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EFFECTS OF AMINE MODIFIERS ON RETENTION AND PEAK SHAPE IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A systematic evaluation of the effects of 15 different amine modifiers on the retention and peak symmetry of three solutes, a primary, a secondary, and a tertiary amine, is presented. Using automated experimentation, mobile phase combinations for each modifier over a pH range of 2.5 to 8 were investigated. The effect of changing the sodium ion concentration of the mobile phase was also examined. The importance of hydrophobic, ion exchange, and hydrogen bonding interactions as mechanisms for retention and peak symmetry of positively charged solutes is discussed.

INTRODUCTION

The widespread popularity of reversed-phase high-performance liquid chromatography (RP-HPLC) is partially due to the chromatographer's ability to vary mobile phase composition to enhance selectivity or to improve other separation characteristics. Solutes with amine functional groups often exhibit poor chromatographic performance on silica-based HPLC columns. The chromatography of these solutes can be improved by adding a modifying agent to the mobile phase. One group of mobile phase modifying agents was termed "ion-pairing" reagents because it was thought that ion-pairs were formed between the modifier and the solute molecules in the mobile phase[1]. Ion-pairing was visualized as masking the ionic portions of the solute molecules, making them more hydrophobic and thus increasing the retention of ionic solutes in RP-HPLC. Several researchers have recently questioned this retention mechanism and have provided alternative mechanisms[2–4].

Bidlingmeyer *et al.*[2] proposed a surface ion interaction mechanism of solute retention in which modifying agents form an active primary layer in close contact

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with the stationary phase. The retention of solutes could then be increased or decreased depending on the charge of the solute and the charge of the ion interaction reagent (IIR). Bij *et al.*[3] and Nahum and Horváth[4] proposed a retention model based on a dual adsorption mechanism in which both hydrophobic and silanophilic interactions on the surface of the RP stationary phase dictate the chromatographic behavior of solutes. This model describes the role of silanol masking agents in decreasing the retention of charged solutes.

While these mechanisms explain the effect IIR's have on the retention of charged solutes, they do not clearly explain improvements in peak symmetry that occur when IIR's are added to the mobile phase. The question of which types of IIR's will be most effective in improving peak shape for charged solutes has also not been addressed. Several researchers have previously reported that peak shape and other chromatographic characteristics of tricyclic amines are determined by the amine group on the side chain of the molecule[5,6]. Peak shape (and thus the resolution) of tricyclic amines can be improved by the addition to the mobile phase of an organic amine modifier in low concentration (Fig. 1). In this article, we report a systematic investigation of the effects of 15 different amine modifiers on the chromatographic behavior of three tricyclic amines. The test solutes include a tertiary amine (amitriptyline), a secondary amine (nortriptyline), and a primary amine (desmethylnortriptyline). The structures of these three solutes are shown in Fig. 1.

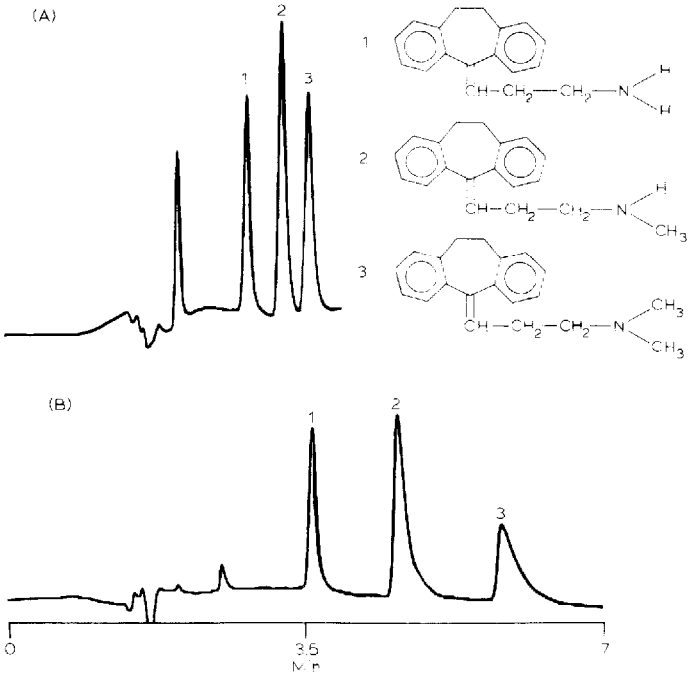

Fig. 1. A chromatogram of the three solutes: (1) desmethylnortriptyline, (2) nortriptyline, and (3) amitriptyline, with the mobile phase 50% acetonitrile, pH 4 with (A) no amine modifier and (B) 25 m*M* triethylamine added to the mobile phase.

EXPERIMENTAL

Reagent grade methylamine HCl, dimethylamine HCl, trimethylamine HCl, tetramethylammonium bromide, ethylamine HCl, diethylamine, triethylamine, tetraethylammonium bromide, *n*-propylamine, *n*-dipropylamine, *n*-tetrapropylammonium bromide, *n*-butylamine, *n*-dibutylamine, *n*-tetrabutylammonium bromide, and *n*-pentylamine were obtained from Aldrich (Milwaukee, WI, U.S.A.). Reagent grade sodium hydroxide, HPLC grade acetonitrile, and orthophosphoric acid were purchased from Fisher Scientific (Atlanta, GA, U.S.A.). Amitriptyline (AMI), nortriptyline (NOR), and desmethynortriptyline (DES) were provided by Merck, Sharpe, and Dohme Research Labs. (Rahway, NJ, U.S.A.).

Experiments were carried out on a Waters HPLC system (Waters Assoc., Milford, MA, U.S.A.) equipped with a Model 441 UV absorbance detector (operated at 254 nm), three Model 6000A pumps, a Model 710B autoinjector, a Model 720 integrator, and a Model 730 system controller. A Supelcosil C_8 reversed-phase column (250 × 4.6 mm) packed with 5-μm packing, fitted with a C_8 guard column (50 × 4.6 mm) packed with 40 μm packing (Supelco, Bellefonte, PA, U.S.A.) was used. Automated experimentation was carried out using the three pumps working together under control of the system controller. Three pumping solutions were prepared (A, B, and C) and delivered isocratically to the chromatographic system by pumps A, B, and C, respectively. Solution A consisted of 0.1 *M* phosphoric acid in acetonitrile–water (50:50), solution B was 0.1 *M* sodium hydroxide in acetonitrile–water (50:50), and solution C was 0.1 *M* amine modifier in acetonitrile–water (50:50). By adjusting the flow-rates of the three pumps, the pH of the mobile phase and concentration of amine modifier in the mobile phase could be set. The total flow-rate was held at 2 ml/min and the flow-rate of pump C was adjusted to 25% of total flow, thus fixing the concentration of amine modifier at 25 m*M* for all experiments. The system controller was preprogrammed to perform the set of experiments for each amine modifier varying the pH of the mobile phase by changing the flow-rates for pumps A and B. A delay of 20 min (approximately 10 column volumes) after adjustment of the mobile phase composition was found to be sufficient for the column to reach equilibrium before the next experiment was initiated, as evidenced by a stable detector signal.

The pH of each mobile phase combination was monitored with a Model 601A ion analyzer (Orion Research, Cambridge, MA, U.S.A.) equipped with a semi-micro combination pH electrode (Corning, PA, U.S.A.) encased in a glass flow cell located immediately after the UV detector. The ion analyzer signal was recorded continuously on a strip chart recorder for later reference.

The retention time for each peak was measured manually on each chromatogram. The dead volume (t_0) of the system was measured as the first distortion of the baseline after injection of water. The capacity factor, k', for each solute under the given experimental conditions was calculated. Each solute data set for each discrete modifier over the continuous pH range was fitted to a quadratic model using a matrix least squares method[7]. The capacity factor response (k') was then plotted as a function of pH and type of amine modifier using a pseudo-three dimensional plotting program[8]. The measurement of chromatographic peak symmetry has been discussed recently by Foley and Dorsey[9]. In our work, the symmetry of each chromatographic peak was evaluated as shown in Fig. 2. Symmetry data was also fitted to quadratic models for each of the solutes as a function of pH and the resulting curves plotted.

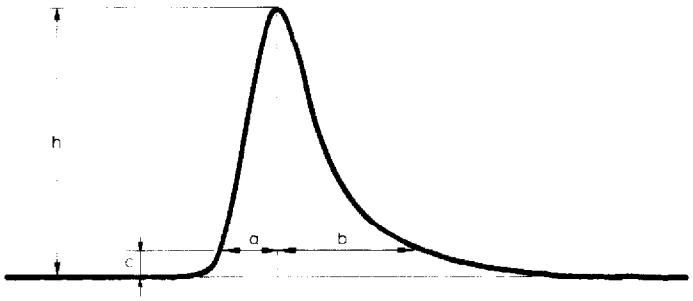

Fig. 2. Peak symmetry factor = $a/b \times 100$, with a and b measured at 10% peak height (h).

RESULTS AND DISCUSSION

The influence of mobile phase pH on the retention of AMI, NOR, and DES is shown in Fig. 3. When the pH of the mobile phase is greater than 5.5, the retention of the tertiary amine, AMI, is increased compared to that at lower pH levels. The retention of the secondary amine, NOR, and the primary amine, DES, are less influenced by changes in the mobile phase pH above 5.5. These amine solutes have relatively high pK_a values and are protonated at mobile phase pH levels below 8. Increased retention at high pH has been attributed to ion exchange effects between the positively charged protonated amine solutes and the negatively charged unprotonated silanol sites on the stationary phase[3,4]. At levels of mobile phase pH below 5.5, silanol sites are well protonated and the retention of amine solutes is influenced by the ability of the solutes to compete for the remaining ion exchange sites on the silica surface of the stationary phase. Lowering the mobile phase pH below 5.5 should not,

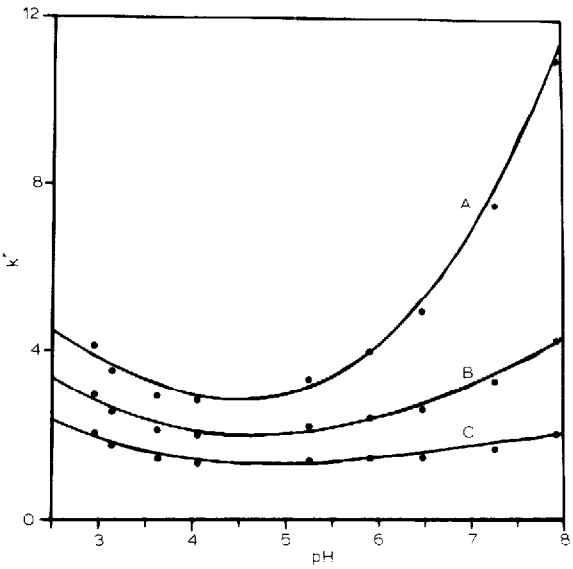

Fig. 3. The relationship between k' and pH for the three solutes with no amine modifier added: (A) amitriptyline, (B) nortriptyline, (C) desmethylnortriptyline.

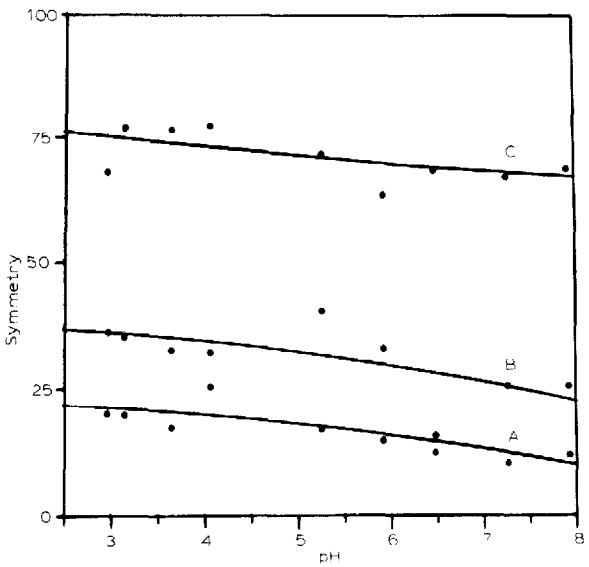

Fig. 4. Relationship between symmetry factor (S) and pH for the three solutes with no amine modifier added: (A) amitriptyline, (B) nortriptyline, (C) desmethylnortriptyline.

however, further affect the ion exchange sites or the retention of ionic solutes. The increase in the retention of the solutes at pH levels below 4 (see Fig. 3) is due to the decrease in sodium ion concentration in the mobile phase and will be discussed below.

Fig. 4 shows the variation in symmetry factor for each of the three solutes over a mobile phase pH range from 2.5 to 8.0. The tertiary amine solute, AMI, exhibits the greatest peak asymmetry over the pH range examined and there is little improvement in peak shape when the mobile phase is made more acidic. The secondary amine, NOR, also shows substantial peak tailing with the primary amine, DES, exhibiting the most symmetric peak shape over the pH range investigated.

Effect of sodium ion concentration

The experimental procedure employed adjusted the pH of the mobile phase from basic to acidic conditions by decreasing the sodium hydroxide concentration and increasing the phosphoric acid concentration in the mobile phase. A decrease in sodium ion concentration in the mobile phase should increase the retention of high pK_a amines because of cationic competition for the ion exchange sites on the silica surface[9]. To confirm this hypothesis, additional experiments were conducted using a single pump delivering mobile phases containing sodium ion concentrations at three different levels (10, 25, and 50 mM) as sodium phosphate in acetonitrile–water (50:50). The mobile phase pH was adjusted to 4 different levels (pH 3, 4, 5, 6) by adding phosphoric acid. This procedure insured that the effect of pH and sodium ion could be independently assessed. The results from this 3 × 4 factorial design were fitted by a full second order model in the two factors[7]. Fig. 5 presents a plot of the fitted model for the retention of one of the three tricyclic amine solutes (AMI) at different sodium ion concentrations over the mobile phase pH range of 3 to 6.

The retention of the other two solutes is similar to that shown for AMI: the

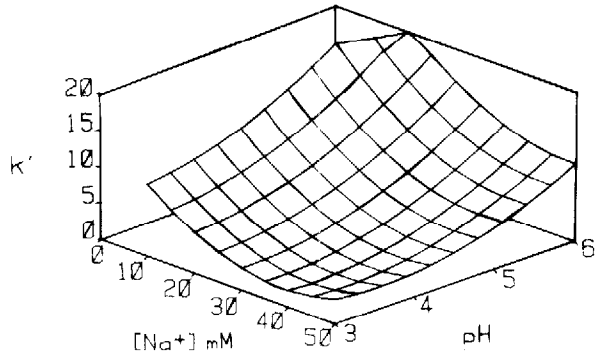

Fig. 5. Capacity factor (k') for amitriptyline as a function of mobile phase sodium ion concentration and pH.

k' values are strongly influenced by both sodium ion concentration and the pH of the mobile phase. Fig. 5 does not show the increase in retention for AMI at low mobile phase pH that is seen in Fig. 3. These results indicate that the increase in retention at low pH seen in Fig. 3 is due to the decreased sodium ion concentration in the mobile phase. The symmetry factor for AMI as a function of sodium ion concentration and pH is plotted in Fig. 6. The peak symmetry of NOR and DES was similarly unaffected by changes in pH or sodium ion concentration.

Modifying agents and peak retention

The effect of several silanol masking agents (IIR's) on the chromatography of the three basic amine solutes was investigated. Because the tertiary amine solute, amitriptyline, exhibited the greatest dependence of retention time and peak symmetry on variations in the mobile phase conditions (pH, sodium ion concentration, type of IIR), in the following sections we discuss these effects for AMI alone. The primary and secondary amine solutes, DES and NOR exhibit similar, but less dramatic effects, with variations in mobile phase conditions. Fig. 7 illustrates the variations in retention time (actually k') for amitriptyline as a function of pH and hydrocarbon side chain length when primary, secondary, tertiary, and quaternary amine modifiers are added to the mobile phase at a concentration of 25 mM. In each of these pseudo-

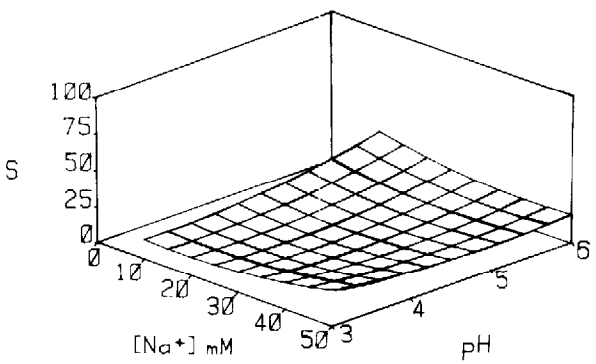

Fig. 6. Peak symmetry (S) for amitriptyline as a function of mobile phase sodium ion concentration and pH.

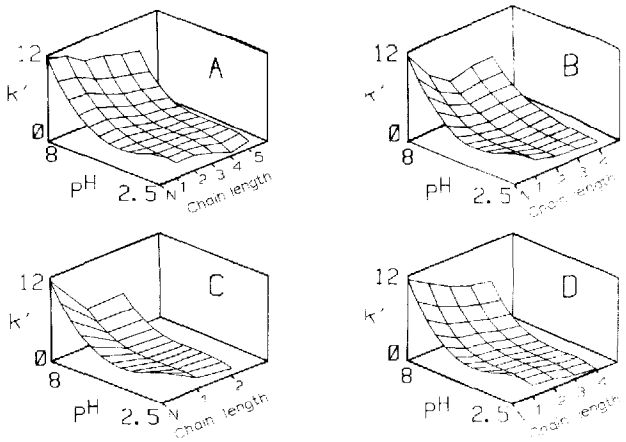

Fig. 7. The relationship between capacity factor (k') for amitriptyline and mobile phase pH and different amine modifiers: A = primary amine modifiers with side chains of one to five carbons, B = secondary amine modifiers with side chains of one to four carbons, C = tertiary modifiers with side chains of one or two carbons, D = quaternary modifiers with side chains of one to four carbons.

three dimensional plots, the line depicting the dependence of k' on pH with no modifier added to the mobile phase is shown as the "N" curve on the "chain length" axis.

It can be seen that, in case of primary modifiers (Fig. 7A), the effect of pH on k' when no modifier was added to the mobile phase and for when the shortest chain (chain length equal to 1) modifier was added was virtually identical at pH levels greater than 4. The addition of longer chain primary amine modifiers (up to *n*-pentylamine) decreases k' at all levels of pH. A stronger effect of reduced k' is observed when secondary amine modifiers are added to the mobile phase (Fig. 7B), with the longer chain length amine modifiers decreasing the k' of AMI the greatest. The effect of two tertiary amine modifiers on the k' of AMI is illustrated in Fig. 7C [longer chain tertiary modifiers were not miscible at a concentration of 0.1 *M* in acetonitrile–water (50:50)]. Trimethylamine and triethylamine appear to be equally effective in decreasing the retention time of the solute. The effect of quaternary amine modifiers is shown in Fig. 7D; again the longer chain modifiers decrease solute retention more than the shorter chain quaternary amine modifiers.

Fig. 8 shows a comparison of the k' values for AMI when methyl or ethyl amine modifiers with differing degrees of side chain substitution are added to the

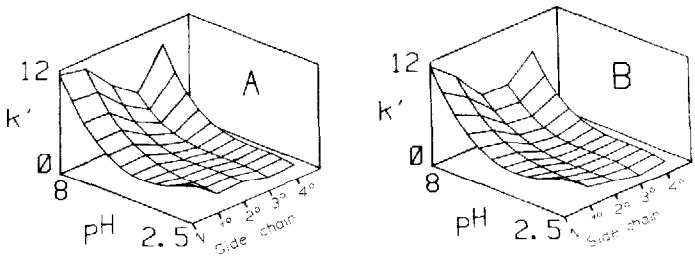

Fig. 8. The relationship between capacity factor (k') for amitriptyline and mobile phase pH and different amine modifiers: (A) 1 = methylamine, 2 = dimethylamine, 3 = trimethylamine, 4 = tetramethylamine; (B) 1 = ethylamine, 2 = diethylamine, 3 = triethylamine, 4 = tetraethylamine.

mobile phase. The effectiveness of methyl and ethyl amine modifiers follow a similar trend with the tertiary modifying agents more effective in reducing retention of the amitriptyline solute.

Modifying agents and peak symmetry

The effect of different IIR's on the peak symmetry of the three amine solutes was also examined. As before, the data for AMI will serve as the focus of our discussion. The addition of primary amine modifiers to the mobile phase resulted in only a slight improvement in peak symmetry for AMI (Fig. 9A). There is no apparent difference between the effects on peak shape for the primary amine modifiers of different chain length. When a secondary amine modifier is added to the mobile phase, however, a noticeable difference is found between the modifiers of different chain length (Fig. 9B). Dimethylamine and diethylamine are substantially better for improving the peak symmetry of AMI than either dipropylamine or dibutylamine. The tertiary modifying agents (trimethylamine and triethylamine) were equally effective in improving peak symmetry (Fig. 9C). None of the quaternary modifiers substantially improved peak symmetry for AMI (Fig. 9D). Changing the chain length of the quaternary modifiers had no effect on peak symmetry.

Fig. 10 shows the effect primary, secondary, tertiary, and quaternary amine modifiers of the same chain length (methyl and ethyl amines) had on the peak symmetry of AMI. It is clear from these data that the tertiary amine modifiers were the most effective in improving the peak symmetry of amine solutes. Secondary amine modifiers are also effective in improving the peak shape of amine solutes, with primary and quaternary amine modifiers improving peak symmetry only to a small extent.

Mechanisms in ion interaction reversed-phase HPLC

The behavior shown by the amine solutes in our studies is predicted well by the two site mechanism of retention on reversed phase packing materials described

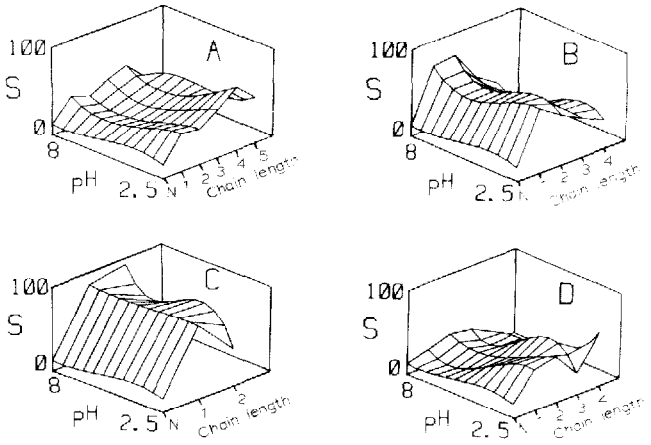

Fig. 9. The relationship between symmetry factor (S) for amitriptyline and mobile phase pH and different amine modifiers: A = primary amine modifiers with side chains of one to four carbons, B = secondary amine modifiers with side chains of one to four carbons, C = tertiary modifiers with side chains of one or two carbons, D = quaternary modifiers with side chains of one to four carbons.

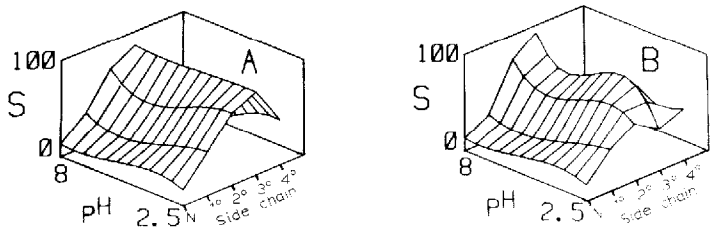

Fig. 10. The relationship between symmetry factor (S) for amitriptyline and mobile phase pH and different amine modifiers: (A) 1 = methylamine, 2 = dimethylamine, 3 = trimethylamine and 4 = tetramethylamine; (B) 1 = ethylamine, 2 = diethylamine, 3 = triethylamine, 4 = tetraethylamine.

by Horváth and co-workers[3,4]. A solute molecule encounters a complex environment as it interacts with the silica based hydrocarbonaceous surface and with the components of the mobile phase (modifying agents, organic solvents, water, inorganic ions, etc.) which themselves have the potential to form an adsorption layer in close contact with the stationary phase. Our discussion will be limited to the surface interactions between solute molecules and the stationary phase and support as shown in Fig. 11.

Hydrophobic interactions between nonpolar moieties of the solute molecule and the nonpolar carbon chain of the stationary phase (Fig. 11A) might contribute only slightly to the selectivity between similarly structured solutes. The role of other mechanisms for the retention of charged solutes, such as ion exchange, has been addressed by several investigators[9-12]. Such silanophilic interactions may dictate potential differences in chromatographic retention much more than the hydrophobic effects. The positively charged solute competes with mobile phase solvent molecules for interaction on ion exchange sites of the silica support with solute retention depending on how well the solute displaces other adsorbed cations (Fig. 11B). Hydrogen bonding of solutes to silanol sites on the silica based support is a third mechanism for peak retention (Fig. 11C). Tertiary and secondary amine solutes have greater hydrogen bonding characteristics compared to primary amines and, thus can be expected to have a greater hydrogen bonding interaction at the support surface. A positively charged organic IIR added to the mobile phase is affected by the same three types of interactions with the stationary phase and support. These hydrophobic,

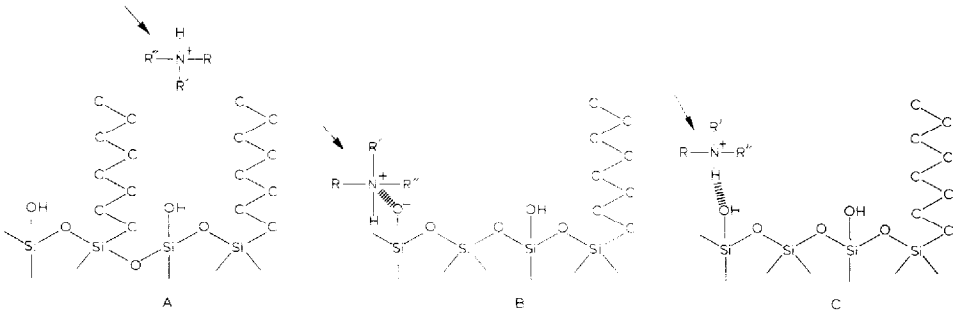

Fig. 11. Potential interactions of a tertiary amine molecule with the reversed phase surface and silica support: (A) hydrophobic interaction, (B) ion exchange, (C) hydrogen bonding.

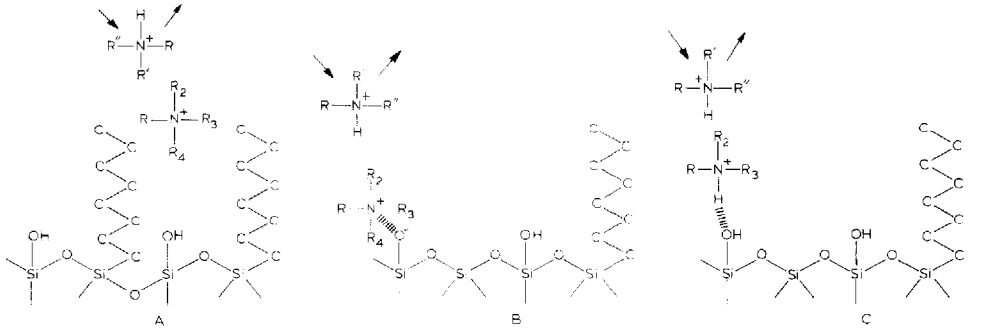

Fig. 12. Potential interactions of amine modifiers on the reversed-phase surface and silica support: (A) charged surface coating the hydrocarbonaceous moieties repelling solute molecules, (B) amine modifiers blocking an ion exchange site on the silica support, (C) a tertiary amine modifier blocking a hydrogen bonding site on the silica support.

ion exchange, and hydrogen bonding interactions may be visualized as depicted in Fig. 12.

Hydrophobic interactions of IIR molecules with the alkyl chains of the stationary phase depend on the length of the alkyl portion of the IIR molecule and the number of alkyl groups attached to the amine group of the IIR. The longer the alkyl chain on the IIR, the stronger the affinity will be for the hydrocarbon chain of the stationary phase. Increasing the alkyl chain length of the IIR will produce a denser charge layer around the hydrophobic portions of the stationary phase which might block positively charged solute molecules from hydrophobic interactions. Increasing the number of nonpolar substituent groups on the IIR molecule will increase the IIR's hydrophobic character and interaction with the alkyl portions of the reversed phase material causing an increased localized charge layer on the stationary phase. With positively charged IIR's (such as the amine modifiers investigated here), a positively charged layer will be formed on the reversed phase packing which will tend to repel any positively charged solutes from interaction with the stationary phase, thereby decreasing the solute retention time (Fig. 12A).

Ion exchange interactions at the surface of the support material in RP-HPLC will also be affected by the addition of a positively charged IIR to the mobile phase (Fig. 12B). Interactions between the IIR molecules and the silanol sites on the silica support will be determined to a large extent by the balance between the hydrophobic and silanophilic effects. IIR molecules with shorter hydrophobic side chains will be less strongly attracted to the stationary phase carbon chain and will tend to adsorb better onto the silanol sites of the support.

The main cause of peak asymmetry for the amine solutes examined in this study involves hydrogen bonding interactions with silanol sites on the silica support material. The addition to the mobile phase of modifying reagents which have the strongest hydrogen bonding characteristics controlled the tailing of amine solutes, apparently by blocking solute hydrogen bonding interactions with the surface silanol groups (Fig. 12C). The short chain tertiary amine modifiers, trimethylamine and triethylamine, were the most effective in reducing peak tailing of the amine solutes, by efficiently penetrating to the support silanol groups and blocking potential solute interactions (Figs. 9 and 10). In the case of quaternary modifying agents, because the

nitrogen group is not protonated, these modifiers can not compete for hydrogen bonding sites and thus have little effect on peak symmetry.

CONCLUSION

A critical evaluation of the effectiveness of several mobile phase amine modifiers for ion interaction reversed-phase liquid chromatography (RP-LC) has been presented and the behavior of ionic solutes in the presence of these ion interaction reagents has been discussed. The best mechanism for charged solute retention in RP-LC is a mixture of hydrophobic, ion exchange, and hydrogen bonding interactions. Hydrophobic effects appear to be of secondary importance for the retention of some basic solutes in RP-HPLC; ion exchange and hydrogen bonding interactions with the silica surface of the support are probably of greater importance. The poor peak symmetry seen for amine solutes in RP-LC without mobile phase modifiers may be due to mixed mode retention. The chromatographic behavior of positively charged solutes is affected in two ways by the addition of ion interaction reagents to the mobile phase. The retention time of positively charged solutes is decreased by the formation of a positively charged layer of IIR sorbed on the alkyl chain of the stationary phase, with the most hydrophobic IIR's reducing solute retention the greatest. The peak symmetry of amine solutes in the presence of mobile phase modifiers is controlled by ion exchange and hydrogen bonding characteristics. Amine modifiers with shorter hydrocarbon chains interact less with the hydrocarbon chains of the stationary phase and to a greater extent with silanol groups on the support material. While the interaction of charged solutes with the silanol sites on reversed phase support materials can produce poor chromatographic peak shape for certain solutes, the ability to alter the nature of these interactions by the addition of modifying agents allows control of both chromatographic peak shape and retention time.

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