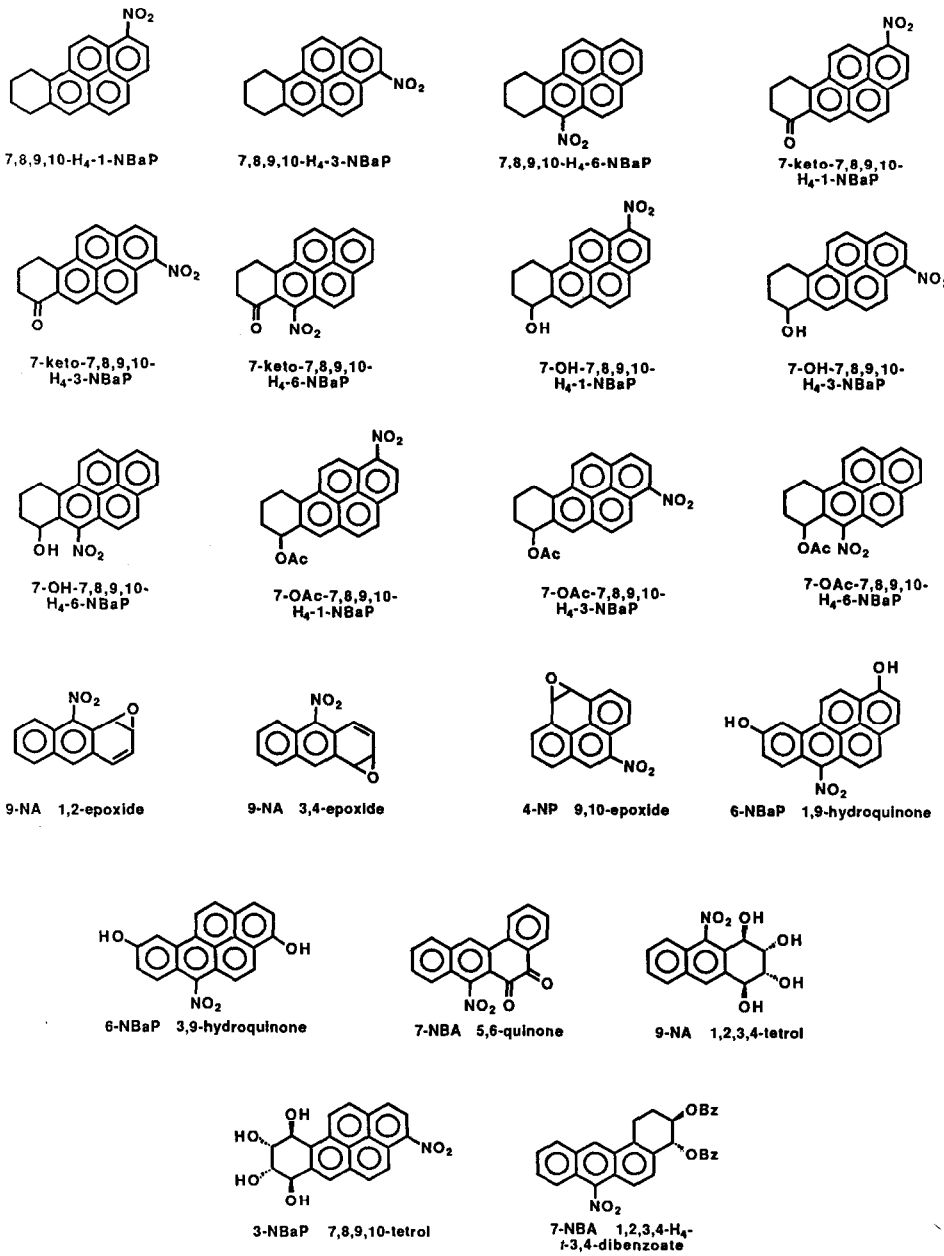

Fig. 2. Structures and abbreviations of 7-substituted nitro-7,8,9,10-tetrahydrobenzo[*a*]pyrene, nitro-PAH epoxides and other ring-oxidized derivatives.

were prepared by acetylation of the corresponding *cis*-dihydrodiols with acetic anhydride and pyridine[12]. 7-OAc-7,8,9,10-H_4-1-NBaP and 7-OAc-7,8,9,10-H_4-3-NBaP were prepared under similar conditions. 4-Hydroxy-9-nitroanthracene (4-OH-9-NA), 5-hydroxy-7-nitrobenz[*a*]anthracene (5-OH-7-NBA), 12-hydroxy-6-nitrochrysene(12-OH-6-NC), 5-hydroxy-3-nitrobenzo[*e*]pyrene (5-OH-3-NBeP), and 12-acetoxy-6-nitrochrysene (12-OAc-6-NC) were synthesized by acid-catalyzed dehydration of the corresponding *cis*-dihydrodiol precursors with *p*-toluenesulfonic acid in ben-zene[13]. All the *trans*-dihydrodiols, the remaining phenolic compounds and derivatives, epoxides, hydroquinones, and tetrahydrotetrols were obtained after metabolism of the corresponding parent nitro-PAHs by rat liver microsomes, as previously described[14–18]. The structures of the compounds synthesized were well characterized by spectral analysis of their UV absorption, mass, and high-resolution proton NMR data. The configuration and conformation of the dihydrodiol compounds and the orientation of the nitro substituents of each compound were also characterized by spectral analysis.

Chromatography

The HPLC system was composed of two Model 510 pumps, a Model 680 gradient controller, a U6K injector, a Model 440 absorbance detector set at 254 nm (all from Waters Assoc.), a Varian Associates (Walnut Creek, CA, U.S.A.) Model A-25 dual-pen strip-chart recorder, and optionally, a Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 3390A reporting integrator. In addition, a Hewlett-Packard 1040A detection system with the data processing unit option was available to replace the Model 440 detector and the strip-chart recorder. The columns used were: Zorbax ODS (250 × 4.6 mm I.D.); Zorbax SIL (250 × 4.6 mm I.D.); Vydac 201TP54 ODS (250 × 4.6 mm I.D.); Deltabond C_8 (250 × 4.6 mm I.D.); Deltabond phenyl (250 × 4.6 mm I.D.); and μBondapak C_{18} (300 × 3.9 mm I.D.). All columns have a particle size of 5 μm except for the Waters μBondapak which has a 10-μm size. The flow-rate was set at 0.5 ml/min. All mobile phases were HPLC grade solvents and were premixed and degassed before use. Each injection was 20 μl or less in volume. The retention time was recorded by the 1040A detection system, and the ultra-violet absorption spectrum of the material in each chromatographic peak was measured, so that the identity of the chromatographic peak could be confirmed. After the retention time of each compound had been determined, a mixture of two or more compounds was chromatographed in order to confirm the relative elution order and reproducibility of retention times. To eliminate the possible UV photolytic decomposition of the compounds, a UV absorbing film was placed above the light diffusion panel.

RESULTS AND DISCUSSION

For studying the relationships of structure and retention order of the ring-oxidized derivatives of nitro-PAHs, a set of these compounds was prepared for HPLC analysis. For comparison, we have determined the retention times of these compounds by employing a Zorbax ODS column, a μBondapak C_{18} column, a Vydac ODS column, a normal-phase Zorbax SIL column, a Deltabond phenyl column, and a Deltabond C_8 column. The Zorbax ODS, the μBondapak C_{18}, and the Vydac ODS columns have a conventional bonding between the silicate hydroxy groups and the

substituents. However, the bonded phase of the Zorbax and μBondapak ODS columns is monomeric and the Vydac ODS is polymeric. The Deltabond reversed-phase phenyl and C_8 columns are packed with a uniform matrix of cross-linked polysiloxane functional groups. Thus, comparison of the separation efficiencies of the columns and the retention order of the compounds will promote an understanding of the mechanisms of interaction between the bonded phase and the solutes. Based on the types of functional groups, the ring-oxidized nitro-PAHs used in this study are divided into four groups. Their retention times on different HPLC columns are summarized in Tables I–IV. We have employed a number of different solvent systems and solvent flow-rates in order to obtain suitable retention times for each compound eluted from the different columns. The final conditions chosen are shown in Tables I–IV.

Separation of the ring-oxidized nitro-PAHs by reversed-phase and normal-phase HPLC

Under the chromatographic conditions used in this study, the monomeric Zorbax ODS column separates the nitro-PAH dihydrodiols and their ester derivatives well, with the retention times ranging from 6.0 to 19.7 min (Table I). As an illustration, the separation of a mixture of six nitro-PAH dihydrodiols and diacetates on a Zorbax ODS column is shown in Fig 3. The polymeric Vydac ODS column gave poor separation of the compounds shown in Table I and, with only one exception, gave retention times which were all within a range of 1.3 min (from 5.4 to 6.7 min). These results are in contrast to previous observations[19] that the Vydac ODS column resolves the monohydroxylated derivatives of benzo[*a*]pyrene, benz[*a*]anthracene and chrysene with a wider range of retention times than the monomeric Zorbax ODS column. Locke[20] suggested that the relative solubility of each PAH in the polar mobile phase was the basis of reversed-phase selectivity. Oxidation of nitro-PAHs to the ring-oxidized derivatives generates additional polar functional group(s), thus increasing the water solubility of the molecules, and affecting their partition coefficients between the stationary and the mobile phases. This may be the reason for the poor selectivity of the polymeric Vydac ODS column for ring-oxidized nitro-PAHs.

Neither the Deltabond phenyl column nor the Deltabond C_8 column separates these compounds well. All had retention times within 2.6 min (from 6.4 to 9.0 min). A similar lack of efficiency of these two columns was observed in the separation of other ring-oxidized nitro-PAHs (Tables II, III and IV). These columns are fully packed with a uniform matrix of cross-linked polysiloxane functional groups, which may well shield the remaining active sites in the silica support. Thus, our results suggest that interaction between the polar active sites of the silica support and the ring-oxidized nitro-PAH substrates is involved in the separation mechanism.

The phenolic nitro-PAHs and their derivatives are well separated by both the Zorbax and the Vydac ODS columns (Table II). The efficient separation by the Zorbax ODS column is illustrated in Fig 4. Although an acetoxy group is less polar than a hydroxy group, it can be eluted earlier than a hydroxylated nitro-PAH, if the former has a larger molecular size (*e.g.*, 3-OAc-6-NBaP and 11-OH-5,6-H_2-7-NBA). As expected, 7-NBA 4,5-quinone, which contains two keto functional groups, has a shorter retention time than the other compounds, which have only one hydroxyl or acetoxy group (Table II), The retention times of the compounds eluted from the μBondapak C_{18} column and the Zorbax SIL column were all close together.

To test the effect of a polar functional group in a nitro-PAH on the HPLC

TABLE I

HPLC RETENTION TIMES OF NITRO-POLYCYCLIC AROMATIC HYDROCARBON DIHYDRODIOLS AND THEIR DERIVATIVES ON DIFFERENT REVERSED- AND NORMAL-PHASE COLUMNS

The eluent for all reversed-phase HPLC analyses was methanol–water (95:5, v/v) at a flow-rate of 0.5 ml/min. The eluent for normal-phase HPLC was 30% of tetrahydrofuran in hexane at a flow-rate of 0.5 ml/min. ND = not determined. Due to apparent column inefficiency, the retention times of the compounds marked with a "–" were not determined.

	Retention time (min)					
	Zorbax ODS	*Vydac ODS*	*μBondapak ODS*	*Deltabond*		*Zorbax SIL*
				phenyl	C_8	
9-NA *t*-1,2-dihydrodiol	6.1	5.4	6.3	7.3	6.5	23.9
9-NA *t*-3,4-dihydrodiol	6.6	5.5	6.7	7.4	6.6	31.7
2-NA 5,6,7,8-H_4-7-keto-*t*-5,6-diol	6.0	5.4	6.3	7.2	6.4	12.7
6-NC *c*-11,12-dihydrodiol	6.8	5.7	7.0	7.5	6.7	25.0
7-NBA *c*-5,6-dihydrodiol	6.8	5.6	6.9	7.6	6.6	14.5
1-NBaP *t*-7,8-dihydrodiol	8.3	6.7	7.7	7.6	6.8	ND
1-NBaP 7,8,9,10-H_4-*t*-7,8-diol*	7.8	6.7	7.4	–	–	ND
1-NBaP *t*-9,10-dihydrodiol	6.3	5.4	6.5	–	–	ND
3-NBaP *t*-9,10-dihydrodiol	6.4	5.5	6.6	7.4	6.5	ND
1-NBeP *c*-4,5-dihydrodiol	6.8	5.7	7.1	7.6	6.6	12.8
3-NBeP *c*-4,5-dihydrodiol	7.1	5.8	7.3	7.7	6.7	16.5
6-NC *c*-11,12-diacetate	9.0	6.1	8.3	8.3	7.2	11.4
7-NBA *c*-5,6-diacetate	8.3	5.9	7.8	8.2	6.9	13.4
6-NBaP *c*-4,5-diacetate	9.7	6.7	8.8	8.7	7.3	14.3
1-NBaP 7,8,9,10-H_4-*t*-7,8-dibenzoate*	19.7	12.4	13.7	–	–	13.0
3-NBeP *c*-4,5-diacetate	9.2	6.5	8.3	–	–	15.6

* It has not been determined yet whether or not the nitro group is at the C_1 or the C_2 position.

retention time, a series of 7-substituted mononitrated 7,8,9,10-tetrahydrobenzo[*a*]-pyrene was used for HPLC analysis (Table III). The good separation of these compounds by a Vydac ODS column is shown in Fig. 5. The three mononitrated

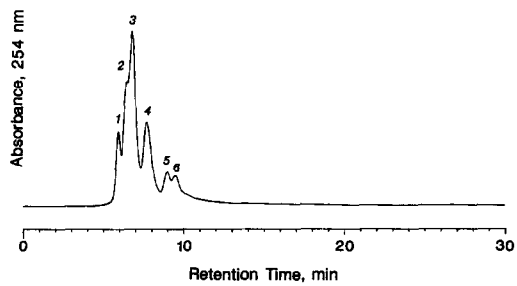

Fig. 3. Reversed-phase HPLC of some dihydrodiols and derivatives of nitro-polycyclic aromatic hydrocarbons on a Zorbax ODS column (250 × 4.6 mm I.D.), eluted with methanol–water (19:1, v/v) at a flow-rate of 0.5 ml/min. The compounds in the chromatographic peaks are: (1) 9-NA *t*-1,2-dihydrodiol; (2) 9-NA *t*-3,4-dihydrodiol; (3) 6-NC *c*-11,12-dihydrodiol; (4) 1- or 3-NBaP 7,8,9,10-H_4-*t*-7,8-diol, (5) 7-NBA *c*-5,6-diacetate; and (6) 6-NBaP *c*-4,5-diacetate.

TABLE II

HPLC RETENTION TIMES OF THE PHENOLIC AND ACETOXY DERIVATIVES OF NITRO-PO LYCYCLIC AROMATIC HYDROCARBONS ON DIFFERENT REVERSED- AND NORMAL-PHA SE COLUMNS

The eluent for all reversed-phase HPLC analyses was methanol–water (95:5, v/v) at a flow-rate of 0.5 ml/min. The eluent for normal-phase HPLC was 30% of tetrahydrofuran in hexane at a flow-rate of 0.5 ml/min. Due to apparent column inefficiency, the retention times of the compounds marked with a "–" were not determined. ND = not determined.

	Retention time (min)					
	Zorbax ODS	*Vydac ODS*	*μBondapak ODS*	*Deltabond*		*Zorbax SIL*
				phenyl	C_8	
4-OH-9-NA	7.8	5.9	7.5	–	6.9	ND
5-OH-7-NBA	8.4	6.1	7.7	–	–	14.2
11-OH-7-NBA	10.5	6.9	8.9	7.9	7.1	13.1
11-OH-5,6-H_2-7-NBA	7.8	5.9	7.6	7.9	6.8	13.5
12-OH-6-NC	11.3	7.4	9.2	7.8	7.2	ND
1-OH-6-NBaP	12.5	11.9	ND	–	–	ND
3-OH-6-NBaP	12.6	11.3	9.9	9.1	7.3	15.4
7-OH-6-NBaP	11.0	8.7	8.9	8.1	7.7	14.5
5-OH-3-NBeP	7.4	5.9	7.3	–	–	ND
11-OAc-7-NBA	11.1	7.2	9.2	8.3	7.5	12.5
12-OAc-6-NC	11.1	7.3	9.1	7.7	7.2	14.0
3-OAc-6-NBaP	13.5	9.8	10.2	8.6	7.7	13.9

H_4-BaPs (23, 24 and 25) have longer retention times than their 7-substituted derivatives. While the hydroxy-substituted derivatives (14, 15 and 16) and the acetoxy-substituted derivatives (18, 19 and 20) exhibit a poorer separation than the parent nitro-H_4-BaPs (23, 24 and 25), the keto derivatives (17, 21 and 22) are better separated than the parent compounds. Thus, these results exemplify that increasing the solubility in the polar mobile phase by introducing a functional group into a solute

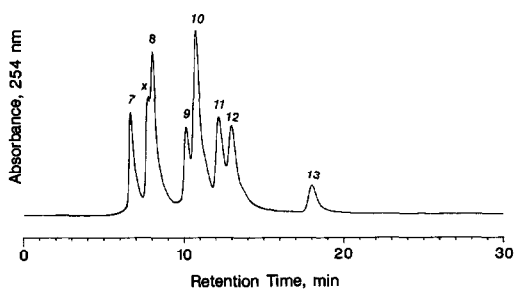

Fig. 4. Reversed-phase HPLC of some phenolic and acetoxy derivatives of nitro-polycyclic aromatic hydrocarbons on a DuPont Zorbax ODS column (250 × 4.6 mm I.D.), eluted with methanol–water (19:1, v/v) at a flow-rate of 0.5 ml/min. The compounds in the chromatographic peaks are: (7) 7-NBA 4,5-quinone; (8) 5-OH-7-NBA; (9) 11-OH-7-NBA; (10) 11-OAc-7-NBA; (11) 3-OH-6-NBaP; (12) 3-OAc-6-NBaP; and (13) 11-OH-5,6-H_2-7-NBA. The chromatographic peak marked with "X" contains an impurity.

TABLE III

HPLC RETENTION TIMES OF 1-, 3-, AND 6-NITRO-7,8,9,10-TETRAHYDROBENZO[*a*]PYRENE AND THEIR 7- AND 8-SUBSTITUTED DERIVATIVES ON DIFFERENT REVERSED- AND NORMAL-PHASE COLUMNS

The eluent for all reversed-phase HPLC analyses was methanol–water (95:5, v/v) at a flow-rate of 0.5 ml/min. The eluent for normal-phase HPLC was 30% of tetrahydrofuran in hexane at a flow-rate of 0.5 ml/min. Due to apparent column inefficiency, the retention times of the compounds marked with a "–" were not determined. ND = not determined.

	Retention time (min)					
	Zorbax ODS	*Vydac ODS*	*μBondapak ODS*	*Deltabond*		*Zorbax SIL*
				phenyl	C_8	
7,8,9,10-H_4-1-NBaP	47.3	25.1	ND	–	–	ND
7,8,9,10-H_4-3-NBaP	51.6	27.0	ND	–	–	ND
7,8,9,10-H_4-6-NBaP	20.5	22.7	ND	–	–	ND
7-Keto-7,8,9,10-H_4-1-NBaP	14.3	11.6	10.4	8.8	7.9	12.5
7-Keto-7,8,9,10-H_4-3-NBaP	14.1	13.4	10.7	9.0	8.0	12.1
7-Keto-7,8,9,10-H_4-6-NBaP	9.9	5.5	6.5	9.0	7.4	ND
7-OH-7,8,9,10-H_4-1-NBaP	10.5	8.1	8.9	8.0	7.2	23.4
7-OH-7,8,9,10-H_4-3-NBaP	10.3	7.8	8.9	8.0	7.2	20.6
7-OH-7,8,9,10-H_4-6-NBaP	10.0	7.2	8.6	8.1	7.2	12.2
7-OAc-7,8,9,10-H_4-1-NBaP	14.4	12.4	10.4	–	–	13.0
7-OAc-7,8,9,10-H_4-3-NBaP	16.4	10.7	11.3	–	–	12.2
7-OAc-7,8,9,10-H_4-6-NBaP	14.9	11.0	10.7	–	–	11.4
1-NBaP 7,8,9,10-H_4-*t*-7,8-dibenzoate	19.7	12.4	13.7	–	–	13.0
1-NBaP 7,8,9,10-H_4-*t*-7,8-diol	7.8	6.7	7.4	–	–	ND

does not always decrease the selectivity of the polymeric Vydac ODS column. HPLC analysis of additional nitro-PAHs with different types of substituents will be required to determine how substituents can affect the column selectivity.

The Zorbax ODS reversed-phase column gave a better separation of a number of

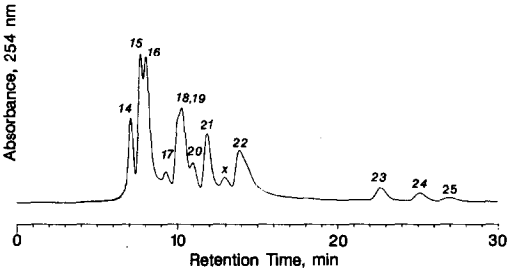

Fig. 5. Reversed-phase HPLC of 1-, 3, and 6-nitro-7,8,9,10-tetrahydrobenzo[*a*]pyrene and their 7- and 8-substituted derivatives on a Vydac ODS column (250 × 4.6 mm I.D.), eluted with methanol–water (19:1, v/v) at a flow-rate of 0.5 ml/min. The compounds in the chromatographic peaks are: (14) 7-OH-7,8,9,10-H_4-6-NBaP; (15) 7-OH-7,8,9,10-H_4-1-NBaP; (16) 7-OH-7,8,9,10-H_4-3-NBaP; (17) 7-keto-7,8,9,10-H_4-6-NBaP; (18) 7-OAc-7,8,9,10-H_4-6-NBaP; (19) 7-OAc-7,8,9,10-H_4-1-NBaP; (20) 7-OAc-7,8,9,10-H_4-3-NBaP; (21) 7-keto-7,8,9,10-1-NBaP; (22) 7-keto-7,8,9,10-H_4-3-NBaP; (23) 7,8,9,10-H_4-6-NBaP; (24) 7,8,9,10-H_4-1-NBaP; and (25) 7,8,9,10-H_4-3-NBaP. The chromatographic peak marked with "X" contains unidentified material.

TABLE IV

HPLC RETENTION TIMES OF RING-OXIDIZED NITRO-POLYCYCLIC AROMATIC HYDROCARBONS ON DIFFERENT REVERSED- AND NORMAL-PHASE COLUMNS

The eluent for all reversed-phase HPLC analyses was methanol–water (95:5, v/v) at a flow-rate of 0.5 ml/min. The eluent for normal-phase HPLC was 30% of tetrahydrofuran in hexane at a flow-rate of 0.5 ml/min. Due to apparent column inefficiency, the retention times of the compounds marked with a "–" were not determined. ND = not determined.

	Retention time (min)					
	Zorbax ODS	*Vydac ODS*	*μBondapak ODS*	*Deltabond*		*Zorbax SIL*
				phenyl	C_8	
9-NA 1,2-epoxide	7.7	5.5	6.7	7.9	–	12.2
9-NA 3,4-epoxide	7.7	5.9	7.4	7.9	7.0	13.6
4-NP 9,10-epoxide	7.1	6.1	7.1	7.7	6.7	14.0
6-NBaP 1,9-hydroquinone	7.9	6.7	7.8	–	–	ND
6-NBaP 3,9-hydroquinone	9.0	6.6	7.6	–	–	ND
9-NA 1,2,3,4-tetrol	5.7	5.3	6.1	7.1	6.4	ND
3-NBaP 7,8,9,10-tetrol	6.2	ND	6.5	–	–	ND
7-NBA 1,2,3,4-H_4-*t*-3,4-dibenzoate	21.1	12.2	13.8	–	–	14.0

ring-oxidized nitro-PAHs, as shown in Tables I–III. However, this column cannot separate 9-NA 1,2-epoxide from 9-NA 3,4-epoxide, by either isocratic or gradient elution. On the other hand, both the monomeric μBondapak C_{18} and the Vydac ODS column can effect this separation (Table IV). Although both Zorbax and μBondapak columns are packed with monomeric octadecyl-bonded phases on a silica gel support, the slight difference in support materials and/or phase preparation can result in different selectivity for polar functional groups[21,22]. Similar observations on the different selectivities for nitro-PAHs on monomeric ODS columns of different manufacturers have been reported elsewhere[23,24]. When chromatographed on the Zorbax SIL column with 30% of tetrahydrofuran in hexane, 9-NA 1,2-epoxide and 9-NA 3,4-epoxide were eluted at 12.2 and 13.6 min, respectively. We have previously found on many occasions that a mixture of two or more ring-oxidized PAHs inseparable by a reversed-phase HPLC column, can be well resolved in a normal-phase HPLC system[14,17,25,26]. Thus, the separation of many compounds, including PAHs, nitro-PAHs, and their ring-oxidized derivatives by HPLC will be facilitated by employing a combination of both reversed-phase and normal-phase HPLC systems.

Relationships between structures and HPLC retention times

(A) The polarity of the molecule is an important factor in determining the HPLC retention time. This can be well supported by the following observations.

(1) In the reversed-phase HPLC system, the general elution order of these compounds is: parent nitro-PAHs > tetrahydro-ketones = acetates > phenolic derivatives > epoxides > dihydrodiols > diol-ketones > tetrahydrotetrols. A specific example is the 9-nitroanthracene (9-NA) and its ring-oxidized derivatives, chromatographed on a Zorbax ODS analytical column:

9-NA tetrahydrotetrol < 9-NA *t*-1,2-dihydrodiol < 9-NA *t*-3,4-dihydrodiol <
(5.7 min) (6.1 min) (6.6 min)

9-NA 1,2-epoxide = 9-NA 3,4-epoxide < 4-OH-9-NA < 9-NA
(7.7 min) (7.7 min) (7.8 min) (8.8 min)

(2) Among the geometric isomers, *trans*-dihydrodiols with both hydroxyl groups in quasiaxial positions were eluted earlier than those with the hydroxyl groups in quasiequatorial positions. Examples include: 9-NA *t*-1,2-dihydrodiol < 9-NA *t*-3,4-dihydrodiol (as shown above) and 1-NBaP *t*-9,10-dihydrodiol < 1-NBaP *t*-7,8-dihydrodiol (see Table I).

(3) A compound having a nitro group perpendicular or nearly perpendicular to the aromatic ring was eluted earlier than the isomer(s) with the nitro group parallel or nearly parallel to the aromatic ring. For example, 7,8,9,10-H_4-6-NBaP and its derivatives were eluted earlier that their analogues having the nitro group at the C_1 and C_3 positions (see Table III).

(4) A phenolic compound having a hydroxyl group *peri* to the nitro substituent was eluted earlier than its isomers. For example, 7-OH-6-NBaP was eluted earlier than 1-OH-6-NBaP and 3-OH-6-NBaP.

(B) In general, retention times increase as the molecular size of the compound increases.

(C) For separation of ring-oxidized derivatives of nitro-PAHs by reversed-phase HPLC, the Zorbax ODS column gave the best resolution.

The results of this study provide increased understanding of the relationships between the structural features and the HPLC retention times of ring-oxidized derivatives of nitro-PAHs. The relation between HPLC retention times and structures of these has never before been studied. Since structural features have been found to be important factors in correlation with bacterial mutagenicity, DNA binding capability, and possibly the tumorigenicity of nitro-PAHs, our study may also facilitate our mechanistic study on the metabolic activation of nitro-PAHs.

REFERENCES

1 H. S. Rosenkranz and R. Mermelstein, *Mutat. Res.*, 114 (1983) 217.
2 C. M. White (Editor), *Nitrated Polycyclic Aromatic Hydrocarbons*, Hiedelberg, Huthig, 1985.
3 H. Tokiwa and Y. Ohnishi, *CRC Crit. Rev. Toxicol.*, 17 (1986) 23.
4 C. M. King, L .J. Romano and D. Schuetzle (Editors), *Carcinogenic and Mutagenic Responses to Aromatic Amines and Nitroarenes*, Elsevier, Amsterdam, 1988.
5 P. P. Fu, M. W. Chou and F. A. Beland, in S. K. Yang and B. D. Silverman (Editors), *Polycyclic Aromatic Hydrocarbon Carcinogenesis: Structure-Activity Relationships*, Vol. II, CRC Press, Boca Raton, FL, 1988, pp. 37–65.
6 S. A. Wise, W. J. Bonnett, F. R. Guenther and W. E. May, *J Chromatogr. Sci.*, 19 (1981) 457.
7 M. A. Stadalius, H. S. Gold and L. R. Snyder, *J. Chromatogr.*, 296 (1984) 31.
8 M. W. Chou, R. H. Heflich, D. A. Casciano, D. W. Miller, J. P. Freeman, F. E. Evans and P. P. Fu, *J. Med. Chem.*, 27 (1984) 1156.
9 P. P. Fu, M. W. Chou, D. W. Miller, G. L. White, R. H. Heflich and F. A. Beland, *Mutat. Res.*, 143 (1985) 173.
10 Y. S. Wu, C. C. Lai, P. P. Fu and L. E. Unruh, *J. Chinese Chem. Soc.*, 35 (1988) 159.
11 K. El-Bayoumy and S. S. Hecht, *Cancer Res.*, 44 (1984) 3408.
12 P. P. Fu, C. C. Lai and S. K. Yang, *J. Org. Chem.*, 46 (1981) 220.

13 P. P. Fu, C. Cortez, K. B. Sukumaran and R. G. Harvey, *J. Org. Chem.*, 44 (1979) 4265.
14 P. P. Fu, M. W. Chou, S. K. Yang, F. A. Beland, F. F. Kadlubar, D. A. Casciano, R. H. Heflich and F. E. Evans, *Biochem. Biophys. Res. Commun.*, 105 (1982) 1037.
15 P. P. Fu and S. K. Yang, *Biochem. Biophys. Res. Commun.*, 115 (1983) 123.
16 P. P. Fu, L. S. Von Tungeln and M. W. Chou, *Carcinogenesis*, 6 (1985) 753.
17 P. P. Fu, R. H. Heflich, L. S. Von Tungeln, D. T. C. Yang, E. K. Fifer and F. A. Beland, *Carcinogenesis*, 7 (1986) 1819.
18 M. W. Chou, R. H. Heflich and P. P. Fu, *Carcinogenesis*, 7 (1986) 1837.
19 M. Mushtaq, Z. Bao and S. K. Yang, *J. Chromatogr.*, 385 (1987) 293.
20 D. C. Locke, *J. Chromatogr. Sci.*, 12 (1974) 433.
21 H. Colin and G. Guichion, *J. Chromatogr.*, 141 (1977) 289
22 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, NY, 1979.
23 M. W. Chou, in W. M. Cooke and A. J. Dennis (Editors), *Ninth International Symposium on Polynuclear Aromatic Hydrocarbons*, Battelle Press, Columbus, OH, 1986, pp. 145–153.
24 M. W. Chou, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 8 (1985) 304.
25 M. W. Chou, D. W. Miller, J. P. Freeman and P. P. Fu, *Chem. Res. Toxicol.*, (1988) 115.
26 P. P. Fu, R. H. Heflich, L. S. Von Tungeln, H. Z. Miranda and F. E. Evans, *Carcinogenesis*, 9 (1988) 951.