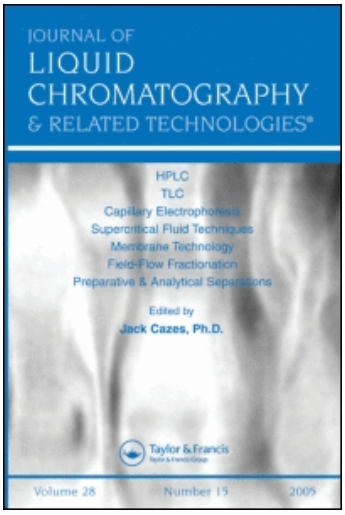


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# Reversed-Phase High-Performance Liquid Chromatography of Unsubstituted Aminobenzoic Acids

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# REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF UNSUBSTITUTED AMINOBENZOIC ACIDS*

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ABSTRACT

High-performance liquid chromatographic (HPLC) characteristics of three position isomers of aminobenzoic acids (potential metabolites of important anesthetic drugs), were delineated with respect to their interactions with various mobile phases and stationary phases. HPLC with five hydrocarbonaceous phases, β-cyclodextrin silica (CDS), macrophase MP-1 polymer (MP), macroporous polystyrene/divinylbenzene (MPD), octadecylsilica (ODS), and propylphenylsilica (PPS), yielded results explicable in terms of substituent effects derived from the bifunctional amino- and carboxy groups. For cases where mobile phases contained sulfonates or quaternary ammonium salts both having longer chain alkyls, retention of analytes on all but CDS appeared to proceed predominantly via an ion-pairing mechanism. The extent of the corresponding counter-ion effects decreased in the order: MPD > ODS > PPS > MP, while the analyte retention order paralleled their $pH_2$ values. On the other hand, an inverse relationship between the magnitude of capacity factors (k') and $pK_1$ values of the title compounds was observed in experiments that produced retention data incompatible with ion-pair interaction rationales. The unique HPLC results obtained with the CDS phase are compared with those obtained with other phases.

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## INTRODUCTION

Ethyl esters of aminobenzoic acdis (ABA) are anesthetic drugs widely used in veterinary medicine. Position isomers of the basic aromatic drugs have been used as local fish anesthetics. Upon metabolic transformation, these ethyl aminobenzoates are known to be hydrolyzed to significant amounts of the ABA products. Reliable quantification of the chemical residues in animal tissues necessitates a comprehensive investigation of the chromatographic behavior of this class of aminoacids.

In view of the highly polar nature of aminobenzoic acids in which an amino group and a carboxy group are both present on the benzene ring, direct analysis of these compounds by high-performance liquid chromatography (HPLC) should be more advantageous than by gas chromatography (GC). If sensitivity requirements are not critical, tedious derivatization procedures often encountered in GC analysis can then be avoided in HPLC assays. A few sporadic reports on HPLC analysis of ABA's have appeared in a number of published works (1-4). However, little is known about the comparative retention behavior of the three position isomers of ABA's in unique chromatographic systems, which have not been described in the literature. Moreover, the title compounds represent a group of HPLC analytes that require special modifications of mobile phase conditions before reasonable success in the normal elution of the ABA solutes can be realized.

Hence, it is a challenge to conduct a systematic study of the influence of reversed-phase HPLC variables on the chromatographic

outcome of ortho-, meta-, and para-aminobenzoic acids (or 2-ABA, 3-ABA, and 4-ABA). In this study, five stationary phases (two of polystyrene/divinylbenzene resins and three of silica-based packings) were chosen for evaluation of stationary phase effects on chromatographic characteristics of ABA's in conventional ion-pairing and ion-interaction systems.

## MATERIALS AND METHODS

### Materials

Ultrapure aminobenzoic acids and sodium alkyl sulfonates were obtained form Aldrich Chemical Co. (Milwaukee, WI.) and were used as received. Sodium perchlorate was acquired from Alpha Products (Danvers, MA.). Other mobile phase electrolytes including buffer salts and HPLC solvents were analytical reagent-grade and were supplied by J. T. Baker (Phillipsburg, NJ.). Quaternary ammonium phosphates were the pure products of Regis Chemicals (Morton Grove, IL.).

Prepacked analytical HPLC columns of five different stationary phases were purchased from various commercial sources: (i) β-cyclodextrin silica (CDS), a product of Advanced Separation Technology, Whippany, NJ. (ii) macrophase MP-1 polymer (MP), a product of Interaction Chemicals, Mountain View, CA. (iii) macroporous polystyrene/divinylbenzene (MPD), a product of Polymer Laboratories, Amherst, MA. (iv) octadecylsilica (ODS), a product of Altex, Berkeley, CA. and (v) propylphenylsilica (PPS), a product of Analytical International, Harbor City, CA. With the

exception of the MP column (15 cm x 4.6 mm ID, 10 um particle size), all other columns used in this study had dimensions of 25 cm x 4.6 mm ID and the column packings were made of 5 um particle size.

Methods

In all HPLC work, a Varian Model LC-5020 liquid chromatograph interfaced with a variable-wavelength UV-Vis detector (Varian Model 110) and a Varian Model 9176 strip chart recorder were used. For chromatographic analysis of aminobenzoic acid samples, the UV detector wavelength was set at 230 nm corresponding to the absorption maxima of the analytes. In a normal procedure, samples (50-100 ppm) were injected via a 10-ul loop of a Valco injector (Model CV-6-UHPa-N60). Mobile phases containing various concentrations of electrolytes in methanol-water systems were pumped (200-250 atm) at a flow rate of 1 ml/min for all but the MP column. The flow rate for the latter column was restricted to 0.5 ml/min as suggested by the manufacturer. For pH studies, 0.05M phosphate buffer solutions (acetonitrile-water mobile phases) were prepared as described elsewhere (5). The void volumes ($v_o$) were determined for the unretained solutes and capacity factors (k') were calculated from the expression: $k' = (v/v_o)-1$, where v is the retention volume of a retained solute (6-8).

## RESULTS AND DISCUSSION

Reversed-phase HPLC of three aminobenzoic acids 2-ABA, 3-ABA, and 4-ABA on each of five stationary phases CDS, MP, MPD, ODS, and

PPS (see MATERIALS AND METHODS for abbreviations) revealed a predictable but nevertheless interesting trend of retention characteristics. Among the ABA's studied, the ortho-isomer (2-ABA) was most strongly retained by most of the phases evaluated. However, in HPLC on CDS with smaller alkyl sulfonates in mobile phases (alkyl < octyl), the para-isomer (4-ABA) appeared to be more strongly retained on this phase than the other two isomers (Table 1).

As shown in Table 1, the retention of ABA's on all but the CDS phase increased with increasing alkyl chain length of sodium alkyl sulfonates (sodium butane sulfonate = SBS, sodium hexane sulfonate = SHS, sodium octane sulfonate = SOS, and sodium decane sulfonate = SDS) added to mobile phases. The magnitude of this sulfonate counter-ion effect was notably dependent on the stationary phase employed and varied in the order MPD > ODS > PPS > MP (the length of the MP column: 15 cm; that of all others: 25 cm). When the results obtained with the silica-based phases ODS and PPS are compared, it is apparent that donor-acceptor interactions between the phenyl groups of ABA analytes and those of PPS phase might be negligible during the chromatographic processes. On the other hand, such pi-pi interactions involving the aromatic moieties of ABA's and the non-silica-based MPD phase must be considerably more important, as greater enhancement in the k'values of ABA's was observed. It is noteworthy that the effect of alkyl sulfonates on the retention of the subject analytes on CDS was distinctly different from the rest of stationary phases

TABLE 1

Retention Data for Aminobenzoic Acids Obtained Under Various HPLC Conditions

| | Capacity factor, k' | | | | | |
|---|---|---|---|---|---|---|
| | Mobile phase electrolyte* | | | | | |
| Compound | $KH_2PO_4$ | $NaClO_4$ | SBS | SHS | SOS | SDS |
| (I) ODS | | | | | | |
| 2-ABA | 1.60 | 1.82 | 1.39 | 2.53 | 3.40 | 10.6 |
| 3-ABA | 0.22 | 0.34 | 0.34 | 1.54 | 2.51 | 8.69 |
| 4-ABA | 0.34 | 0.35 | 0.34 | 1.08 | 1.62 | 3.94 |
| (II) PPS | | | | | | |
| 2-ABA | 1.17 | 1.21 | 1.10 | 1.90 | 2.40 | 3.98 |
| 3-ABA | 0.18 | 0.22 | 0.24 | 0.93 | 1.78 | 3.22 |
| 4-ABA | 0.30 | 0.32 | 0.31 | 0.93 | 1.33 | 2.22 |
| (III) MPD | | | | | | |
| 2-ABA | 22.5 | 16.8 | 15.7 | 18.6 | 21.4 | 29.9 |
| 3-ABA | 0.83 | 0.70 | 0.83 | 3.54 | 6.87 | 16.4 |
| 4-ABA | 3.87 | 2.78 | 2.74 | 4.09 | 5.61 | 10.3 |
| (IV) MP | | | | | | |
| 2-ABA | 11.1 | 3.79 | 4.21 | 4.30 | 4.38 | 4.48 |
| 3-ABA | 0.15 | 0.00 | 0.03 | 0.48 | 0.88 | 2.55 |
| 4-ABA | 1.21 | 0.52 | 0.58 | 0.76 | 1.00 | 1.79 |
| (V) CDS | | | | | | |
| 2-ABA | 0.83 | 0.57 | 0.55 | 0.50 | 0.48 | 0.43 |
| 3-ABA | 0.19 | 0.17 | 0.17 | 0.18 | 0.19 | 0.26 |
| 4-ABA | 1.36 | 0.74 | 0.73 | 0.52 | 0.48 | 0.40 |

*All mobile phases: methanol-water (40:60) with 0.01 M electrolyte concentration at pH 2.5.
For column and salt abbreviations, see RESULTS AND DISCUSSION.

studied. In this instance, lower k' values of 2-, and 4-isomers were obtained from experiments where larger alkyl sulfonates (longer alkyl chains) were used in the mobile phases. This phenomenon is much like the one reported for long-chain high molecular-weight quaternary ammonium salts (7). Unfortunately, the corresponding variation in the k' values of the 3-isomer with the sulfonates tended to be erratic, though there was some indication of a slight increase in retention of the ABA's with the increasing chain length of the alkyl sulfonate additives. In agreement with previous findings on the elution order (2,3), elution on CDS with mobile phases containing lower members of alkyl sulfonates (alkyl < octyl) led to the first emergence of the meta-isomer, followed successively by the ortho-, and para-isomers.



HPLC of ABA's on MPD with a series of quaternary ammonium phosphates [tetrabutylammonium phosphate (TBAP), hexyltriethylammonium phosphate (HXTEAP), heptyltriethylammonium phosphate (HPTEAP), and octyltriethylammonium phosphate (OTEAP)] in mobile phases led to counter-ion effects (Table 2) parallelling to the cases with alkyl sulfonates. Careful examination of the retention data in Tables 1-2 showed a general pattern of elution order depending heavily on chromatographic conditions. In reversed-phase HPLC under conditions compatible with ion-pair retention mechanisms, the k' values of 3-ABA were generally greater than those of 4-ABA. The strongest acid in the series is the 4-isomer, which was found to be the least retained analyte of

TABLE 2

Effects of Quaternary Ammonium Ions on the Retention of Aminobenzoic Acids on MPD

| | Capacity factor, k' | | | |
|---|---|---|---|---|
| | Quaternary ammonium coounter-ion* | | | |
| Compound | HXTEAP | HPTEAP | OTEAP | TBAP** |
| 2-ABA | 3.87 | 5.97 | 6.57 | 5.97 |
| 3-ABA | 1.22 | 1.83 | 2.17 | 1.39 |
| 4-ABA | 0.91 | 1.48 | 1.52 | 1.48 |

*All mobile phases: methanol-water (40:60) with 0.01 M ammonium salt concentration at pH 7.
**The concentration of TBAP was 0.005 M.
For column and salt abbreviations, see RESULTS AND DISCUSSION.



the three isomers. Thus, the elution order of these aminoacids has the same sequence as their $pK_2$ values:

$$k'_{2\text{-ABA}} > k'_{3\text{-ABA}} > k'_{4\text{-ABA}}$$

$$pK_2\ (2\text{-ABA}) > pK_2\ (3\text{-ABA}) > pK_2\ (4\text{\_ABA})$$

In situations where ion-pairing processes were apparently not favorable, the above elution order of the 3-, and 4-isomers was reversed ($k'_{4\text{-ABA}} > k'_{3\text{-ABA}}$). Then, the elution order of the three ABA's is in opposite direction to that of their $pK_1$ values:

$$k'_{2\text{-ABA}} > k'_{4\text{-ABA}} > k'_{3\text{-ABA}}$$

$$pK_1\ (2\text{-ABA}) < pK_1\ (4\text{-ABA}) < pK_1\ (3\text{-ABA})$$

This reversal in the elution order might reflect a change in the retention mechanism and its occurrence in different HPLC systems depended much on the mobile phase conditions and the four stationary phases MP, MPD, ODS, and PPS used (Tables 1-3).

The concentration effects of mobile phase counter-ions (alkyl sulfonates and alkylammonium phosphates) on capacity factors of ABA's are demonstrated in Table 3. An increase in the concentration of either of the two types of salts gave rise to an increase in the retention (higher k' values) of the analytes on either MPD or ODS (also on MP, only when SDS was used ). Conversely, hydrophobic interactions between the analyte solutes and the MP phase were weaker as the concentrations of TBAP or SOS became higher. A contrary to this observation was found in the retention behavior of 3-ABA. In general, there were many chromatographic variables (such as the nature of mobile phase additives, the size of ion-pairing reagents, the type of stationary phases, and mobile phase pH ) contributing to the observed concentration effects of counter-ions in HPLC of ABA's.

Fig. 1 shows pH-dependence of capacity factors of the three isomeric ABA's on stationary phases (A) MP, (B) MPD, and (C) ODS. These familiar downward trends of the k' values with increasing mobile phase pH follow common observations as described in similar studies on Amberlite XAD resins (1) and on various CDS phases (2,3). The neutral analyte species present at lower mobile phase pH presumably interacted more strongly with hydrophobic phases than the dissociated counterparts produced at higher pH values of

TABLE 3

Counter-ion Effects on the Capacity Factors, k', Aminobenzoic Acids on Various Stationary Phases

| HPLC* electrolyte concentration ($M \times 10^3$) | Capacity factor, k' | | |
|---|---|---|---|
| | Compound | | |
| | 2-ABA | 3-ABA | 4-ABA |
| (I) SOS (pH 2.5) | | | |
| 0.5 | 17.8 | 1.48 | 3.21 |
| 1.0 | 18.2 | 2.13 | 3.56 |
| 5.0 | 20.2 | 5.09 | 4.74 |
| 10.0 | 21.4 | 6.87 | 5.61 |
| 15.0 | 21.8 | 8.13. | 5.96 |
| (II) HPTEAP (pH 7) | | | |
| 5.0 | 4.78 | 1.61 | 1.26 |
| 10.0 | 5.97 | 1.83 | 1.48 |
| 15.0 | 7.39 | 2.08 | 1.52 |
| 20.0 | 9.09 | 2.30 | 1.63 |
| (III) SOS (pH 2.5) | | | |
| 10.0 | 4.38 | 0.88 | 1.00 |
| 20.0 | 3.67 | 1.03 | 0.91 |
| (IV) SDS (pH 2.5) | | | |
| 10.0 | 4.48 | 2.55 | 1.79 |
| 20.0 | 5.22 | 3.00 | 2.15 |
| (V) TBAP (pH 7) | | | |
| 5.0 | 1.27 | 0.39 | 0.37 |
| 10.0 | 1.09 | 0.27 | 0.23 |
| (VI) SDS (pH 2.5) | | | |
| 1.0 | 4.21 | 3.22 | 1.55 |
| 5.0 | 7.53 | 5.92 | 3.26 |
| 10.0 | 10.6 | 8.69 | 3.94 |

*All mobile phases: methanol-water (40:60). Columns used were of MPD (I and II), MP (III,IV and V), and ODS (VI).
For column and salt abbreviations, see RESULTS AND DISCUSSION.

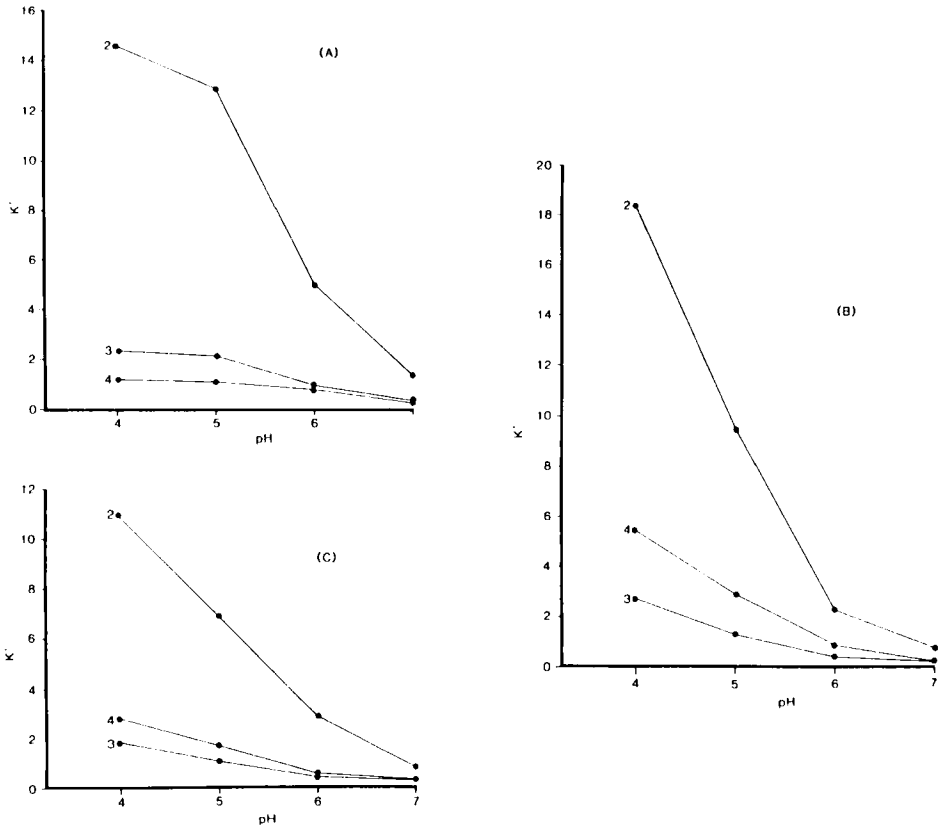


Fig. 1.

Effects of pH on the capacity factor, k', of isomeric aminobenzoic acids 2-ABA, 3-ABA, and 4-ABA. HPLC conditions: 0.05M phosphate buffers in acetonitrile-water in ratios (A) 3:7 and (B,C) 1:9; stationary phases, (A) MP, (B) MPD, and (C) ODS; flow rates, (A) 0.5 ml/min, and (B,C) 1 ml/min.See text for column abbreviations.

mobile phases. The curvature of the k'-pH plots of MP and MPD as well as the elution order of the three isomers are somewhat different, although both these columns were made of similar packing materials of polystyrene divinylbenzene resins. Taking advantage of their stability in basic eluents, pH studies were further extended to the pH 8-12 region (not shown). No drastic changes in the retention curves were detectible for both MP and MPD phases. As the eluent pH was raised beyond pH 7, the retention curve for the former showed a slight decline in the k' values, while the other curve in the latter case began to level off.



Reversed-phase HPLC on each of MP, MPD, ODS and PPS phases showed that, invariant with the chromatographic conditions used, the ortho-isomer was always retained longer than others. This might be attributed to the hydrogen bonding between the bifunctional amino $NH_2$ group and the carboxy COOH group on adjacent aromatic ortho-carbons forming a six-membered partially bonded-ring system of less polarity. Accordingly, hydrophobic interactions between the ortho-analyte solutes and hydrocarbonaceous phases should be much more enhanced than those of meta-, and para-isomers. Fig. 2 gives typical examples of chromatograms for comparing separations of ABA' mixtures on (A) MP and (B) MPD , both of nonpolar aromatic resins. The mobile phase conditions including flow rates (an intermediate pH 4.4 was used instead) employed in theses instances are different from those discussed so far. Additional retention data obtained at this pH

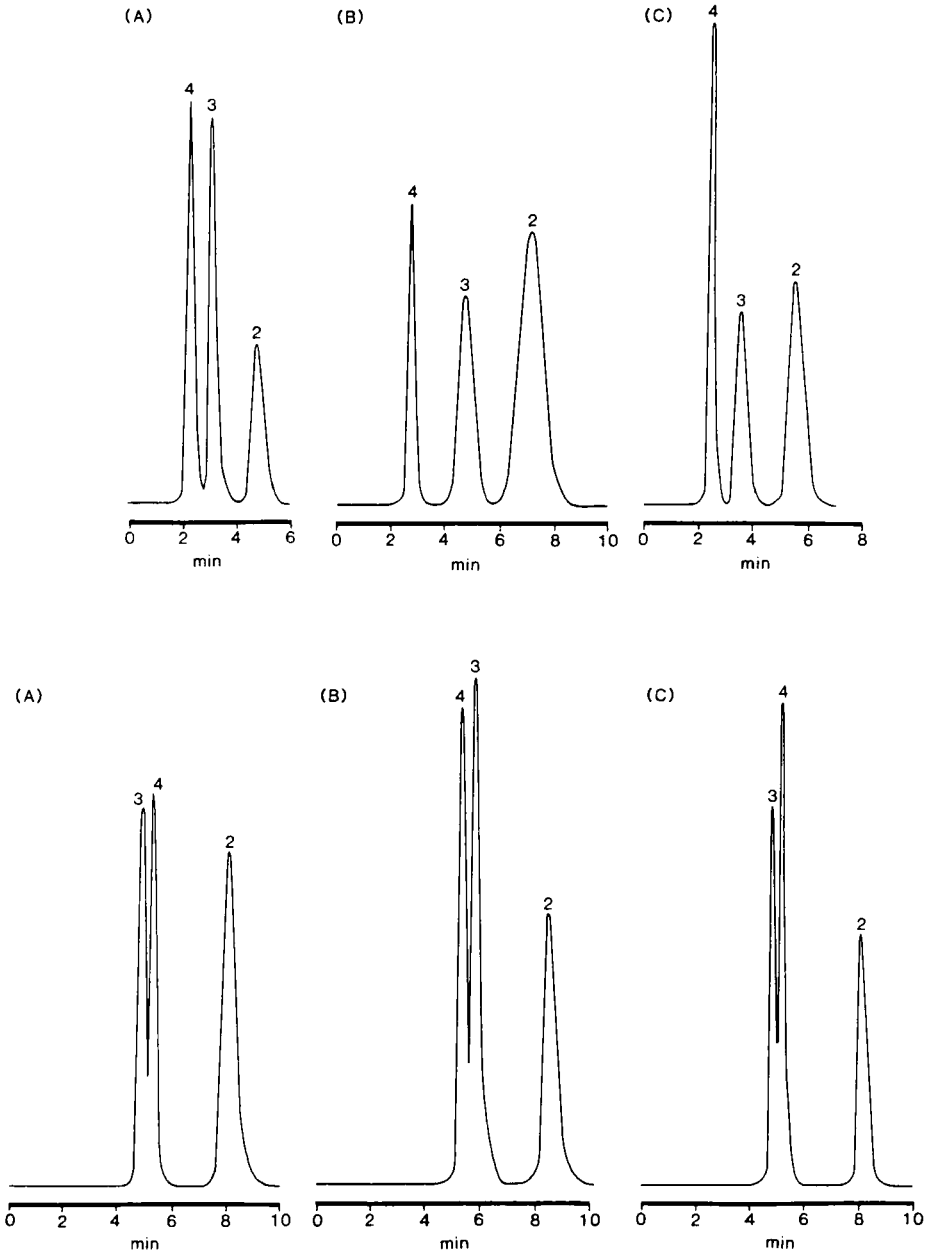


Fig. 2.

Typical chromatograms showing separation of isomeric aminobenzoic acids 2-ABA, 3-ABA, and 4-ABA. HPLC conditions: mobile phases, methanol-water (4:1) containing (A) 0.01M SA + 0.01M SHPS, (B) 0.01M SA, and (C)0.01M SA + 0.01M (top) or 0.02M (bottom) $NaClO_4$; flow rates, 1 ml/min (top) and 0.7 ml/min (bottom). SA = sodium acetate. SHPS = sodium heptane sulfonate.

are shown in Table 4 to illustrate the mobile phase effects entailing various electrolyte compositions on retention characteristics of ABA's. The elution order of ABA's on MP tended to be as follows: $k'_{2ABA} > k'_{3-ABA} > k'_{4-ABA}$, whereas increasing concentrations of either SOS or $NaClO_4$ reduced the k' values. On the other hand, the elution order in the case of the MPD phase was noted as follows: $k'_{2-ABA} > k'_{4-ABA} > k'_{3-ABA}$. A gradual increase in k' values was noticeable in response to an increase in the concentration of SOS (Table 4).

The results of this study suggest that the retention behavior of the three ABA's isomers in reversed-phase systems (excluding the CDS variant), where mobile phases employed long chain alkyl-sulfonate- or quaternary ammonium counter-ions for the respective charged bifunctional groups $NH_2$ and COOH, can be interpreted based on hydrophobic ion-pair interaction rationales (9-11). Evidently, the HPLC variables in both mobile phases and stationary phases play crucial roles in determining the modes of chromatographic processes. The difference in chromatographic outcome of ABA's on the two polymeric resin phases MP and MPD can be partly ascribed to the relatively less retentive property of the former because of its extra octadecyl groups covalently bonded to the polystyrene divinylbenzene resins. The unique HPLC results obtained with the CDS phase are indicative of a unique retention mechanism characterized by inclusion complex formation (12-14).

The versatile reversed-phase HPLC techniques presented here can be applied to the simultaneous separation and quantification

TABLE 4

Comparisons of the Retention Behavior of Aminobenzoic Acids on Nonpolar Phases MP and MPD at an Intermediate pH 4.4

| Mobile phase* | Capacity factor, k' | | |
|---|---|---|---|
| Methanol-water (80:20) | Compound 2-ABA | 3-ABA | 4-ABA |
| (I) MP | | | |
| 0.01M SA<br>0.01M SOS | 6.57 | 4.23 | 3.36 |
| 0.01M SA<br>0.05M SOS | 4.84 | 3.45 | 2.82 |
| 0.01M SA | 6.30 | 3.81 | 3.03 |
| 0.01M SA<br>0.01M $NaClO_4$ | 7.83 | 5.o8 | 3.44 |
| 0.01M SA<br>0.05M $NaClO_4$ | 5.30 | 3.52 | 2.81 |
| (II) MPD | | | |
| 0.01M SA<br>0.01M SOS | 5.70 | 3.31 | 3.62 |
| 0.01M SA<br>0.05M SOS | 6.43 | 3.97 | 4.26 |
| 0.01M SA | 5.83 | 3.64 | 3.72 |

* Flow rates were 0.7 ml/min (I) and 1 ml/min (II).
SA = sodium acetate
For column and salt abbreviations, see RESULTS AND DISCUSSION.

of the three position isomers of aminobenzoic acids. With emphasis on the optimization of HPLC conditions, HPLC elution of the title compounds can be met with much success without undesirable peak tailing, peak broadening or analyte adsorption on a column.

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