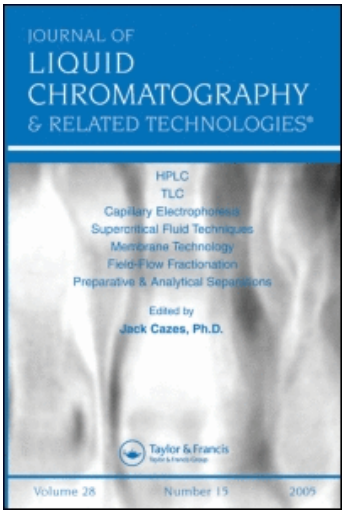

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Influence of the Structure of the Amine Modifiers on the Mechanism of the Separation of Basic Compounds by Reversed-Phase High-Performance Liquid Chromatography

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INFLUENCE OF THE STRUCTURE OF THE AMINE MODIFIERS ON THE MECHANISM OF THE SEPARATION OF BASIC COMPOUNDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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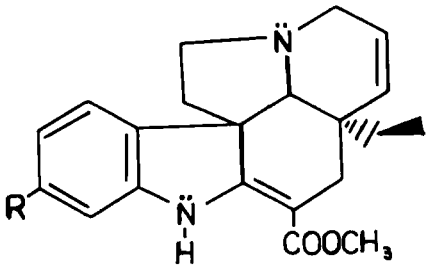
ABSTRACT

Peak shapes and chromatographic retention for a couple of basic compounds (tabersonine and methoxy - tabersonine) in reversed-phase high-performance li - quid chromatography have been studied by the addition of different amine modifiers to the eluent. A discus - sion on the chromatographic mechanism is presented. It is suggested that the amine modifiers adhere to the hydrocarbon ligands of the bonded phase, thus promoting the mass transfer process of basic solutes between the stationary and liquid phases. A change of the initial hydrophobicity of the octyl ligands occurs, influencing the retention times. The mechanism advanced as being responsible for the unusual variation of elution times was confirmed by the chromatographic behaviour of twelve basic compounds.

INTRODUCTION

Recently a method for the quantitative determination of the alkaloids tabersonine (T) and methoxytabersonine (MT) by reversed-phase high-performance liquid chroma-

tography (RP-HPLC) was developed and reported (1). Amine modifiers in the mobile phase were employed as silanol group masking agents. The paper treated the separation and the related problems from a practical point of view, while no consideration was given to the theoretical aspects of the chromatographic process. The latter are an object of the present study.



R=H-T

R=OCH$_3$-MT

Typical for the analysis of basic compounds by RP-HPLC is their adsorption by the silica support which is due to the unreacted silanol groups - up to 50% (2,3). Because the acidity of the unreacted silanol groups is too strong (4,5,6) they cause severe peak tailing and band broadening during the chromatographic separation of basic compounds (7,8,9). Capping residual silanols by the further treatment of the column packing has been only to reduce interference from the silica (3). Another method to overcome the adsorption is the addition of a competing base to the mobile phase. Large improvements in peak shape, respectively in the number of theoretical plates and resolution are demonstrated by the addition of some amines (10,11,12,13). Several studies have examined the effect of solute structure for the RP-HPLC separation of basic solutes

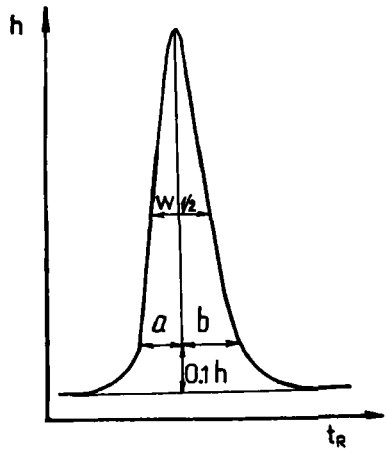


FIGURE 1. Asymmetry factor As=b/a presenting deviations from Gaussian peak.

(7,11,14,15,16). Recently a new packing material was introduced having low adverse silanol reactivity by attaching chemically competing nucleophilic groups close to the silica surface (17).

These articles have not discussed the mass transfer processes that take place between the stationary phase and the sorbate in the presence of amine modifiers in the liquid phase. An elucidation of these mechanisms would serve as a guideline for selecting the amine additives and organic modifiers of the mobile phase. This would lead to greater efficiency of the stationary phase.

Deviations from theoretical Gaussian peaks are usually expressed by the asymmetry factor As=b/a (Fig.1). At a minimum dead volume of the chromatographic system the value of b represents mainly the degree of adsorption, while the value of a reflects the mass transfer processes if there is no adsorption (18,19). The resolution between the peaks of a pair of compounds :

$R = 2\Delta t/w_1+w_2$

is determined by peak width, while peak shape is not taken into account. Thus it is necessary not only to prevent adsorption (decreasing b value), but also to improve mass transfer (decreasing a value). The values of a and b in different chromatograms are compatible if they are referred to the quantity of the solute (h).

The present paper shows the results of our studies regarding the role of the amine additives on mass transfer in the system sorbent - sorbate - eluent, which might prove to be helpful for the separation ofbasic compounds by RP-HPLC.

EXPERIMENTAL

Chromatographic analyses were carried out on an Isco model 2350 pump and a V^4 Variable Absorbance Detector (Isco, Lincoln, Nebraska, U.S.A.). T and MT were detected at 228 nm. The rest of the basic com - pounds were detected at 254 nm. Samples were injected with a Valco C6W(Valco Instr. Co. Inc.,Houston,Texas, U.S.A.) equipped with a 25 µl loop. Chromatographic data was collected and analyzed on an IBM-PC-AT with Chemresearch Data Management Software. The dead volume of the chromatographic system was determined by methanol as an unretained component for all mobile phases.

Two RP-HPLC columns were employed for the analyses: a home-packed (250 x 4.5 mm I.D.) with 5 µm Nucleosil (Macherey-Nagel,Duren,Germany) and a Perkin-Elmer (150 x 4.5 mm I.D.) C_8, 5 µm (Norwalk,Connecticut,U.S.A.). All chromatography was performed at ambient tempera - ture (22°C).

Gas chromatographic analyses were carried out on a Perkin-Elmer, Sigma 300 (Norwalk,Conn.,U.S.A.) with an electronic integrator Shimadzu C-R1B. A home-made capillary column 25 m/0.3 mm with OV-1701 stationary phase was used at 65 C. The velocity of nitrogen was

25 cm/sec. The temperature of the injector (split ratio 1:60) and the N,P-detector was 260 C. The quantitative analysis was carried out by the external standard method.

All organic solvents were of HPLC-grade and were obtained from Merck (Darmstadt,Germany). Water was doubly distilled and filtered through a 0.5 μm Millipore membrane filter (Millipore,Milford,MA,U.S.A.). All mobile phases were degassed in an ultrasonic bath prior to use. After mixing the organic solvent with a 15 mM aqueous potassium buffer and adding the amine modifier, the pH was adjusted with phosphoric acid. The pH measurements were made with a PraciTronic MV870 Digital pH-meter.

Triethylamine (TEA), diethylamine (DEA), dibuthylamine (DBA), ammonium hydroxide and sodium carbonate (Merck) all of purities better than 99% were used as modifiers of the mobile phase. Analytical-grade potassium phosphate, dibasic was obtained from Aldrich (Milwaukee, WI,U.S.A.).

T and MT were isolated from Vinca herbacea and purified by column liquid chromatography to afford a purity of over 99% (1). The other basic compounds(presented in Table 3) nicotinic acid, nicotinamide, pyridine, piperidine, 2,6-dimethylaniline, phenethylamine and caffeine were obtained from Aldrich. Amphetamine, codeine, chinin, cocaine and emetine (all of purity 99+ %) were gifts from the Institute of Pharmacy and Pharmacology, Medical Academy, Sofia, Bulgaria.

RESULTS AND DISCUSSION

Owing to the fact that during the separation of organic compounds by RP-HPLC the capacity factors k obtained are dependent on pH of the mobile phase it was determined that k values of T and MT do not depend on pH in the range of pH 3.00 to 3.60 (1). Due to this

relationship, the mobile phase employed as an eluent had a pH = 3.2±0.1 . The silanol groups possess a pKa value of 4.5 (17), which means that they would not be dissociated at pH=3.2 .

Owing to adsorbance it was imposible to separate T and MT by a methanol-water or acetonitrile-water mobile phase without the addition of an amine modifier. Separation was inadequate when employing tetrahydrofuran-water (30:70, v/v) as an eluent without any amine. The absence of tailing in this case, however, demonstrates that tetrahydrofuran masks the active silanols to a great extent but owing to the eluent's low selectivity the resolution achieved was unsatisfactory.

The reduction of peak tailing was negligible when ammonium hydroxide or carbonate were utilized as odiers of the mobile phase. It is evident that Na^+ and NH_4^+ ions do not block the silanol groups since the latter are not dissociated. Also the size of these ions is small enough so that hindrance of the silanols cannot occur.

Peak tailing was sharply reduced and separation improved when employing such amines as DEA, TEA or DBA as competing bases (Fig. 2).

It was observed that retention times for T and MT decreased 15% upon increasing DEA's concentration from 0.1 to 0.2%. The minimum elution time was registered for 0.2% DEA. A further increase in the concentration of DEA up to 0.5% led to an increase in retention times. Above this concentration, however, the elution times began to decrease in direct proportion to the increase in the amine content in the mobile phase. The same variation in retention times upon increasing the amine content in the eluent was observed for twelve other basic compounds (Table 1). Dolan (20) recommends never to work with mobile phase additives (amines) at concentrations lower than 20-25 mM, since below these levels,

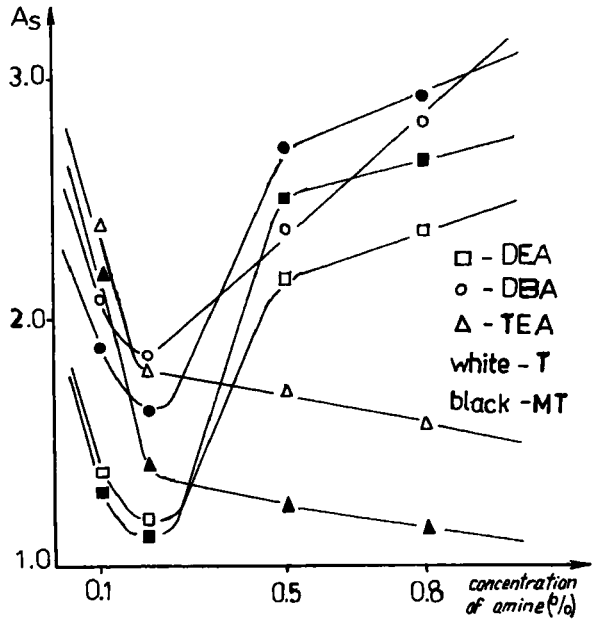


FIGURE 2. Dependence of As upon the concentration of the amine.

retention times could be affected by small changes in additive concentration. No comment, however, is made on the observed fact. These results suggest a combined retention mechanism by hydrophobic and silanophilic interactions similar to the one proposed by Melander et. al. (10).

The initial decrease in retention times could be due to the weaker retention of the basic analytes by the DEA adsorbed on the silanol groups in relation to the active ones. Since tailing is sharply reduced at 0.2% DEA in the mobile phase, it is apparent that the overlapping of the reactive silanols by this concentration of the amine has completed and the effect of the silanol groups on retention is minimum.

TABLE 1

Retention Times (in minutes) of Basic Solutes for Different Concentrations of Diethylamine in the Mobile Phase Obtained With Nucleosil C_8 (250 × 4 mm I.D.), 5 μm, Methanol-Water (50:50, v/v), pH=3.2, 1 ml/min, λ =254 nm.

No.	Compound	Concentration of DEA in the Eluent (%)				
		0.2	0.5	1.0	1.5	2.0
1.	Pyridine	4.95	4.78	5.22	5.10	4.73
2.	Piperidine	5.00	4.80	5.25	5.10	4.75
3.	Nicotinic acid	5.43	5.00	4.78	5.75	5.10
4.	Nicotineamide	5.70	5.30	5.10	6.15	5.54
5.	Amphetamine	6.00	5.38	5.85	5.80	5.60
6.	1 - Phenylethylamine	5.20	5.00	5.54	5.10	5.00
7.	2,6 - Dimethylaniline	6.83	6.60	6.00	7.40	6.65
8.	Cocaine	8.25	8.05	8.65	8.45	8.00
9.	Caffeine	8.20	7.80	8.20	8.45	8.00
10.	Chinin	6.60	6.45	7.60	7.33	7.00
11.	Codeine	5.15	5.00	5.42	5.35	5.20
12.	Emetine	5.40	5.65	6.10	6.00	5.60

The observed increase in elution times for T and MT at concentrations of DEA over 0.2% could be explained by the following mechanism. At such concentrations part of the amine continues to deactivate the silanols until a dynamic equilibria is achieved. A portion of the additional DEA adheres to the octyl ligands of the bonded phase. Methanol has already bound to the hydrocarbonaceous chains. The other portion of the amine is directed towards the molecules of methanol and sticks to them due to their acidic properties though very weak. Interactions between methanol and DEA could arise due to hydrogen bonding between the hydrogen atom of the hydroxyl group and the free electron pair at the nitrogen atom of DEA. Part of the amine continues to deactivate the silanols, while the other part binds directly to the octyl ligands. These interactions alter the initial hydrophobicity of the C_8 chains. The ethyl chains of DEA lead to an increased hydrophobic nature on the surface of the octyl radicals where the effect of methanol's hydroxyl groups is decreased to a certain extent. There is also a presence of nitrogen atoms on the hydrocarbon surface of the stationary phase similarly to the way solutes are bound to the hydrocarbonaceous ligands (21). This change in the properties of the ligands improves their interaction with the basic solutes. As a consequence, mass transfer is facilitated. It is likely that DEA displaces the methanol and takes their place on the octyl ligands (Fig.3).

In order to verify our supposition regarding the role of DEA for facilitating mass transfer, we performed several experiments. First, we attempted by gas chromatography to determine the amount of the amine modifier adsorbed on the stationary phase. The employed reversed-phase (C_8) column was initially conditioned by a pure water-methanol mixture (50:50, v/v; 1 ml/min). After that the liquid phase was replaced by an eluent

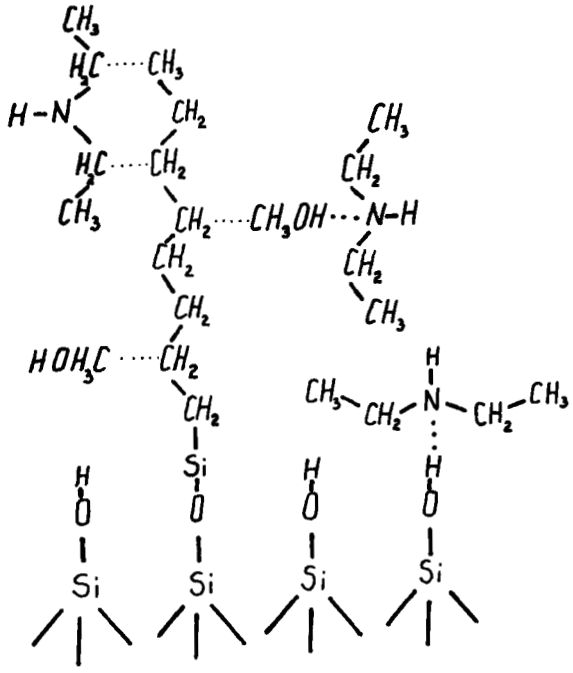


FIGURE 3. Surface structure of C_8 reversed-phase and behaviour of methanol-water mobile phase modified with diethylamine.

concisting of the same water/methanol ratio plus 0.2% DEA. The pH was adjusted to 2.9 with phosphoric acid. With the introduction of the new mobile phase we began collecting 2 ml-fractions. Immediately upon collection, these fractions were subjected to gas chromatographic analyses and pH measurements. As a result, an exponential increase in the concentration of DEA in the mobile phase was established by constructing a %DEA vs time plot. A similar mode of decrease in the pH of the fractions was demonstrated by the pH vs time plot.

The same experiment was repeated with a mobile phase containing 0.5% DEA. The accuracy and precision,

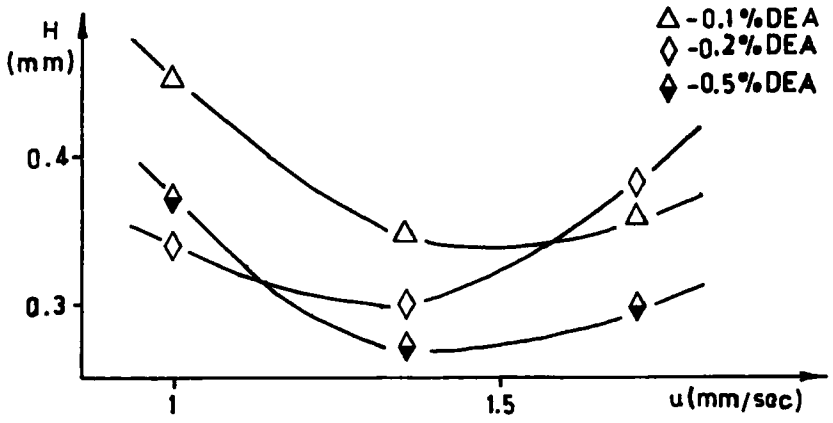


FIGURE 4. Plate height versus mobile phase velocity (Knox) plots for three amine concentrations.

as well as the detection limit , were unsatisfactory, however, for pointing out the expected difference between the plots (%DEA vs time; pH vs time) constructed for 0.2% and 0.5% DEA. The difference would have been attributed to the amount of DEA adsorbed on the stationary phase.

An indirect evidence for the attaching of the amine to the octyl radicals would be the Knox plot (efficiency vs mobile phase velocity) C term, expressing the mass transfer processes for the chromatographic system. For that purpose we eluted T with three mobile phases containing 0.1%, 0.2% and 0.5% DEA , respectively, at different linear velocities of the eluent. The other chromatographic conditions remained constant (Fig. 4).

The plots obtained show that the C term decreases upon increasing DEA's concentration. At 0.1% amine a slight tailing was observed, which is an evidence that most of the silanols have been masked. Tailing did not

occur at 0.2% and 0.5% DEA, indicating that silanol group masking has completed. In such a case the silanols no longer act as interaction sites for the DEA. Therefore the decrease in the C term on going from 0.2% to 0.5% amine should be due to the increased DEA concentration. On the grounds of the increased retention time, observed for 0.5% DEA, we advance the presumption that the amine molecules adhere to the octyl ligands of the stationary phase and increase their hydrophobic nature.

The addition to the mobile phase of DEA over 0.5% generates a portion of the amine, which is not engaged in either silanol masking, nor in the process of adhering to the C_8 chains of the bonded phase. This "free" portion of DEA leads to an increase in the organic content of the eluent and a decrease in its polarity, respectively. Thus mobile phase strength is increased resulting in decreased retention times.

Table 2 shows that upon increasing the concentration of DEA from 0.2% to 0.5% the value of the ratio a/h for T and MT decreases more than twofold, while that of b/h decreases only about 25%. This reflects the idea that mass transfer has been improved by the formation of a surface layer of DEA molecules around the octyl ligands. Furthermore the adsorption due to silanophilic interactions decreases which indicates that the process of masking the silanols by the DEA molecules is still carried out though to a small extent. Owing to the improved mass transfer, which is reflected in the decreased peak widths, the resolution R values increase from 1.33 to 1.54 in spite of the higher asymmetry factor As.

It is noteworthy that when using DBA and TEA as competing bases the values of a/h and b/h respectively, are smaller in relation to those obtained when applying DEA. According to this, the resolution achieved must be

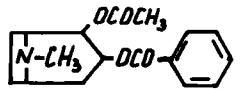
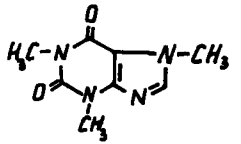
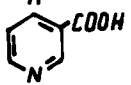
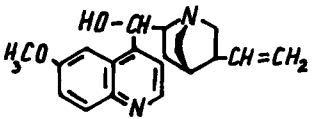
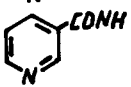
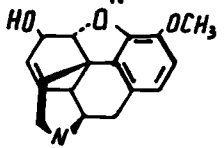
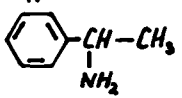
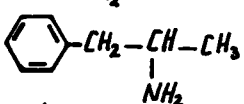
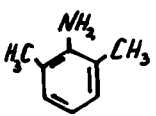
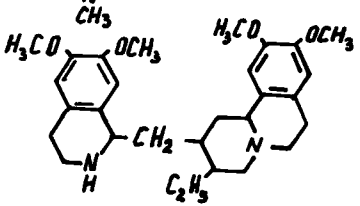
TABLE 2

Influence of the Structure of the Amine Modifiers on the Selectivity and the Mechanism of Separating Tabersonine and Methoxytabersonine by Reversed-Phase High-Performance Liquid Chromatography

amine		a_1/h_1	b_1/h_1	A_s	α	R_s	a_2/b_2	b_2/h_2	A_s	α	R_s
0,2% amine in methanol/water = 50/50							0,5% amine in methanol/water = 50/50				
DEA	T	0.029	0,034	1.17	1.40	1.33	0,010	0.025	2.50	1.30	1.54
	MT	0.047	0.053	1.13			0.021	0.045	2.14		
DBA	T	0.015	0.025	1.67	1.30	1.22	0.007	0.019	2.71	2.28	1.67
	MT	0.027	0.050	1.85			0.019	0.046	2.42		
TEA	T	0.018	0.025	1.39	1.25	1.20	0.019	0.024	1.26	1.26	1.40
	MT	0.028	0.050	1.79			0.031	0.051	1.65		
0.2% DEA + 0.1% DBA in methanol/water = 50/50											
	T	0.016	0.040	2.50	1.38	1.70					
	MT	0.026	0.067	2.58							
THF/water = 30/70											
	T	0.036	0.036	1.90	1.18	1.15					
	MT	0.063	0.137	2,18							
0.2%DEA + 0.1%DBA in AcCN/THF/H_2O = 15/15/70											
	T	0.013	0.012	2.80	1.11	0.50					
	MT	0.012	0.038	3.07							

TABLE 3

Structures of Basic Compounds Employed in This Study

No.	Name	Structure	No.	Name	Structure
1.	PYRIDINE		8.	COCAINE	
2.	PIPERIDINE		9.	CAFFEINE	
3.	NICOTINIC ACID		10.	CHININ	
4.	NICOTINAMIDE		11.	CODEINE	
5.	α-PHENETHYLAMINE				
6.	AMPHETAMINE				
7.	2,6-DIMETHYL - ANILINE		12.	EMETINE	

larged than the one in the case of DEA but it is not. Since R is related to selectivity α by the known equation :

$$R = \sqrt{N}/4 \ (\alpha - 1/\alpha)(k/k+1) \qquad (2)$$

we calculated the values of α exhibited by the chromatographic systems differing in the amine modifier. It was determined that the largest α value was obtained with a mobile phase containing DEA (Table 2). Therefore the type of the amine modifier affects the selectivity. It should be noted that the largest α value is observed at 0.5% DEA which is not owed to further blocking of active silanol groups, since at this concentration of the modifier they are masked already. In other words, the higher selectivity is due to the presence of DEA not associated by any means with the silanols.

Considering the chemical structure of the amines, it can be pointed out that the ratio of number of nitrogen atoms/carbon atoms (N/C) is the largest for DEA, smaller for TEA and the smallest for DBA. The higher nitrogen concentration on the octyl ligands is a prerequisite for a more clearly expressed basicity and hence higher selectivity. A similar relation has been determined by studying the influence of the relative nitrogen content of the amine modifiers on the silanol masking (11). Since the ratio N/C for TEA is greater than for DBA, selectivity must be higher for the first amine. The case is different, however. The smaller selectivity for TEA suggests that the structure of the amine affects selectivity. DBA is a secondary amine which is a prerequisite for the increased role of the free electron pair of the nitrogen atom due to the less steric hindrance. At the same time DBA is more hydrophobic than TEA owing to the higher carbon content. The considerable decrease in a/h upon changing from DEA to TEA suggested that a mixture of DEA and DBA would prove to be the best as an amine additive. At a concentration 0.1% DBA and 0.2% DEA, respectively in the mobile phase, the resolution achieved

between T and MT was 1.70 - considerably higher than the value obtained in the case of adding the either modifier individually. Table 2 shows that when the amine mixture is employed, selectivity is almost as high as with DEA and the values of a/h for T and MT are low and close to those obtained with DBA by itself.

The role of the increased nitrogen content on the octyl ligands of the bonded phase suggests that instead of methanol, acetonitrile could be employed as a mobile phase modifier with an amine mixture (0.1% DBA, 0.2% DEA). In order to maintain the same solvent strength as with methanol the strength of the eluent was determined by referring to the equation (22) :

$$\phi_c = \phi_b (P_b / P_c) \qquad (3),$$

where P_b, ϕ_b and P_c, ϕ_c are polarity and volume fraction values for methanol and acetonitrile, respectively. The resolution and the selectivity achieved for T and MT were R=0.90 and α =1.27, respectively, while the values of a/h did not differ considerably from the ones obtained with a methanol-water mobile phase. It is apparent that the separation of basic compounds is influenced only by the nitrogen containing substances with basic character surrounding the octyl ligands. In support of this statement is the separation of T and MT by employing a liquid phase acetonitrile/tetrahydrofuran/water (15:15:70,v/v/v) (Table 2). The a/h and b/h values are not high. Although acetonitrile is present as a nitrogen carrier the selectivity and the resolution achieved are smaller than those for a mobile phase containing 30% THF in water.

CONCLUSION

It was confirmed that the type and concentration of the amine modifiers of the mobile phase have a considerable influence on the separation of basic compounds by

RP-HPLC. At the low concentrations, the amine overlaps the active silanols. Then together with the methanol in the mobile phase, it forms a layer around the hydrocarbon ligands of the stationary phase. Depending on the properties of the basic solutes, the separation can be improved by employing a combination of amine modifiers with complementary properties. The results obtained demonstrate once again the complex nature of the mass transfer processes taking place in RP-HPLC and that their elucidation is a significant prerequisite for improving such separations.

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