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SEPARATION OF AMINO- AND ACETYLAMINO-POLYCYCLIC AROMATIC HYDROCARBONS BY REVERSED- AND NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

In the field of chemical carcinogenesis, amino- and acetylamino-polycyclic aromatic hydrocarbons (PAHs) are among the most studied compounds. Many of these compounds have recently been detected in the environment. Presently, knowledge permitting predictions of the high-performance liquid chromatographic (HPLC) retention order of amino- and acetylamino-PAHs, particularly among their geometric isomers is lacking. In order to obtain a better understanding of the separation of these types of compounds, we have studied the separation of a series of structurally related amino- and acetylamino-PAHs derived from naphthalene, phenanthrene, anthracene, pyrene, benz[*a*]anthracene, benzo[*a*]pyrene, and benzo[*e*]pyrene by using reversed-phase and normal-phase HPLC columns of different types (monomeric, polymeric, and chiral stationary phase). The results indicate: (i) Pirkle-type chiral stationary phase columns and the Zorbax SIL column can efficiently separate both the amino-PAHs and acetylamino-PAHs; (ii) in general, there was no correlation between retention time and molecular size; (iii) when acetylamino-PAHs were separated on the monomeric Zorbax ODS column, the isomer with the acetylamino group located at the carbon position of higher electron density has a shorter retention time; and (iv) separation of the parent PAHs was better than that of the amino-PAHs and acetylamino-PAHs. Our results thus may provide useful information for the analysis of amino-PAHs, particularly for distinguishing the geometric isomers of environmental samples.

* The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Food & Drug Administration.

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INTRODUCTION

Mechanistic studies of metabolic activation of aromatic amines and their amide derivatives is an important research area in chemical carcinogenesis[1,2]. Recent studies have indicated that large quantities of carcinogenic aromatic amines exist in the environment and in natural energy resources, *e.g.* coal distillates[3–8]. Consequently, it is important to detect and identify toxic aromatic amines in the environment. Amino-polycyclic aromatic hydrocarbons (amino-PAHs) constitute the major type of aromatic amines so far studied. There are many geometric isomers that are difficult to separate by conventional analytical methods, including high-performance liquid chromatography (HPLC). There are several reports describing the use of HPLC for the separation of aromatic amines in coal distillates[4–8]. However, the emphasis has been on the detection of these compounds in environmental samples. Little is known concerning the relationships between structural features, such as polarity and molecular size, and HPLC retention time[9–11]. It is known that when a compound is eluted from a HPLC column, polarity and molecular size of the molecule are important factors in determining the HPLC retention time. Polarity is largely related to the type, number, and location of the functional group(s) in the molecule. It has been reported that structural features of amino-PAHs can affect mutagenic and tumorigenic potencies as well as chemical properties of these compounds[12,13]. Consequently, a study of HPLC retention times of amino-PAHs on different types of HPLC columns will help in correlating their structures with mutagenicity–tumorigenicity. For this purpose, we have measured the HPLC retention time of some amino-PAHs and related acetylamino-PAHs on several different HPLC columns (reversed-phase and normal-phase; monomeric, polymeric, and chiral stationary phases).

EXPERIMENTAL

Materials

The structures and abbreviations of the amino-PAHs used in this study are shown in Fig. 1. The following amino-PAHs were purchased from Aldrich (Milwaukee, WI, U.S.A.): 1-A-N, 2-A-N, 1-A-A, 2-A-Ph, 3-A-Ph, 9-A-Ph, 6-A-Ch, 1-A-Py, 2-A-F, and 4-A-Bp. 4-, 6-, 11-, and 12-A-BaP were prepared as previously described[13]. 7-A-BA, 4-A-Py, 1- and 3-A-BeP, 1-, 3-, and 6-A-H_4-BaP, and 1- and 3-A-H_4-BeP were synthesized by reduction of the corresponding nitro-PAH precursors, either with hydrazine and palladium on charcoal in 95% ethanol, or with zinc powder in tetrahydrofuran, methanol, and ammonium chloride[14–17]. 1-, 3-, and 6-Nitro-7,8,9,10-tetrahydrobenzo[*a*]pyrenes and 1- and 3-nitro-9,10,11,12-tetrahydrobenzo[*e*]pyrenes, which were the precursors for preparing the corresponding amino-PAHs, were synthesized as previously described[17]. 6-A-H_2-BaP was synthesized by nitration of 4,5-dihydrobenzo[*a*]pyrene to yield the 6-nitro-4,5-dihydrobenz[*a*]pyrene, which was reduced with hydrazine and palladium on charcoal[15]. 3-A-Ch and 1-A-BaP were synthesized according to published procedures[15,18]. The acetylamino derivatives are similarily abbreviated by adding an additional "A" as a prefix to the abbreviation of the corresponding amino-PAH. For instance while 4-A-Bp designates 4-aminobiphenyl, 4-AA-Bp represents 4-acetylaminobiphenyl. The amido derivatives of the above described amino-PAHs were subsequently synthesized

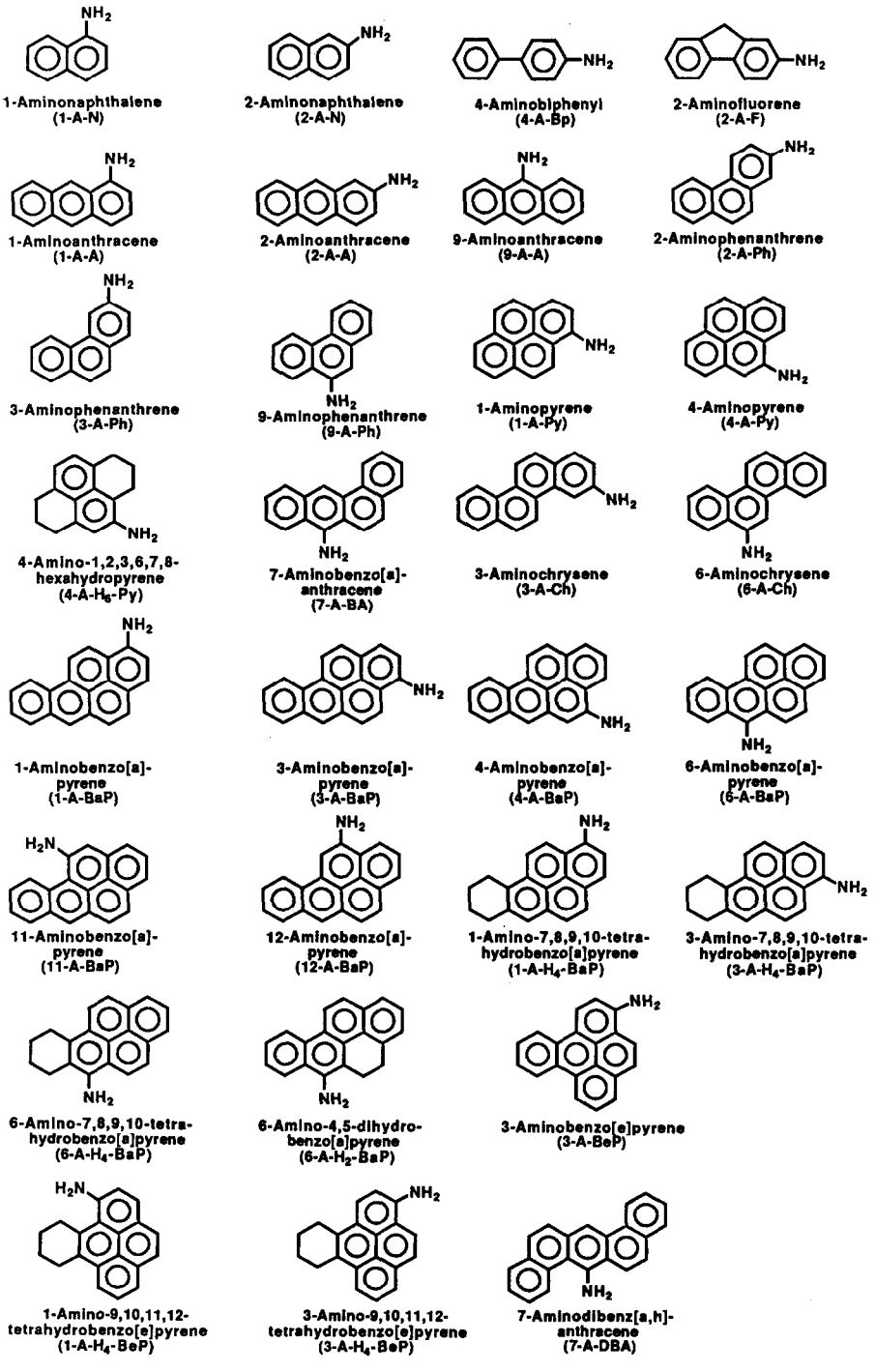

Fig. 1. The names, structure and abbreviations of the amino-PAHs used in this study.

by reacting the amino-PAHs with acetic anhydride and pyridine at ambient temperature for a period of 15 h (ref. 19). All the known, either purchased or synthesized, amino- and acetylamino-PAHs were characterized by comparison of their UV–VIS absorption and mass spectra with the published data. For the identification of the new compounds, high-resolution nuclear magnetic resonance spectral analysis was employed.

Chromatography

The HPLC system was composed of two Beckman/Altex (Fullerton, CA, U.S.A.) Model 100A pumps with prep heads, a Beckman/Altex Model 420 gradient controller, a Beckman/Altex Model 210 injector, a Waters Assoc. (Milford, MA, U.S.A.) Model 440 absorbance detector set at 254 nm, a Kipp & Zonen (Delft, the Netherlands) Model BD41 dual-pen strip-chart recorder, and, optionally, a Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 3390A reporting integrator. A second HPLC system was composed of two Model 510 pump, a Model 680 gradient controller, a U6K injector, a Model 440 absorbance detector (all from Waters Assoc.) set at 254 nm, a Varian (Walnut Creek, CA, U.S.A.) Model A-25 dual-pen strip-chart recorder, and optionally, a Hewlett-Packard Model 3390A reporting integrator. In addition, a Hewlett-Packard 1040A detection system with the Data Processing Unit option was available to be used in place of the Waters Assoc. 440 detector and the appropriate strip-chart recorder. The following columns were used: Zorbax ODS (250 × 4.6 mm I.D.); Zorbax Sil (250 × 4.6 mm I.D.) (DuPont Medical Products, Wilmington, DE, U.S.A.); Vydac 201TP54 (250 × 4.6 mm I.D.) (the Separations Group, Hesperia, CA, U.S.A.); μBondapak Phenyl (250 × 4.6 mm I.D.) (Waters Assoc.); Deltabond C_8 (250 × 4.6 mm I.D.) (Keystone Scientific, State College, PA, U.S.A.) and a Pirkle-type chiral stationary-phase column (250 × 4.6 mm I.D.) (Regis, Morton Grove, IL, U.S.A.) packed with (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine covalently bonded to spherical particles of 5-μm γ-aminopropylsilanized silica. To examine the separation efficiency, a semi-preparative Zorbax ODS column (250 × 21.2 mm I.D.) was employed. All the mobile phases, as described in the footnotes of Tables I and II, were premixed and degassed before use. For each injection 2 μl or less of the solution was used. To eliminate the possible UV photolytic decomposition of the compounds, a UV absorbing film was placed above the lightning diffusion panel.

RESULTS AND CONCLUSION

A set of structuraly related amino-PAHs and acetylamino-PAHs were prepared in order to study the relationships between structure and HPLC retention time. Compounds with slight differences in the structural geometry (*e.g.*, 7-aminobenz[*a*]anthracene *vs.* 6-aminochrysene), molecular size (*e.g.*, 6-aminochrysene *vs.* 6-aminobenzo[*a*]pyrene), and location of the amino/acetylamino functional group served as model compounds for studying the effect of molecular features on HPLC retention time. For comparison, we measured the retention times of these compounds on a monomeric Zorbax ODS column, a polymeric Vydac ODS column, a Deltabond revesed-phase C_8 column, a μBondapak phenyl column, a Zorbax SIL normal-phase column and a Pirkle-type chiral stationary-phase column. Both Zorbax ODS and Vydac ODS columns have a conventional bond between the silicate hydroxyl groups

and the alkyl substituents. Zorbax ODS is a monomeric column and the Vydac ODS is a polymeric column. The Deltabond reversed-phase C_8 column is packed with a uniform matrix of cross-linked polysiloxane functional groups. The findings are summarized below.

Separation of amino-PAHs by reversed-phase and normal-phase HPLC

In general, the Zorbax ODS column, the Zorbax SIL column, and the

TABLE I

HPLC RETENTION TIMES (IN MIN) OF AMINO-PAHs ELUTED FROM DIFFERENT COLUMNS

Compound	*Zorbax ODS**	*Vydac ODS***	*Vydac ODS****	*Zorbax SIL*§	*Deltabond* C_8§§	*Pirkle Ph-Gly*§§§	*Waters phenyl*†
1-A-N	6.5	1.8	2.6	9.7	8.4	4.9	3.2
2-A-N	7.4	1.9	2.7	16.6	8.4	8.0	3.2
4-A-Bp	7.7	2.0	2.8	8.5	8.6	13.1	3.7
2-A-F	9.0	2.1	3.1	9.1	8.8	9.2	3.6
1-A-A	6.2	1.8	2.9	15.6	8.0	8.5	3.2
2-A-A	8.4	2.3	3.4	7.5	8.0	13.6	3.4
9-A-A	6.0	1.7	2.6	19.6	8.0	3.2	2.8
2-A-Ph	8.0	2.4	3.4	9.6	8.4	14.0	3.3
3-A-Ph	8.8	3.6	5.6	9.2	8.4	13.8	3.4
9-A-Ph	7.6	1.9	2.9	14.6	8.6	4.6	3.5
7-A-BA	10.2	6.1	9.2	17.1	–	5.9	3.9
3-A-Ch	10.8	3.6	5.8	10.0	8.8	23.0	4.1
6-A-Ch	9.9	2.5	3.6	8.6	8.7	5.8	3.9
1-A-Py	3.8	8.6	8.0	8.6	8.0	11.4	3.7
4-A-Py	9.2	2.2	3.2	9.0	8.2	12.0	4.0
4-A-H_6-Py	14.3	2.7	3.8	5.7	–	5.5	3.9
1-A-BaP	5.84	1.7	2.6	9.0	–	7.6	3.4
3-A-BaP	5.7	1.7	2.5	15.5	–	8.7	3.4
4-A-BaP	5.8	1.7	2.6	8.9	8.0	9.2	3.4
6-A-BaP	14.2	4.7	7.0	7.8	8.4	41.0	3.8
11-A-BaP	6.2	1.8	2.8	9.6	8.0	9.1	3.4
12-A-BaP	5.6	1.7	2.6	9.1	8.0	9.8	3.4
1-A-H_4-BaP	6.0	1.8	2.6	7.1	10.8	6.3	5.2
3-A-H_4-BaP	5.9	1.7	2.5	9.1	8.0	9.7	3.3
6-A-H_4-BaP	19.1	5.4	8.0	5.8	10.8	20.0	4.2
6-A-H_2-BaP	19.4	4.8	2.7	11.4	–	6.1	3.8
3-A-BeP	5.96	1.9	2.9	9.5	9.6	16.1	3.4
1-A-H_4-BeP	6.1	1.8	2.6	9.6	8.0	10.0	3.1
3-A-H_4-BeP	5.92	1.7	2.5	7.0	10.0	9.5	3.2
7-A-DBA	31.0	3.2	6.6	6.9	–	5.9	–

* 250 × 4.6 mm I.D. column; methanol–water (90:10, v/v); 0.5 ml/min.

** 250 × 4.6 mm I.D. column; methanol–water (90:10, v/v); 1.5 ml/min.

*** 250 × 4.6 mm I.D. column; methanol–water (90:10, v/v); 1.0 ml/min.

§ 250 × 4.6 mm I.D. column; *n*-hexane containing 10% ethanol–acetonitrile (2:1, v/v); 1.0 ml/min.

§§ 250 × 4.6 mm I.D. column; methanol–water (90:10, v/v); 1.0 ml/min. Due to the apparent inefficient separation, the retention times of the compounds marked with "–" were not determined.

§§§ Pirkle-type covalent phenylglycine column (250 × 4.6 mm I.D.); *n*-hexane containing 10% ethanol–acetonitrile (2:1, v/v); 1.0 ml/min.

† 250 × 4.6 mm I.D. column; methanol–water (90:10, v/v); 1.0 ml/min.

Pirkle-type chiral stationary pase column can separate the amino-PAHs (see Table I), but not the Vydac ODS polymeric, the Deltabond column, or the μBondapak phenyl column. This contrasts with the previous report that the Vydac ODS column can separate PAHs and their methyl-substituted and hydroxylated derivatives more efficiently than the monomeric ODS column[20]. Nevertheless, the polymeric Vydac ODS column exhibits a better separation capability for some of the isomeric amino-PAH. For example, while the Zorbax ODS column shows poor separation of 3- and 6-aminochrysene, the Vydac column provides much better separation of these two isomers (Table I). On the other hand, as summarized in Scheme 1, the retention orders of amino-PAHs, separated on the polymeric Vydac ODS column and the monomeric Zorbax ODS column, are the same. These results suggest that the types of interaction of amino-PAHs with the monomeric ODS-bonded-phase adsorbent are similar to those with the polymeric ODS bonded phase adsorbent.

These compounds are not well separated either by the Deltabond C_8 column or by the μBondapak phenyl column (Table I), yielding a very narrow range of retention times of the tested compounds. Since all the active sites of the silica stationary phase in the Deltabond column have been completely shielded by te cross-linked polysiloxane functional groups, this suggests that the active polar sites are important in the separation of the amino-PAHs. The poor separation by the phenyl column indicates the π–π interactions between the bonded phase and amino-PAHs do not facilitate separation.

It is noteworthy to indicate that the separation of amino-PAH will be facilitated by employing a combination of both the reversed-phase and the normal-phase columns. For example, under the experimental conditions, 1- and 3-A-BaP cannot be separated by the Zorbax ODS column, but are well resolved by the Zorbax SIL normal-phase column (Table I and Fig. 2). Our previous reports have shown that on many occasions when a mixture of two or more PAHs, nitro-PAHs, and their ring-oxidized derivatives cannot be separated on a reversed-phase column, they can be nicely resolved on a normal-phase column[21–23].

Separation of the acetylamino-PAHs by reversed-phase and normal-phase HPLC

The results of the separation of acetylamino-PAHs by reversed- and normal-phase HPLC are shown in Table II. The Pirkle-type chiral stationary-phase column exhibits the best separation, and normal-phase HPLC shows a good separation. Like their amino-PAH analogues, these acetylamino-PAHs are not well separated on the Deltabond column. This further indicates that active sites in the silica matrix are involved in the separation mechanism.

Some amino-PAHs are unstable and may undergo air oxidation. Acetylation of an amino-PAH results in a more stable and less polar compound. Comparison of the separation of amino-PAHs and acetylamino-PAHs (Tables I and II) indicates that, in some cases, the acetylamino-PAH isomers are easier to separate than the corresponding amino-PAHs. A good example is the analysis of 3- and 6-A-Ch and 3- and 6-AA-Ch.

Separation of amino-PAHs and acetylamino-PAHs by the Pirkle-type chiral stationary phase column

The Pirkle-type chiral stationary phase column employed was packed with

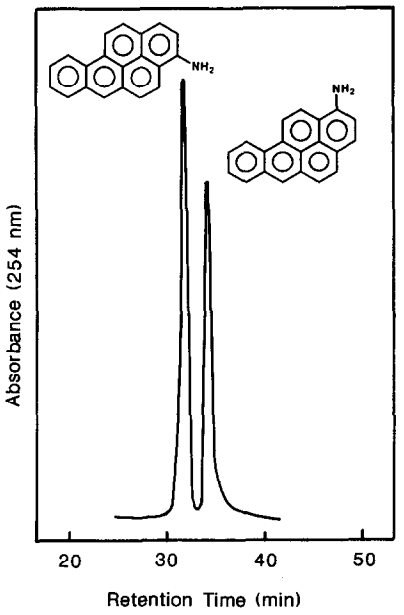

Fig. 2. Separation of 1- and 3-aminobenzo[*a*]pyrene by reversed-phase HPLC employing a semi-preparative Zorbax ODS column (250 × 21.2 mm I.D.).

optically active (R)-N-(3,5-dinitrobenzoyl)phenylglycine, covalently bonded to spherical particles of 5-μm γ-aminopropylsilanized silica. This column has been shown to resolve enantiomers of a large number of compounds, including the ring-oxidized derivatives of PAHs[24–26], and efficiently separates some nitro-PAHs[27,28]. A three-point interaction model was proposed by Pirkle and Finn[29] and others[25,27] for these separations. As shown in Tables I and II, the results indicate that the best separation of both amino-PAHs and acetylamino-PAHs are on the Pirkle-type column. In both cases, the compounds are separated over a wide range of retention times. There is no correlation between the retention time and molecular size of amino-PAH. However, a correlation exists for acetylamino-PAHs: an increased molecular size results in longer retention time. Based on the multiple-recognition-site mechanism, there are four different interaction sites: (i) π-bonding by the aromatic ring; (ii) hydrogen bonding by the amide hydrogen; (iii) hydrogen bonding by the amide carbonyl; and (iv) dipole formation by the amide linkage (Fig. 3A). We propose that there is an additional interaction that facilitates the separation: it is the hydrogen bonding between the nitro groups of the stationary phase and the amino and acetylamino groups of the substrate (Fig. 3B). Additionally, the electron-donating character of the amino and acetylamino groups makes the aromatic rings of the substrate more nucleophilic. This should enhance the π–π interaction of the aromatic ring with the stationary phase, which is electrophilic, due to the two electron-withdrawing nitro substituents. The existence of a correlation between the molecular size and retention time of the acetylamino-PAHs may suggest that acetylamino-PAHs can better fit into the interaction site than the amino-PAHs. Further studies are required for verification of this hypothesis.

TABLE II

HPLC RETENTION TIMES (IN MIN) OF ACETYLAMINO-PAHs ELUTED FROM DIFFERENT COLUMNS

Compound	*Zorbax ODS**	*Zorbax SIL***	*Vydac ODS****	*Deltabond C_8*§*	*Pirkle Ph-Gly*§§*
1-AA-N	10.4	22.6	4.2	8.4	14.5
2-AA-N	15.3	21.4	2.8	8.0	12.1
4-AA-Bp	4.3	22.1	2.6	–	10.9
2-AA-F	10.4	17.1	3.3	7.6	13.9
1-AA-A	8.8	26.5	3.0	8.0	20.5
2-AA-A	10.9	28.8	3.6	8.0	17.2
9-AA-A	8.5	34.2	2.8	8.0	20.8
2-AA-Ph	10.4	29.2	3.4	8.0	18.1
3-AA-Ph	10.5	27.5	3.2	8.4	14.4
9-AA-Ph	8.7	28.6	2.9	8.4	24.1
7-AA-BA	10.0	46.0	10.0	8.4	40.6
3-AA-Ch	15.6	38.0	6.3	8.8	24.5
6-AA-Ch	10.9	34.4	18.6	8.4	27.9
1-AA-Py	10.8	35.2	3.3	8.0	43.6
4-AA-Py	4.5	32.2	2.7	–	37.4
4-AA-H_6-Py	5.3	24.4	2.8	–	13.2
1-AA-BaP	6.1	41.4	5.4	–	64.8
3-AA-BaP	6.1	41.4	5.4	–	64.8
6-AA-BaP	4.8	52.4	3.3	–	65.7
11-AA-BaP	14.8	35.8	4.6	–	53.9
12-AA-BaP	12.8	36.2	4.5	–	54.2
1-AA-H_4-BaP	7.7	31.6	4.8	–	75.4
3-AA-H_4-BaP	7.7	31.6	4.8	–	75.4
6-AA-H_4-BaP	5.7	37.6	3.6	8.4	70.4
3-AA-H_4-BeP	5.5	24.7	3.2	–	

* 250 × 4.6 mm I.D. column; methanol–water (90:10, v/v); 1.0 ml/min.

** 250 × 4.6 mm I.D. column; *n*-hexane containing 20% tetrahydrofuran; 2.0 ml/min.

*** 250 × 4.6 mm I.D. column; methanol–water (90:10, v/v); 1.0 ml/min.

§ 250 × 4.6 mm I.D. column; methanol–water (90:10, v/v); 1.0 ml/min. Due to the apparent inefficient separation, the retention times of the compounds marked with "–" were not determined.

§§ Pirkle-type covalent phenylglycine column (250 × 4.6 mm I.D.); *n*-hexane containing 10% ethanol–acetonitrile (2:1, v/v); 2.0 ml/min.

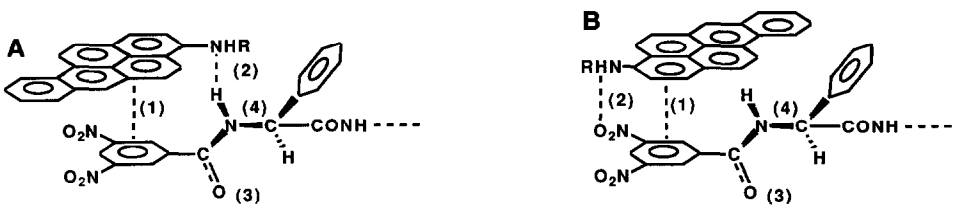

Fig. 3. Proposed three-point interaction model between Pirkle-type chiral stationary phase and the best orientations of 3-aminobenzo[*a*]pyrene (panel A and panel B) for maximum interaction. The number in parenthesis represents the type of interaction, as described in the text.

Relationships between structures and HPLC retention times

The following is a summary of our observations.

(1) For amino-PAHs, retention time in general does not correlate with molecular size (Tables I and II). However, in the case of acetylamino-PAHs, separation by the Pirkle-type chiral stationary-phase column and by normal-phase HPLC, the retention time does correlate with molecular size. Further study on larger series of amino-PAHs is required to determine the factor(s) which decide the retention order.

(2) The retention orders of the isomeric amino-PAHs and acetylamino-PAHs on different columns are summarized in Schemes 1 and 2. When acetylamino-PAHs were separated by the monomeric Zorbax ODS reversed-phase HPLC systems, an interesting phenomenon was observed, as shown in Scheme 2. The isomer having its acetylamino functional group located at the long axis of the aromatic moiety has the longest retention time, and the isomer having its acetylamino group at the shortest axis has the shortest retention time. For example, the retention order of the acetylaminoanthracene series is 2-AA-A > 1-AA-A > 9-AA-A. There exists a correlation in that the compound having its acetylamino group located at the carbon position with the highest electron density of the parent PAH has the sortest retention time. Similar results (with a few exceptions) were obtained with the polymeric Vydac ODS reversed-phase column (Scheme 2) and with the Pirkle-type chiral stationary-phase column. Similar results were also obtained when amino-PAHs were separated on a monomeric Zorbax ODS reversed-phase column.

(3) As shown in Tables I and II, there is no correlation between the retention of the parent PAHs and their amino or acetylamino derivatives. It shows that with an amino or acetylamino group, the resulting compounds are more difficult to separate on the monomeric Zorbax ODS column.

2-A-F > 4-A-Bp > 2-A-N > 1-A-N
2-A-A > 1-A-A > 9-A-A
3-A-Ph > 2-A-Ph > 9-A-Ph
3-A-Ch > 6-A-Ch
6-A-BaP > other A-BaP isomers
6-A-H_4-BaP > 1-A-H_4-BaP = 3-A-H_4-BaP

Scheme 1. HPLC retention times of the isomeric amino-PAHs, eluted from Zorbax ODS and Vydac ODS columns.

1. Eluted from the monomeric Zorbax ODS column
2-AA-N > 1-AA-N ⩾ 1-AA-Bp
2-AA-A > 1-AA-A > 9-AA-A
3-AA-Ph = 2-AA-Ph > 9-AA-Ph
3-AA-Ch > 6-AA-Ch
11-AA-BaP > 12-AA-BaP > 1-AA-BaP = 3-AA-BaP ⩾ 6-AA-BaP

2. Eluted with the polymeric Vydac ODS column
1-AA-N > 2-AA-N
2-AA-A > 1-AA-A > 9-AA-A
2-AA-Ph > 3-AA-Ph > 9-AA-Ph
6-AA-Ch > 3-AA-Ch
1-AA-BaP = 3-AA-BaP > 11-AA-BaP > 12-AA-BaP ⩾ 6-AA-BaP

Scheme 2. HPLC retention times of the isomeric acetylamino-PAHs.

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