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Effects of aliphatic amines on capillary electrochromatographic performance of tricyclic antidepressants on octadecylsilica

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Abstract

Adding aliphatic amines to the mobile phase improves peak symmetry and efficiency in capillary electrochromatography of tricyclic antidepressants on octadecylsilica. The most hydrophobic aliphatic amine studied, dimethyloctylamine (DMOA), was the most efficient. Despite the fact that the amine additives substantially reduced the electroosmotic flow, the retention of the analytes decreased indicating a strong competitive effect of the additives. DMOA gave the largest retention decrease, and simultaneously reduced the resolution, indicating that silanophilic interaction is significant to the separation. Highest efficiencies were obtained at the lowest pH (2.8). Acetonitrile influenced both efficiency and peak symmetry, and best results were obtained at 60%.

Keywords: Electrochromatography; Mobile phase composition; Pharmaceutical analysis; Amines; Antidepressants, tricyclic

1. Introduction

Capillary electrochromatography (CEC) is one of the newest members in the family of micro-scale techniques. CEC combines the vast selectivity possibilities of liquid chromatography (LC) with the high efficiency of capillary electrophoresis (CE). The history and theory of CEC has been thoroughly discussed elsewhere [1,2].

In many of the papers on CEC with reversed-phase columns, the analytes have been neutrals or protolytes in their ion-suppressed form. In the pharmaceutical realm, however, strongly basic substances are common, and analyzing them in their non-ionized form implies pH above 10, which is detrimental to most stationary phases in chromatography. Due to mixed retention mechanisms, analytes, like amitriptyline and nortriptyline, are notorious for giving severe peak tailing in reversed phase systems when chromatographed in their protonated form. Kiel and Morgan [3] describe three different mechanisms: (1) hydrophobic interaction with the alkyl chains; (2) ion-exchange at charged silanol groups; and (3) hydrogen bonding to silanol groups. These silanophilic interactions might have much more influence on the differences in the retention than the hydrophobic effect. Besides the interaction with the stationary phase, the electrophoretic influence on the analytes will be added in CEC. To reduce the tailing of hydrophobic amines, many stationary phase materials are base deactivated, e.g. by end-capping [4]. In CEC, however, these silanol groups are essential for generating the electroosmotic flow (EOF), and hence end-capped stationary phases are not suitable in CEC [5–7]. Therefore it has been stated that analyzing hydrophobic amines on C_{18} phases with CEC results in intolerable chromatographic behavior

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regarding symmetry and efficiency [8,9]. In a paper that attracted much attention, Smith and Evans [10] used a cationic exchanger to separate strongly basic analytes and reported astonishing efficiencies due to a focusing effect. The electrochromatographic behavior on this type of stationary phase, though, has proven to be irreproducible and the focusing effect unexplainable so far [8,11,12], although a hypothesis based on a theoretical approach has been presented [13]. Another way to reduce the influence of the silanol groups, often used in LC, is to add a small amine to the mobile phase as a masking agent [14–18]. Different additives like triethylamine, *n*-butylamine and *N*,*N*-dimethyloctylamine (DMOA) improve the symmetry, shorten the retention time and influence the resolution to different extent [19]. DMOA improved symmetry and efficiency most. In CEC, the use of silanol masking agents, like hexylamine [12,20] and triethylamine or triethanolamine [21], also have proved to be beneficial.

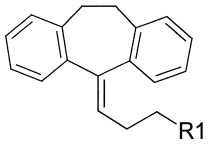
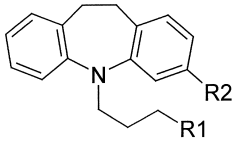
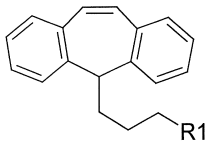
The aim of this study was to investigate the effect of the mobile phase composition on the electrochromatographic performance of tricyclic antidepressants and related quaternary ammonium compounds (Table 1) on reversed-phase capillary columns. The mobile phase was changed regarding its content of different aliphatic amines (ranging from hexylamine to DMOA) in varying concentrations, ionic strength, pH and content of acetonitrile.

2. Experimental

2.1. Chemicals

The buffer stock solutions with pH between 2.8 and 6 were made from an aqueous solution of an aliphatic amine and 1 *M* H_3PO_4 (Merck, Darmstadt, Germany). The final concentration of the amine was 75 m*M*. Mobile phases were made from buffer stock solution, water (Milli-Q water system, Millipore, Bedford, MA, USA) and acetonitrile (ACN) (Fisher Scientific UK, Leicestershire, UK). The pH in the buffer was measured before addition of acetonitrile. The aliphatic amines, *n*-hexylamine (HxA), *n*-heptylamine (HpA), *n*-octylamine (OA) and *N*,*N*-dimethyloctylamine (DMOA) were from Acros (Geel, Belgium). For example, a 50-ml batch of a mobile phase denoted 'DMOA 7.5 m*M*, ACN 60%' was made by mixing 5 ml of DMOA buffer stock solution, 15 ml of water and 30 ml of ACN. When changing the ionic strength without altering the

Table 1
Structures of the analytes

Structure	Name	R1	R2	Short
	Nortriptyline	$-NHCH_3$		Nor
	Amitriptyline	$-N(CH_3)_2$		Ami
	N-Methyl-amitriptyline	$-N(CH_3)_3^{\oplus}$		M.ami
	Desipramine	$-NHCH_3$	–H	Desi
	Imipramine	$-N(CH_3)_2$	–H	Imi
	Clomipramine	$-N(CH_3)_2$	–Cl	Clomi
	*N*,*N*-Dipropyl-protriptyline	$-NCH_3(C_3H_7)_2^{\oplus}$		DP.Pr

content of aliphatic amine, potassium chloride (Kebo, Stockholm, Sweden) was added. Stock solutions of the solutes, amitriptyline hydrochloride (Merck Sharp & Dohme, Rahway, NJ, USA), nortriptyline hydrochloride (Pharmacia, Uppsala, Sweden), desipramine hydrochloride (Ciba-Geigy, Basel, Switzerland), imipramine hydrochloride (Hässle, Mölndal, Sweden), clomipramine hydrochloride (Ciba-Geigy), *N*,*N*-dipropylprotriptyline bromide and *N*-methylamitriptyline bromide were made from 1 mg of the substance dissolved in 500 μl buffer and 1500 μl water. The quaternary ammonium derivatives were synthesized from protriptyline and amitriptyline, respectively, as described by Borg and Schill [22]. As electroosmotic marker 1-phenyl-1,2-ethanediol (Aldrich, Milwaukee, WI, USA) was used, the stock solution was 10 m*M* in water and ACN (80:20). The sample solutions were typically made from 30 μl of each analyte solution, 60 μl EOF marker and 750 μl mobile phase. The packing material, Waters Spherisorb ODS 1, 3 μm, was kindly donated by Dr. Gerard Rozing (Agilent Technologies, Waldbronn, Germany).

2.2. Instrumentation

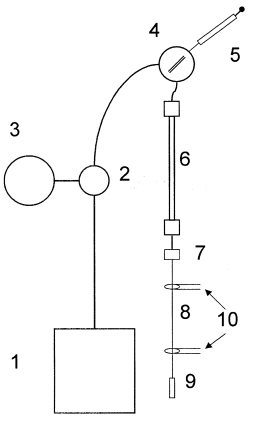
The experiments were performed with packed fused-silica capillaries obtained from Polymicro Technologies (Phoenix, AZ, USA). The dimensions of the capillaries were 100 μm I.D.×360 μm O.D., with an efficient length of 25 cm. The length after the detector was 8.5 cm. The capillaries were mounted in a Hewlett-Packard 3D CE system (Hewlett-Packard, Waldbronn, Germany). To suppress bubble formation both ends of the column were pressurized at 10 bar. Detection was carried out at 210 nm. A Jasco PU-980 HPLC (Jasco, Tokyo, Japan) pump was used to purge the capillary when necessary by using the constant pressure mode (100 kg/cm^2). The samples were electrokinetically injected at 4–6 kV for 4–7 s towards the cathode. The column was thermostated at 25°C. The voltage was 25 kV in all runs and resulted in a current of 3–18 μA depending on the mobile phase.

2.3. Column preparation

To prepare a CEC column with a packed bed of 25 cm, a 53-cm long fused-silica capillary was used. At 13, 39 and 47.5 cm from the upper end the polyimide layer was removed where the inlet end, detection window and outlet end of the final column would be. The capillary was mounted through two heating coils [23], and the upper end was connected to the clean and dry packing chamber (Fig. 1). The heating coils were adjusted to the positions where the inlet and outlet frits would be. A slurry of 35 mg of packing material in 350 μl acetone was filled into the packing chamber and when the slurry started to come out of the capillary the lower end was connected to the filter assembly, and then a pressure of 550 bar was applied. A mixture of acetonitrile–water–sodium chloride (20:80:5 m*M*) was used as packing solvent. The packing continued for 2 h before the outlet frit was made by low heating of the coil for 30 s. Then the filter assembly was cut off and the packing material below the frit was purged out of the capillary. The inlet frit was made and the pump was turned off. The pressure was allowed to drop slowly before the column was cut loose from the packing chamber. The packed capillary was connected to a HPLC-pump and purged with a mixture of acetonitrile–water (50:50) for 1 h at 100 kg/cm^2. Before mounting the column in the CE system the capillary was cut to the right length with a Shortix (Agilent Technologies). The column was slowly equilibrated in the CE system with the mobile phase by applying a low voltage and pressure only on the inlet side. The voltage was stepwise increased and when the current and baseline were steady at the intended voltage, e.g., 25 kV, the system was ready for use.

2.4. Evaluation of electrochromatograms

When examining the effects of the mobile phase composition on peak symmetry, efficiency and resolution the injection was adjusted so that the peak size of an analyte was kept quite constant to avoid comparing a small peak with a larger and, maybe, overloaded peak. Different mobile phases produce different EOF and, hence, different amounts of the sample will be injected. The asymmetry factor (asf) was calculated at 10% of the peak height by dividing the widths at the rear and the front sides of the peak. The values for efficiency (N) and resolution (R_S)



#	Description	Brand	Information	Model
1	Airdriven pump	Haskel	Max 16500 psi	
2	Needle valve	SSI	Three-way, side vent	02-0125
3	Manometer	WIKA	1000 bar	
4	Switching valve	Valco		C6W
5	Syringe	Hamilton	Luer lock, 500 μl	
6	Packing chamber			
	Reducing unions	Valco		ZRU21
	Glasslined stainless steel tubing	SGE	ID 0.5 mm,OD 1/8 inch 30 cm long	0827375
7	Capillary connector			
	Union	Valco		ZU1M
	Special ferrule	Valco	PEEK	FS1.4PK
8	Capillary		fused silica	
9	Filter assembly			
	Mini union	SGE	glasslined	103432
	Filter	hetp	2μm	part 206
	Ferrules	SGE	vespel	072696
10	Heating coils			

Fig. 1. Description of the capillary packing system. Stainless steel capillaries of 0.5 mm I.D.×1/16 in. O.D. were used to connect the different parts of the packing system (1 in.=2.54 cm).

were calculated by HP ChemStation which used the following equations:

$$N = 5.54 \cdot \frac{t^2}{w_{0.5}^2} \quad (1)$$

$$R_S = \frac{2.35(t_B - t_A)}{\frac{1}{2} \cdot (w_{0.5B} + w_{0.5A})} \quad (2)$$

where t is the retention time, $w_{0.5}$ is the width at half height.

3. Results and discussion

3.1. Effects of different C_{18} materials

Some preliminary experiments for this study were performed during a research stay at Agilent Technologies in Waldbronn, Germany. For chemicals and packing procedures see Ref. [12]. Two different packing materials, Hypersil C_{18} and Spherisorb ODS 1, were compared. When analyzing a neutral standard mixture [12] using a mobile phase consisting of 80% acetonitrile (ACN) and 20% 25 m*M* Tris buffer, pH 8, the two different columns behaved in a rather similar way. The Spherisorb material exhibited a slightly higher efficiency and EOF. However, when analyzing three hydrophobic amines at low pH and with hexylamine added to the mobile phase the difference was pronounced (Fig. 2). The more asymmetric peaks and the higher EOF of Hypersil is probably due to the presence of more acidic silanol groups than in Spherisorb ODS 1. A further indication of this was obtained in experiments with lower content of ACN (60%) when the interaction with the Hypersil material became so much higher that the solutes eluted after the EOF marker. On the

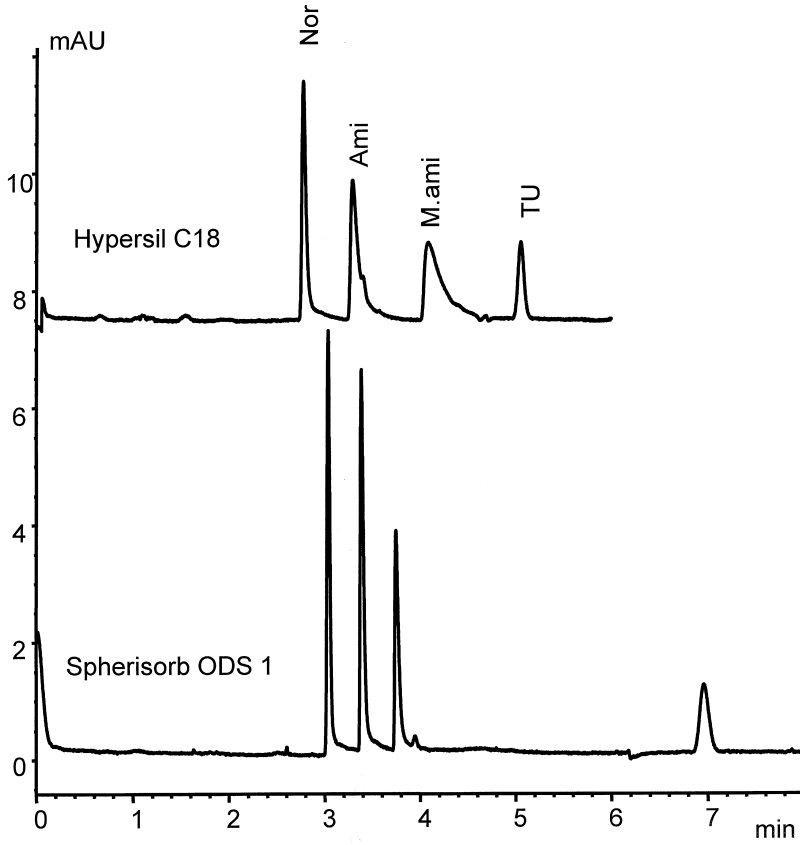


Fig. 2. Different stationary phases give different chromatographic performance for the tricyclic analytes, mostly due to differences in number of and acidity of underivatized silanol groups. Mobile phase: 7.5 m*M* hexylamine in phosphate buffer, pH 2.8, 80% ACN. Injection, 5 kV for 6 s; voltage, 25 kV; current, 13.8 μA on Hypersil and 11.1 μA on Spherisorb. TU, thiourea; the rest of the analytes as defined in Table 1.

Spherisorb column the analytes still eluted before the EOF.

3.2. Effects of different aliphatic amines

The four aliphatic amines used in this study had different influence on the electrochromatographic performance. DMOA improved symmetry (Fig. 3) and efficiency more than equal additions of HxA, HpA, or OA to the mobile phase. The addition of amines to the background electrolyte in CE is often used to reduce or even reverse the EOF [24,25], and a question was how much the EOF would be affected by the addition of the aliphatic amine to the mobile phase in the CEC experiments. The results obtained here showed that the EOF decreased with increasing hydrophobicity of the amine. Using DMOA as additive gave a 31% lower EOF than an equal concentration of HxA. At the same time, however, the retention of all the tricyclic substances decreased. As an example, a 19% shorter analysis time for the analytes was obtained when adding DMOA compared to HxA to the mobile phase, despite a lower EOF. All seven analytes eluted in less than 6 min when using 7.5 m*M* of DMOA and 60% ACN in the mobile phase. The resolution was, however, reduced most with DMOA, which indicates that the silanophilic interaction contributes quite a lot to the separation. At a concentration of 7.5 m*M* of the

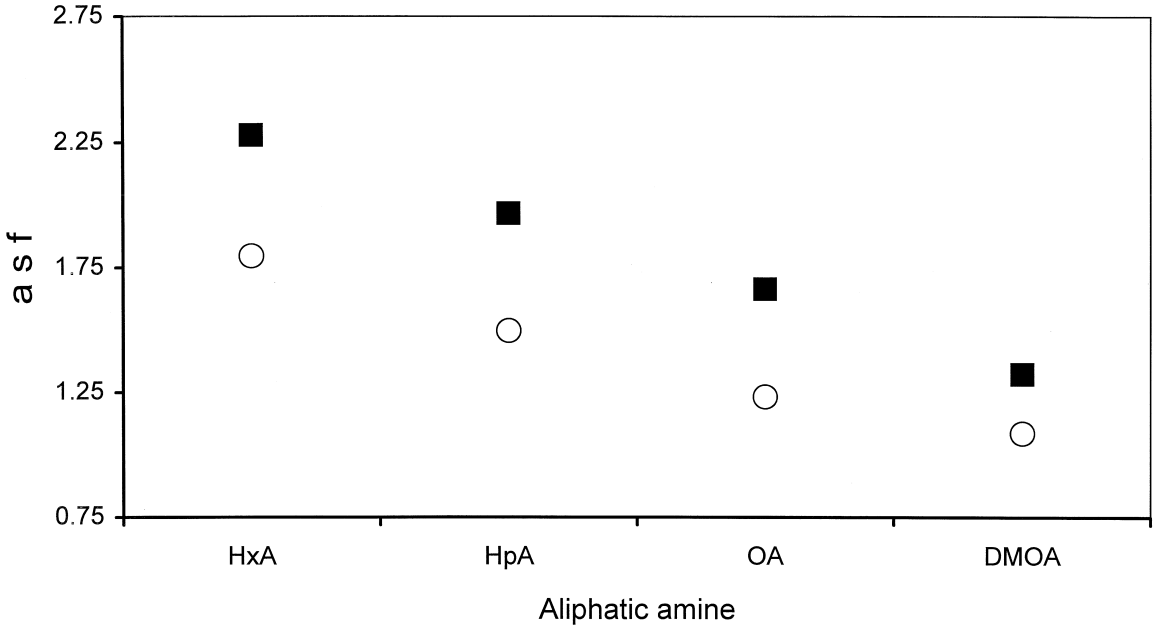


Fig. 3. The effect on asymmetry factor (asf) of the first and last eluting analytes, Desipramine (○) and *N,N*-dipropylprotriptyline (■), respectively, by the aliphatic amine in the mobile phase. The asf is correlated to the hydrophobicity of the additive. The aliphatic amines were HxA, hexylamine; HpA, heptylamine; OA, octylamine; and DMOA, dimethyloctylamine. The mobile phases consisted of 7.5 m*M* aliphatic amine in phosphate buffer, pH 2.8, and 60% ACN. The asymmetry factor (asf) was calculated at 10% of the peak height by dividing the widths at the rear and the front sides of the peak.

aliphatic amine the resolution was sufficient for all the analytes.

3.3. Effects of ionic strength and concentration of the aliphatic amine

It is difficult to correctly calculate the ionic strength in a solution with high content of an organic solvent, since factors like pH and pK_a values will change. But if these effects are ignored the ionic strength in a mobile phase with 7.5 m*M* aliphatic amine was estimated to be 0.0073, and to double the ionic strength without changing the buffering components, 7.3 m*M* potassium chloride was added. The increased ionic strength resulted in lower resolution (Fig. 4), reduced EOF, improved symmetry and loss of efficiency. Doubling the concentration of the amine to 15 m*M* in the mobile phase had a more dramatic effect. Increasing the concentration of DMOA resulted in co-elution of Clomi and M.ami; hence, the decrease in resolution was larger than when the same increase of the other aliphatic amines was used (Fig. 4). At this stage of the study it was decided to examine the effects of DMOA more deeply and leave the other aliphatic amines. Reducing the content of DMOA buffer even further to 5.6 and 3.8 m*M* showed that there was an optimum in the efficiency for the analytes at a DMOA concentration around 5.6 m*M* (Fig. 5).

3.4. Effect of pH

A phosphate buffer is probably not the ultimate system when increasing the pH from 2.8 to 4.4 and 6.0 due to impaired buffer capacity above pH 3 and below pH 6.5. We tried other acids like citric acid, malonic acid, etc., but they all gave baseline disturbances. Moreover, it is not appropriate to have different buffer systems at various pH values when making comparisons, since different types have dissimilar effects on factors like EOF and possible ion-paring with the analytes. We therefore used phosphate buffers throughout. When the pH is changed, both the EOF and the charge of the

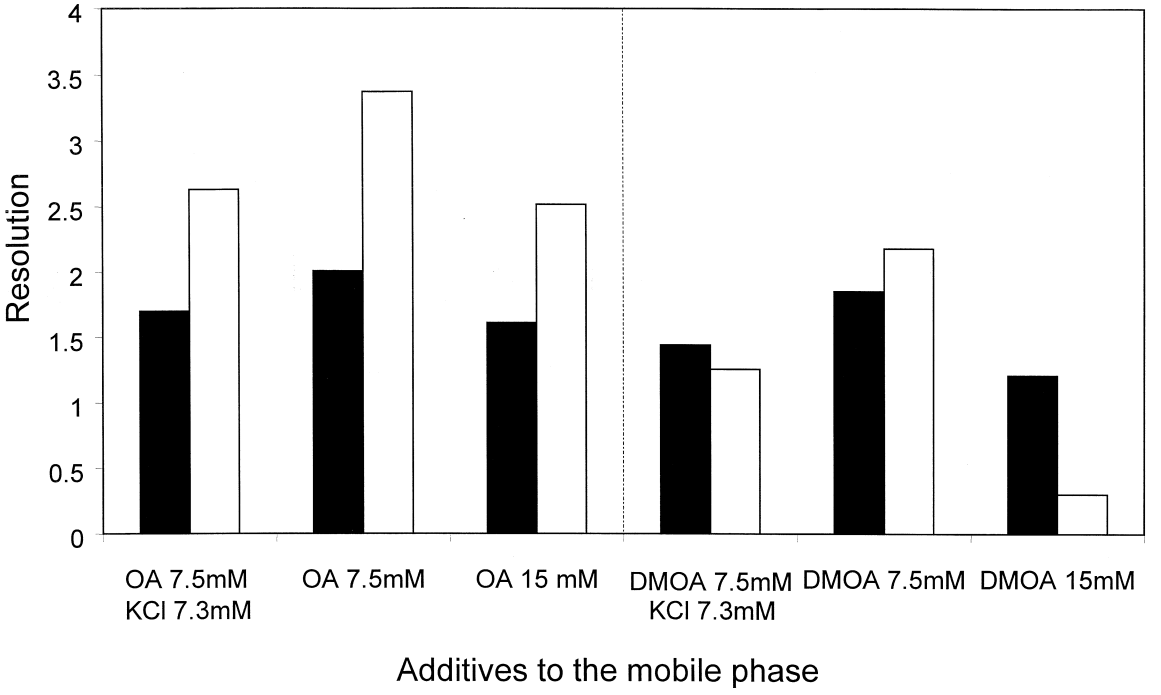


Fig. 4. The influence of ionic strength and concentration of octylamine (OA) and *N*,*N*-dimethyloctylamine (DMOA) in the mobile phase on the resolution between desipramine–nortriptyline (black bars) and clomipramine–*N*-methylamitriptyline (white bars), respectively. The concentration of the aliphatic amines was 7.5 or 15 m*M*. To double the ionic strength without changing the buffering components 7.3 m*M* of potassium chloride were added to the mobile phase with OA and DMOA concentrations at 7.5 m*M*. The ACN content was 60%.

analytes will be influenced. As the pH became higher the silanol groups were dissociated to a higher extent, and hence the EOF increased. The retention of all solutes increased with increasing pH. This is probably a result of a combination of effects. As the silanol groups are dissociated, the retention by silanophilic interaction increases. The extent of protonation of the hydrophobic amines will decrease when the pH is raised, reducing the electrophoretic mobility. Clomi and M.ami co-eluted at pH 4.4, and at pH 6.0 the retention order was reversed compared to pH 2.8. The relative retention order of the other analytes was not changed. The resolution between the secondary (*sec*.-) amine Nor and the tertiary (*tert*.-) amine Imi increased with pH, while the resolution between Desi-Nor (*sec*.-amines) and between Imi-Ami (*tert*.-amines), respectively, did not change substantially. Tertiary amines have lower pK_a values and are more likely to have reduced mobility at lower pH than secondary amines, and they are also more hydrophobic, indicating a larger interaction with the octadecyl ligands (Table 2). In aqueous solutions the pK_a values of the basic analytes are above 9. The results, however, indicate that they behave as protonated to a lower extent in mobile phases containing a high concentration of ACN, which influences resolution and retention order in a way that would not be seen in purely aqueous systems. There are also indications that organic solvents change the pK_a values for *sec*.- and *tert*.-amines to different extent [26,27]. The efficiency was lowered by 16 and 35%, respectively, as pH went from 2.8 to 4.4 and 6.0.

3.5. Effect of acetonitrile content

The content of organic modifier influences the EOF by a combination of effects on the viscosity, the zeta potential and the dielectric constant in the mobile phase. When using ACN, and keeping the buffer concentration constant, the EOF usually increases with higher content of ACN in the mobile

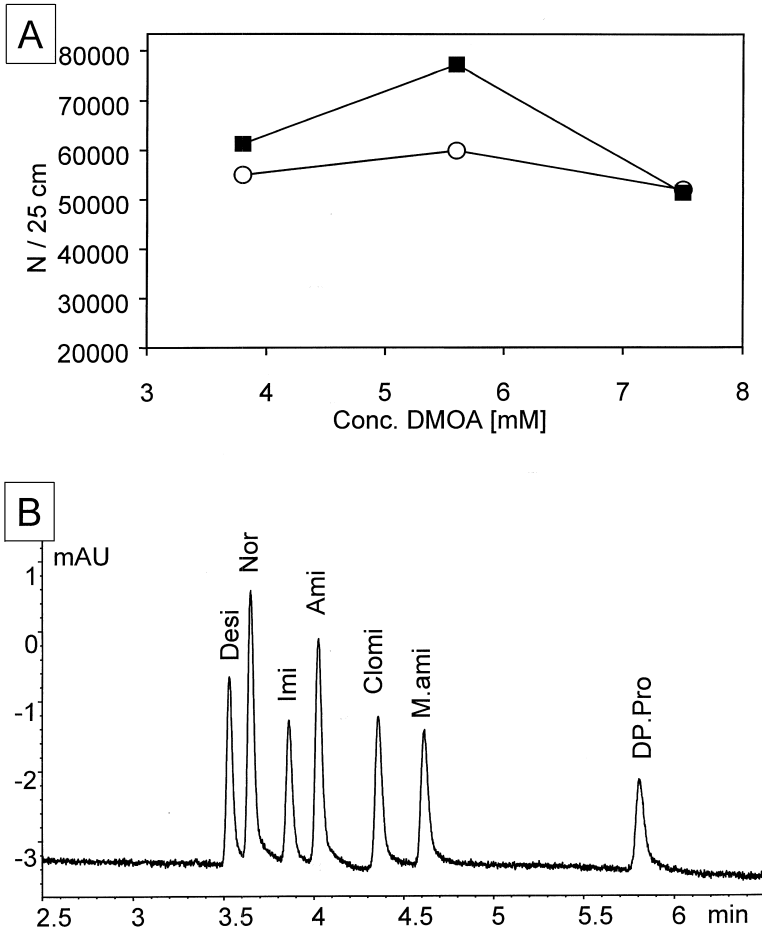


Fig. 5. A: Diagram showing the influence of the concentration of DMOA in the mobile phase on the column efficiency (plate numbers per 25 cm) for the first and last eluting analytes desipramine (○) and *N*,*N*-dipropylprotriptyline (■). B: Electrochromatogram with the optimal mobile phase. Mobile phase: 5.6 m*M* DMOA, pH 2.8, 60% ACN. Injection, 6 kV for 5 s; voltage, 25 kV; current, 4.5 μA. Analytes as defined in Table 1. The electroosmotic marker eluted after 10.7 min.

Table 2
Physicochemical data of the protolytic analytes

Analyte	FW	pK_a	Log K_D [a]	Type of amine
Desi	266.4	10.2	4.2	*sec.*
Nor	263.4	9.7	4.0	*sec.*
Imi	280.4	9.5	4.6	*tert.*
Ami	277.4	9.4	5.0	*tert.*
Clomi	314.9	9.4	5.3	*tert.*

[a] Octanol.

phase, corresponding to our observations. The mobility of the analytes also increased. The effect on the retention and the mobility of the analytes will be a combination of how the polarity of the mobile phase is influenced, to what extent the pK_a values, and hence the charge, is changed, etc. Changing the percentage of ACN had the highest effect on the resolution between Desi and Nor, which decreased as the ACN content was raised. The efficiency was highest at 60% ACN for all the analytes. There was an optimum in the symmetry for the last eluting analyte DP.Pro at 60%. The other analytes had about

the same symmetry at 60 and 70%, but the symmetry was impaired for all at 50%.

4. Concluding remarks

The use of silanol masking agents, such as DMOA, makes analysis of hydrophobic amines on C_{18} materials feasible. By adding low concentrations of aliphatic amines to the mobile phase the asymmetry is successfully reduced and the efficiency is increased. DMOA improved symmetry and efficiency more than equal additions of HxA, HpA or OA, and the effect could be connected to the hydrophobicity of the aliphatic amine. There was an optimum in the effect on efficiency at a concentration of DMOA at 5.6 m*M*. Despite the fact that the EOF decreased, the analytes eluted faster the more hydrophobic the additive was. The aliphatic amine lowered the silanophilic interaction, and the fact that the analytes eluted faster when potassium chloride was added indicates that the silanophilic interaction has an ion-exchange nature. The system had the best efficiency at the lowest pH (2.8) and medium content of ACN (60%).

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