The Effect of Different Amines Added to Eluents as Silanol Masking Agents on the Chromatographic Behavior of Some Diuretics in Reversed-Phase High-Performance Liquid Chromatography Using C_{18} Packings

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Abstract

A preliminary gradient separation in reversed-phase liquid chromatography of a mixture of 25 solutes (diuretics, probenecide, and atenolol) is carried out using several C_{18} columns and an aqueous phosphoric acid solution (pH 3.2)–acetonitrile mobile phase as a control. Using this separation, the chromatographic behavior of these solutes is studied using 11 water-soluble primary, secondary, and tertiary amine modifiers in the range of 0.7–7.5mM and a Spherisorb C_{18} column. This study reveals the presence in the complex sample of two groups of solutes with positive (five typical solutes showed improvements in peak symmetry and retention) or negative responses using these amines as mobile phase modifiers. After experimentation in the presence of amines, these differences are related to solute structure. Hexylamine is found to be an effective masking agent of silanols because of its structure and small required concentration. On these bases, the silanophilic and hydrophobic character of typical solutes and several C_{18} packings are evaluated under isocratic elution and a relative effectiveness index for amines, and a method for their assessment is proposed. The role of the amine structure on solute retention and the importance of selecting amines of suitable hydrophobic character, molecular geometry, and concentration is discussed. A model of the formation and stabilization of the silanol–amine complex based on hydrophobic and ionic interactions is also proposed.

Introduction

Diuretics (DIUs) are compounds with therapeutic value (1) that are banned by the International Olympic Committee (2). The principal groups are thiazides, inhibitors of carbonic anhydrase, those that have a carboxylic acid group, and DIUs that do not have the thiadiazine ring but resemble the thiazides in mechanism and site of action (3). Other compounds such as probenecide (PRO) are used because they reduce the urinary excretion of anabolic steroids. Atenolol (ATN) is associated with DIU in pharmaceuticals because it is a β-blocker, antiarrhythmic, and antihypertensive agent. Some of these drugs have basic properties. The separation and determination of complex mixtures of these compounds are not easy (with a wide variety of functional groups and pK_a values). Although there are numerous chromatographic methods for individual DIUs (4–9), only a few screening methods have been developed (10–13).

Chromatographers often encounter problems in the reversed-phase high-performance liquid chromatographic (RPHPLC) separation of samples containing amines or quaternary ammonium compounds. Typically, such compounds exhibit severe peak tailing and broad bands and give rise to low plate numbers; retention often varies from column to column, and dramatic changes in the separation take place as the column ages. These changes vary with the structure of the basic compounds to be separated and are commonly ascribed to "silanol effects." These effects are mainly due to the interaction of the basic drug with free silanols and/or heavy metals (14–16). Similar effects can also be due to acidic samples (17). Many methods have been developed for reducing (or eliminating) untoward manifestations due to silanophilic retention of several basic solutes in RPHPLC by adding surfactants (18,19) and amines (20–22) to the eluent. There have also been many studies on the role of amines as modifiers (23–31). Nahum and Horváth (23) and Bij et al. (24) proposed a retention model based on a dual mechanism in which both hydrophobic and silanophilic interactions on the surface of the reversed-phase stationary phase dictated the chromatographic behavior of solutes. This model describes the role of silanol masking agents in decreasing the retention of charged solutes but does not clearly explain improvements in peak symmetry when amines are added to the eluent. Kiel et al. (25) studied

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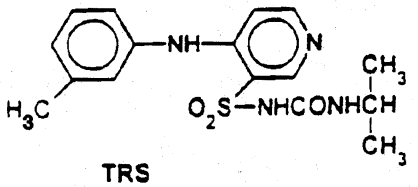
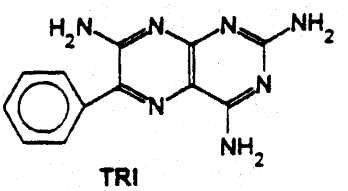
Table I. Structures of DIU, ATN, and PRO

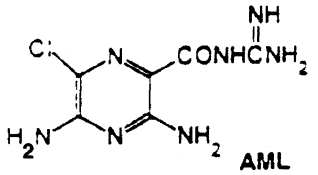
Amine-Eluting Solvents



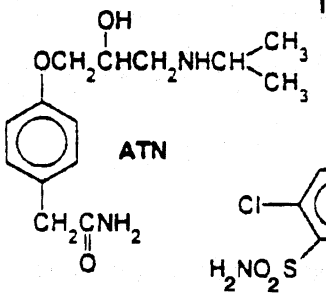
TRS



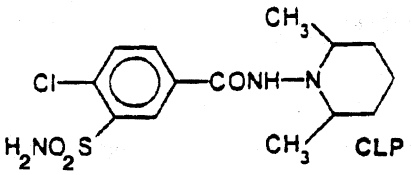
TRI



AML

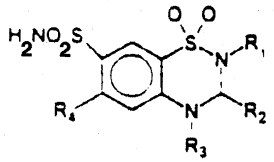


ATN

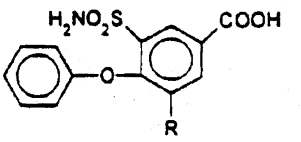


CLP

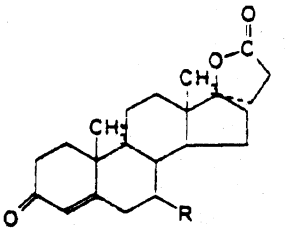
Amine-Noneluting Solvents



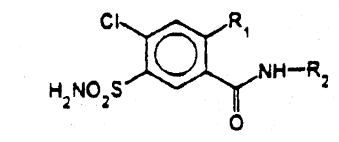
R_1	R_2	R_3	R_4	Δ_{a-b}	Name
H	$-CH_2SCH_2CH{-}CH_2$	H	Cl		ALT
H	$-CH_2SCH_2C_6H_5$	–	Cl	═	BNZ
$-CH_3$	$-CH_2SCH_2CF_3$	H	Cl		POL
H	$-CH_2C_6H_5$	H	CF_3		BND
H	H	–	Cl	═	CT
H	H	H	Cl		HCT
H	H	H	CF_3		HFM
H	$-CHCl_2$	H	Cl		TCM



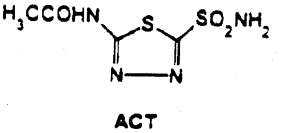
R	Name
$-NHC_4H_9$	BUM
	PIR



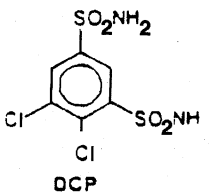
R	Δ_{a-b}	Name
H	═	CAN
$-SCOCH_3$	–	SPL



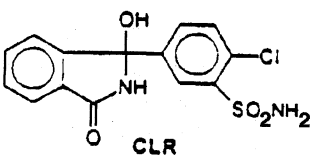
R_1	R_2	Name
		XIP
H		IND



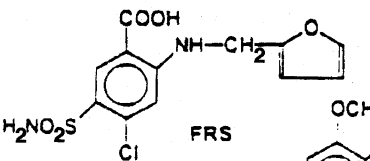
ACT



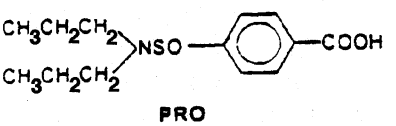
DCP



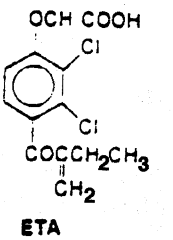
CLR



FRS



PRO



ETA

* Some double bonds in these structures are denoted as Δ_{a-b}.

the effects of 15 different amine modifiers on the chromatographic behavior of amitriptyline derivatives and also proposed a retention model based on a mixture of hydrophobic, ion exchange, and hydrogen bonding interactions. In this mechanism, the hydrophobic effects appeared to be of secondary importance for solute retention in RPHPLC, whereas ion-exchange and hydrogen-bonding interactions were of greater importance.

In the present paper, a complex mixture of DIUs including torasemide, a recently developed synthetic DIU not commercialized in many countries, and two non-DIU compounds, PRO and ATN, were used. The structures of these compounds are shown in Table I. A separation under a gradient elution was initially selected using several C_{18} columns. Using this separation as a control test, the effect of the structure and concentration of 11 water-soluble amine modifiers on the chromatographic behavior of the solutes was studied using a Spherisorb C_{18} column. Two different groups of solutes were detected by the presence of amines in the eluent, and an explanation of this behavior was reported. Hexylamine (HA) appeared to be an effective masking agent that allowed the evaluation of the hydrophobic and silanophilic characteristics of several C_{18} packings and solutes and a definition of a relative effectiveness index for amines. An evaluation of the mechanism and amine effectiveness in ion-interaction RPHPLC was discussed, and a model showing hydrophobic and ionic interactions related to the formation of the silanol–amine complex was also proposed.

Experimental

Apparatus

The chromatographic system consisted of the following components, which were all from Thermo Separation Products (Riviera Beach, FL): a Constametric 4100 solvent delivery system, a spectromonitor 5000 photodiode array detector (DAD) covering the range 190–360 nm interfaced to a computer for data acquisition, and a recorder model CI 4100 data module. A Rheodyne 20-µL-loop injector (Cotati, CA), and a Jones Chromatography block, heated series 7960, for thermostatting columns in the range 30–60°C (Seagate Technology, Scotts Valley, CA) were also used. The following 5-µm (150 × 4.6-mm i.d.) reversed-phase C_{18} columns were used: Hypersil, Ultracarb, Spherisorb, and Nucleosil, which were all from Phenomenex (Torrance, CA), and a polymeric PRP-I column from Hamilton (Reno, NV).

Chemicals

Chlorthalidone (CLR), hydroflumethiazide (HFM), canrenone (CAN), bendroflumethiazide (BND), spironolactone (SPL), acetazolamide (ACT), ethacrynic acid (ETA), dichlorphenamide (DCP), furosemide (FRS), hydrochlorothiazide (HCT), bumetanide (BUM), chlorothiazide (CT), amiloride (AML), triamterene (TRI), trichlormethiazide (TCM), clopamide (CLP), althiazide (ALT), indapamide (IND), benzthiazide (BNZ), atenolol (ATN), xipamide (XIP), polythiazide (POL), piretanide (PIR), and probenecide (PRO) were purchased from Sigma (St. Louis, MO). Torasemide (TRS) (1-isopropyl-3-[4-*m*-toluidino-3-pyridyl] sulphonylurea) was donated by Boehringer-Mannheim (Barcelona, Spain).

The following amines were obtained from Aldrich (Alcobendas, Madrid, Spain): *n*-propylamine (PA), isopropylamine (IPA), *n*-butylamine (BA), isobutylamine (IBA), *n*-hexylamine (HA), cyclopentylamine (CPA); cyclohexylamine (CHA), diethylamine (DEA), piperidine (PPA), triethylamine (TEA), and ethyldiisopropylamine (EDIA). H_3PO_4 and ammonium sulphate were of analytical reagent grade from Merck (Darmstadt, Germany). HPLC-grade acetonitrile was purchased from Promochem (Wesel, Germany). Water was purified with a Milli-Q system (Millipore, Molsheim, France). Millipore 0.45-µm nylon filters (Bedford, MA) were also used. Other chemicals were of analytical reagent grade.

Mobile phase and chromatographic analysis

The mobile phases were adequately prepared by mixing aqueous solutions (solvent A) with acetonitrile (solvent B). For DIU elution in the absence or presence of amines, phosphoric acid was added to pure water or to aqueous solutions containing amines (0.7–7.5mM) or ammonium sulphate (30mM) to adjust to pH 3.2 (solvent A). After conditioning the column with an eluent composed of solvents A and B (90:10, v/v), a linear gradient elution to increase 10, 25, 35, and 60% of solvent B in 0, 7, 20, and 23 min, respectively, was used by programming the pumps. All solvents and eluents were first filtered under a vacuum through nylon filters and degassed using a helium sparge.

Once the column was conditioned with the eluent at 40°C, chromatograms were obtained. A methanolic solution containing a single compound or an appropriate mixture of them in the range of 1–10 µg/mL was injected (20 µL). The flow rate was 1.0 mL/min, and ultraviolet (UV)–diode array (DAD) detection in the range of 190–360 nm was used. The Hamilton PRP-1 column used a flow rate of 0.5 mL/min. Solutes were identified by comparison between the UV spectra of the chromatographic peaks and the UV spectra of compounds previously registered by injection of each one. Analyses were monitored at 220, 245, 275, and 300 nm.

Results and Discussion

Theory

The silanophilic retention of basic charged solutes (SH^+) with acidic sites (silanols, SiOH) on silica-based HPLC columns may be described by the following ion-exchange process, assuming that the site (SiOH) is strongly acidic (14):

$$SH^+ + -SiO^-H^+ \leftrightarrows H^+ + -SiO^-SH^+ \qquad \text{Eq 1}$$

Based on this equilibrium, solutes with either amine or other similar functional groups often exhibit broad and asymmetric peaks and do not follow a regular hydrophobic retention behavior. To improve their chromatography (retention

times, peak symmetry, etc.), amines added to the eluent as masking agents can compete with SH^+ for the silanols on the silica surface of the stationary phase according to the equilibrium:

$$-SiO^-SH^+ + AH^+ \leftrightarrows -SiO^-AH^+ + SH^+ \qquad \text{Eq 2}$$

where SH^+ and AH^+ are, respectively, the solute and amine under cationic form. The formation of silanol–amine complex is favored by lowering the dielectric constant of the medium (32). According to Equations 1 and 2, retention time of solutes in the absence of amines (t_R) can be expressed by the equation:

$$t_R = t_S + t_H \qquad \text{Eq 3}$$

where t_H is the retention time in the absence of silanol effects, and t_S is the increase in retention time due to silanols in the presence of amines (assuming 100% amine effectiveness, t_S = 0) by the equation $t_R = t_H$.

DIU retention without amines under gradient elution

Owing to the different structures (Table I), basicity, and hydrophobicity and the great number of compounds under study, a gradient elution was required. Prior to the use of amines as mobile phase modifiers, a preliminary study of the separation was carried out. A phosphoric acid solution (pH 3.2)–acetonitrile mobile phase and several silica-based C_{18} columns of the same length (Spherisorb, Hypersil, Nucleosil, Ultracarb [with

Table II. DIU Retention Times Without the Addition of Amines to the Eluent

Compound	pK_a	Retention time (min) Spherisorb	Hypersil	Nucleosil	Ultracarb	Hamilton
ACT	7.2/9.0	3.52	1.63	3.74	3.28	2.56
CT	6.7/9.5	3.90	1.95	4.23	4.03	4.28
HCT	7.0/9.2	4.86	2.31	5.33	4.58	5.10
HFM	8.9/10.7	7.62	4.32	8.39	7.76	7.34
DCP	7.4/8.6	9.81	6.59	10.57	9.97	8.35
CLR	9.4	10.86	8.36	11.22	10.85	8.34
TCM	8.6	12.19	9.12	13.39	12.31	10.84
FRS	3.9	16.28	12.58	17.34	17.02	12.72
ALT	8.0	16.38	12.06	17.42	16.55	13.30
CLP	8.0	16.57	> 40	13.72	13.08	9.82
IND	8.1	18.59	14.08	20.10	19.86	6.82
BNZ	4.9	19.43	14.84	21.15	20.18	14.50
ATN	9.6	19.05	> 40	3.75	3.03	1.80
POL	9.1	22.35	14.39	24.57	23.58	14.95
CAN	4.2	21.57	16.32	23.24	22.50	13.60
BND	8.5	23.05	17.67	24.71	23.99	15.00
XIP	4.8	23.83	18.44	24.82	24.99	15.40
BUM	4.4	25.33	22.66	26.18	26.19	15.20
PRO	3.4	25.43	22.97	26.38	26.30	15.53
ETA	3.5	25.52	19.85	25.08	25.28	15.67
PIR	4.2	27.37	24.20	28.00	26.54	14.88
SPL	—	26.76	29.20	30.71	30.60	17.62
AML	8.7	> 40	> 40	5.75	4.15	1.80
TRI	6.2	> 40	> 40	10.33	9.17	4.96
TRS	6.2	> 40	> 40	18.50	17.00	9.33

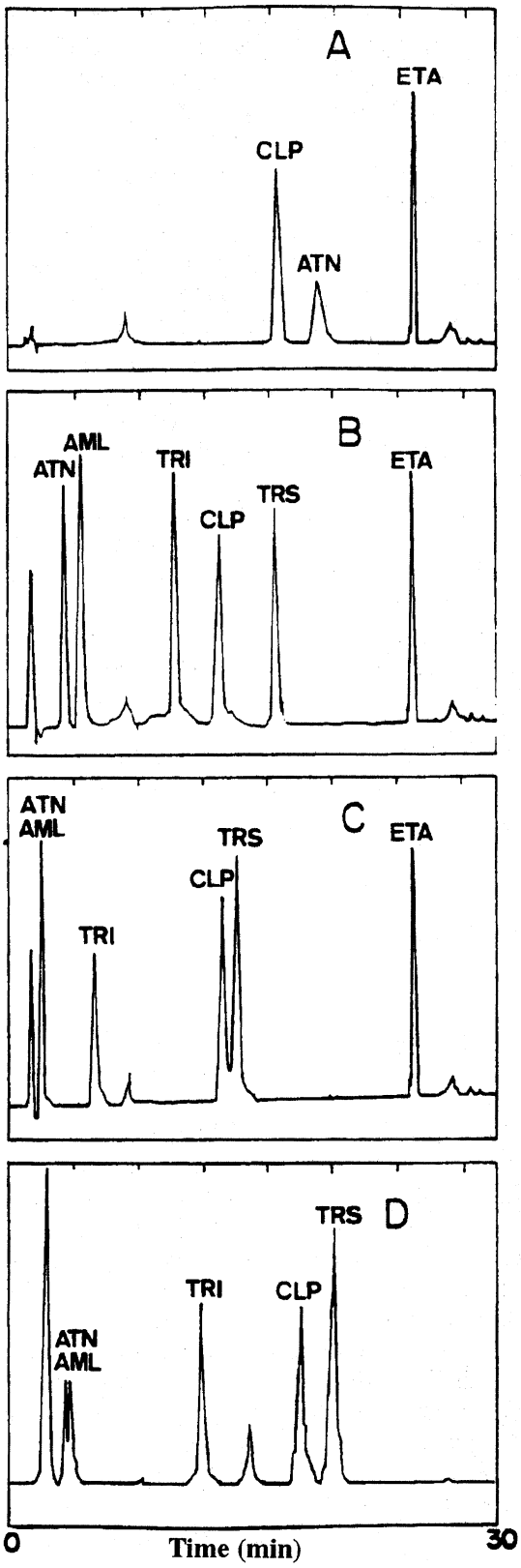


Figure 1. Typical chromatograms for AESs and ETA using a Spherisorb C_{18} column obtained under gradient elution (A) in the absence of amines, (B and C) in the presence of 4.5mM TEA or HA, and (D) with the Hamilton polymeric column. Injection for all compounds was 5 µg/mL, and UV detection was 220 nm.

a flow rate of 1.0 mL/min], and the polymeric Hamilton [with a flow rate of 0.5 mL/min]) were selected. Although the polymeric Hamilton column provides a completely different chemical environment for the solutes than C_{18} packings and perhaps involves a different retention mechanism, it was used only as a reference column because of a lack of silanol functions and a certain similarity with C_{18} packings. In order to avoid DIU coelution with the system peak, pH 3.2 was used because some of the solutes have pK_a values very close to this pH value (33,34, A.I. Gasco-López and R. Izquierdo-Hornillos. Unpublished work.) (Table II), and ionization can take place by increasing the pH (FRS, BUM, PRO, ETA, and PIR are partially ionized). Several gradient elutions were tried (run time was about 40 min) by varying the acetonitrile concentration in the range of 5–60%; to carry out further experiments, the gradient described in the Experimental section in absence of

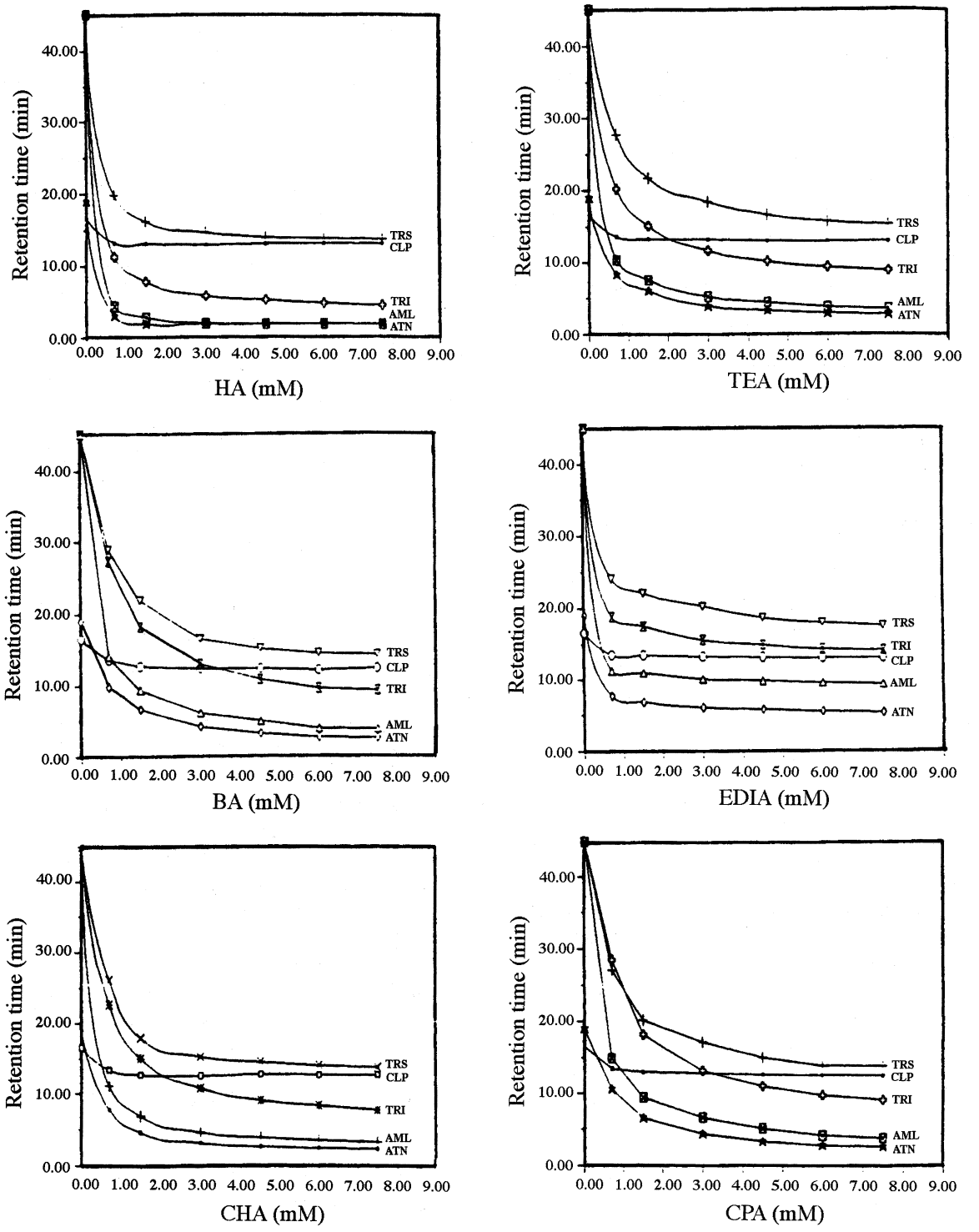


Figure 2. Influence of amine concentration on AES retention (Spherisorb C_{18} column).

Table III. Typical Amine Concentrations for AES Elution Using the Spherisorb Column and Relative Effectiveness Index for Amines Obtained from TRI Retention Data* Under Gradient Elution

Amine	$Amine_T$ (mM)	NCA	Type	Relative effectiveness index (%) (4.5mM)	Relative effectiveness index (%) (7.5mM)
HA	3.0	6	P	86	**100**
CHA	4.5	6	P	49	57
TEA	6.0	6	T	44	51
BA	6.0	4	P	41	48
CPA	> 7.5	5	P	41	48
IBA	7.5	4	P	39	45
PPA	7.5	5	S	37	43
DEA	> 7.5	4	S	34	39
EDIA	4.5	8	T	30	35
PA	> 7.5	3	P	28	33
IPA	> 7.5	3	P	26	31

* See Figure 2.

Table IV. Hydrophobic* and Silanophilic Retention for AES†

AES	Retention time (min) Spherisorb		Hypersil		Nucleosil		Ultracarb	
	t_H	t_S	t_H	t_S	t_H	t_S	t_H	t_S
CLP	13.10	3.47	9.60	> 20.4	13.91	0.03	13.74	0.01
ATN	1.90	17.15	1.20	> 28.8	2.15	1.60	2.49	0.54
AML	1.90	> 38.1	1.20	> 38.8	2.21	3.54	2.52	1.63
TRS	14.20	> 25.8	11.40	> 28.6	13.90	4.60	13.37	3.63
TRI	5.30	> 34.7	2.60	> 37.3	4.73	5.60	4.62	4.55

* After adding 7.5mM HA to the eluent.
† Retention times follow the equation (2): $t_R = t_s + t_H$. t_R values are given in Table II, and t_H values were obtained from the HA plot (see Figure 2).

Table V. Some Technical Characteristics of C_{18} Packings

	Hypersil	Spherisorb	Nucleosil	Ultracarb
Pore size (Å)	120	80	100	90
% Carbon	10.0	12	14	22
Coating (μ mol C_{18}/m^2)	2.34	2.72	2.06	3.53
Coating (μ mol C_{18}/g)	488	598	721	1306

amines was selected. In such circumstances, the pH of the hydroorganic eluent during the gradient and, obviously, other equilibrium characteristics are practically unchanged (35). These elution conditions and retention data (t_R) were used as a control test. For this reason, the effect of solute concentration was not studied. Retention times (t_R) for these compounds and columns are listed in Table II (retention time data for the Hamilton column was normalized for a flow rate of 1.0 mL/min). Some of these compounds eluted at retention times higher than 40 min (e.g., AML, TRI, and TRS) using the Spherisorb column.

DIU retention with amines under gradient elution

The Chemicals section lists the 11 amine modifiers that were added to the eluent in the concentration range of 0.7–7.5mM. These compounds affected only the retention of five solutes (AML, CLP, TRI, TRS, and ATN) with regard to the control test. These solutes were termed AESs (amine-eluting solutes), and the remaining solutes were termed ANESs (amine-noneluting solutes). Figure 1 illustrates the chromatographic behavior of AESs and ANESs (ETA was chosen as a representative example) in the absence (Figures 1A and 1D) and presence of amines (Figures 1B and 1C for TEA and HA). As can be seen, some of the AESs (TRI, TRS, and ATN) were not present in the chromatogram (Figure 1A) because of strong silanophilic interactions; however, they were definitively observed in the chromatograms (Figures 1B and 1C) due to the presence of amine modifiers in the eluent, decreasing the overall analysis run time.

In Figure 2, the variation of AES retention time with the type and amine concentration studied using the Spherisorb column is depicted. A strong decrease in AES retention was observed using small amine concentrations. However, when amine concentrations were further increased, only a slight decrease in the retention times of the AESs was observed. Thus, in order to reach a constant retention for all compounds, a typical amine concentration ($Amine_T$) higher than those required for the initial stabilization of the retention times was used (Table III). These concentrations can represent valuable data of amine effectiveness in blocking silanols. Retention for AESs at this amine concentration or higher than those considered in Table III is probably due to hydrophobic interactions.

From Table II and Figures 1 and 2, several observations can be drawn: (*a*) The elution order of AESs in the absence of amines follows the sequence CLP, ATN, AML, TRI, and TRS. However, in the presence of amines, the sequence was ATN, AML, TRI, CLP, and TRS, except when EDIA was used (TRI and CLP peaks' elution order was inverted). (*b*) When HA was used, the lowest concentration was required for reaching constant and lowest AES retention (Table III and Figure 2).

The influence of the ionic strength on AES retention was studied in the range of 5–25mM Na_2SO_4 for 3.0mM HA. Under such conditions, the retention times of these compounds did not differ significantly with regard to the same study done in the absence of Na_2SO_4. Thus, HA appeared to be more effective for silanol suppression than sodium ions, and the shapes of the curves in Figure 2 were mainly due to the increased amine concentration.

The role of the ammonium ion was also compared with HA added to the eluent. The gradient elution of AESs with 30mM ammonium ion and the Spherisorb column gave retention times of 4.83, 6.85, 12.36, 15.50, and 17.4 min for ATN, AML, TRI, CLP, and TRS, respectively. As expected (14), these values were higher than those obtained for HA as a consequence of its hydrophobicity.

The sample size effect was studied in the range of 1–10 μg/mL in the presence of amines (3.0–7.5mM). The experi-

mental t_R values varied up to 2.8%, so that t_R values were not practically affected by sample size.

AES retention using different packings

Table IV lists the retention times for AESs obtained in the presence of 7.5mM HA using four different columns. In this table, separate hydrophobic (t_H) and silanophilic (t_S) retention data assessed using Equation 3 were considered. From t_S retention data, the following sequence was obtained for the packings: Hypersil > Spherisorb > Nucleosil > Ultracarb, which indicates the relative activity (acidity) of their SiOH groups for solutes. This relative activity was correlated to the carbon percentages of packings (36). In other words, the activity of SiOH groups decreased when the carbon percentages increased (Table V).

Moreover, based on the t_S data of the Nucleosil and Ultracarb columns, the following sequence for AES solutes was also obtained: TRI > TRS > AML > ATN > CLP, showing their relative activity (basicity). The following can also be drawn: (*a*) CLP showed no interactions with the Nucleosil or Ultracarb columns; however, a stronger retention was observed with the Hypersil column. (*b*) The Hypersil column presented the strongest silanophilic retention for solutes; however, once this interaction was suppressed, the Hypersil column exhibited the lesser hydrophobic retention with solutes. (*c*) For all the columns tested, ATN and AML coeluted, exhibiting lower retention times, which indicated that 7.5mM HA suppressed mainly silanophilic interactions. In other words, before using a column for an application, a test involving the amine chosen and its concentration is suggested. Dolan (28), however, recommends never working with amines at concentrations lower than 20–25mM because below these levels, retention could be affected by small changes in amine concentration.

Influence of amine structure on AES retention and peak symmetry

Figures 1A, 1B, and 1C show the differences found for AES retention under the control test and using TEA and HA as examples. In order to explain AES retention, several correlations (*a–i* in Figure 3) were obtained for the amines studied, which are related to their structural characteristics such as type, hydrophobicity (expressed by the number of the carbon atoms (NCA)], and steric hindrance. Amines basicity was not considered because, under working conditions (pH 3.2), all of them were in the cationic form. Figure 3 shows several of these AES correlations for a 4.5mM amine concentration added to the

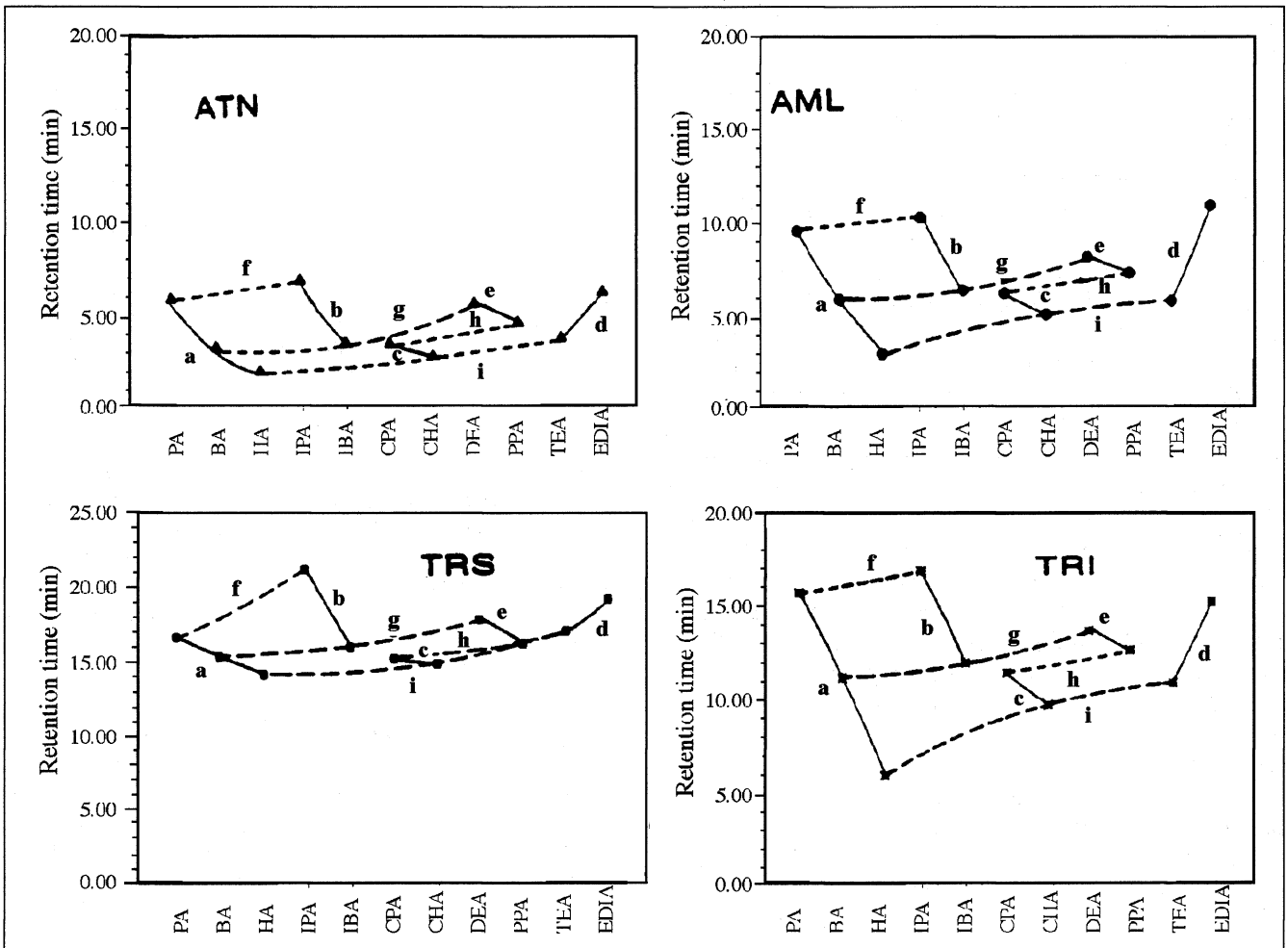


Figure 3. Correlations *a–i* between amine structure and AES retention. Full lines *a–c* for primary (linear chain, branched, and cyclic), *e* for secondary (linear chain), and *d* for tertiary amines with variable NCA. Dotted lines *f–i* also included differences between primary, secondary, and tertiary amines with constant NCA. Amine concentration was 4.5mM, and a Spherisorb C_{18} column was used. Retention times were obtained from Figure 2.

eluent (retention times for AESs were obtained from Figure 2 data). Similar plots were observed for each AES, which indicated that the equilibrium given by Equation 2 was shifted by amines to a different extent, providing typical retention times (amine effectiveness). However, AES retention was also affected to a different extent due to a different solute interaction with silanols. Taking into account the amines' characteristics (Table III) and the data from Figure 3, the following conclusions can be suggested to explain the AES retention. When primary amines were used as modifiers, the AES retention decreased as NCA increased (see full lines in Figure 3: *a* for PA, BA, and HA [linear chain], *b* for IPA and IBA [branched], and *c* for CPA and CHA [cyclic]), and the hydrophobic effect (NCA) overcame the steric effect. Using secondary amines, the results were similar to those obtained with primary amines (see the full line *e* in Figure 3 for DEA and PPA [linear chain]). On the contrary, when tertiary amines were used, the AES retention increased as NCA increased (see the full line *d* in Figure 3 for TEA [linear] and EDIA [branched]). In this case, the effect of the steric hindrance was more important than the hydrophobic effect.

When NCA remained constant, the following was observed. The AES retention increased as the amine steric hindrance increased (dotted lines *f* for NCA = 3, which includes PA and IPA; *g* for NCA = 4, BA, IBA, and DEA; *h* for NCA = 5, CPA, and PPA; and *i* for NCA = 6, HA, CHA, and TEA). Some of these lines included differences between primary, secondary, or tertiary amines (e.g., lines *g* and *i* show the difference between primary and secondary and primary and tertiary, respectively). Figure 3 also shows the steric effect in the case of branched amines (e.g., primary amines PA and IPA as well as BA and IBA). Other correlations can be obtained for comparing different amines.

Gradient elution is a poor way of studying peak symmetry caused by silanol effects because gradients tend to compensate tailing. AES solutes were eluted without adding amine additives to the eluent under gradient elution showing poor peak characteristics (e.g., see the chromatogram in Figure 1A for CLP and ATN [asymmetry factor values were 2.2 and 1.6] in which TRS, TRI, and AML were not observed). However, important improvements in retention and peak characteristics were observed when the amine concentration added to the eluent increased. In such conditions, AESs exhibited better peaks when using HA than TEA as a consequence of their effectiveness. Figure 1B shows typical chromatograms obtained using TEA (asymmetry factor [ASF] values were 1.8 and 1.4 for CLP and ATN) and in Figure 1C using HA (ASF values were 1.2 and 1.1 for CLP and ATN). Moreover, TRS, TRI, and AML were definitively observed. Like other ANESs, ETA (peak 20 in Figures 1A–1C) did not modify symmetry peak characteristics. The observed tailing effects were thus selective for AESs.

Isocratic elution is the common way of studying retention and peak asymmetry caused by silanol effects. These studies have been well-described (16,29,30) and reported plots (ASF versus amine concentration) similar to those obtained in Figure 2 (t_R versus amine concentration). These plots indicate that the improvements in retention and peak

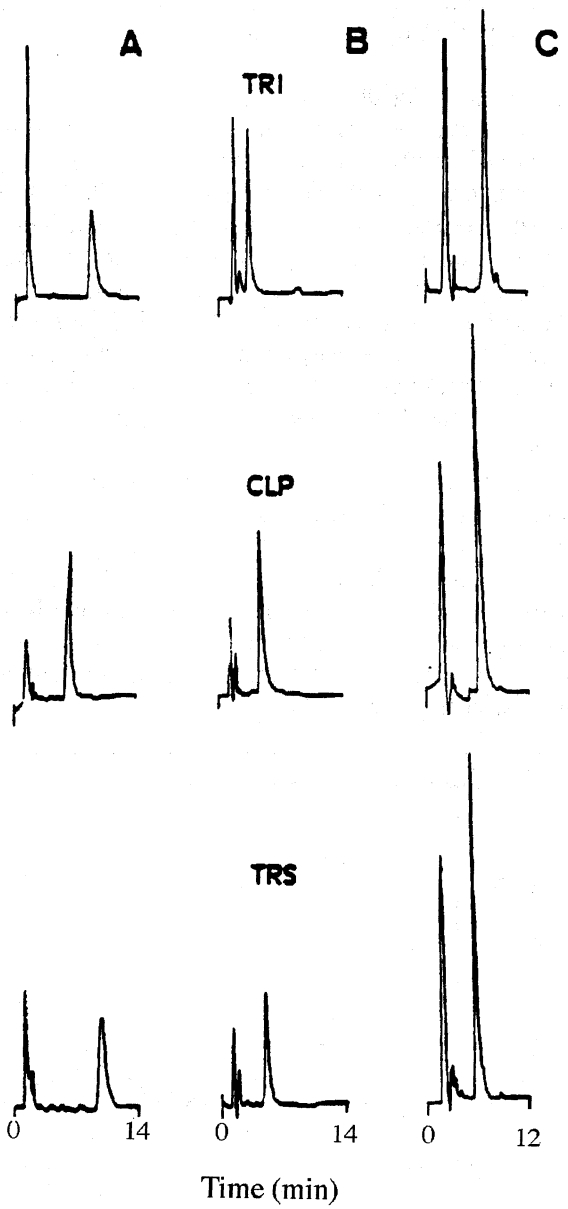


Figure 4. Typical chromatograms for some AESs obtained under isocratic elution with the following eluents: an aqueous phosphoric acid solution (pH 3.2)–acetonitrile (90:10, v/v) for TRI and another (75:25, v/v) for CLP and TRS. Figures 4A and 4B were obtained in the absence and the presence, respectively, of 4.5mM HA added to the eluent, and Figure 4C was obtained using the Hamilton column. Other conditions were as in Figure 1.

Table VI. Retention Factors (*k*) and ASF Values for Some AESs Obtained Under Isocratic Elution*

		TRI		CLP		TRS	
Column	HA	*k*	ASF	*k*	ASF	*k*	ASF
Nucleosil	—	3.30	3.4	2.0	1.5	4.05	2.0
	4.5mM	0.71	1.1	1.43	1.4	1.58	1.5
Hamilton	—	2.29	1.5	1.58	1.4	1.78	1.2

* Conditions given in Figure 4.

symmetry take place within the same process (Equation 2) because the amine concentration was increased. For this reason, lower retention times and ASF values using an appropriate amine concentration were obtained. On these bases, TRI and CLP or TRS (as the focus of our discussion) were eluted isocratically with two mobile phases of phosphoric acid (pH 3.2) and acetonitrile (90:10, v/v or 75:25,v/v) in the absence and in the presence of 4.5mM HA added to phosphoric acid (pH 3.2) (Figures 4A and 4B) using the Nucleosil column. The Hamilton column was also used but in the absence of amines (Figure 4C). Table VI shows the retention factors (k) ASF values found for CLP, TRI, and TRS (calculated as in reference 37). As expected, improvements in peak shape and retention were obtained for these compounds in the presence of HA. These results were very similar to those obtained using the Hamilton column (absence of SiOH groups), showing that HA not only exhibited the best effectiveness for AES retention but also gave appropriate peak characteristics.

Relative effectiveness index for amines

As explained above, HA proved to be the most effective masking agent for a given column, using an adequate concentration. Several facts corroborated this statement: the concentration data for amines (Table III), the lowest retention times achieved for AES, and coelution for AML and ATN using the Spherisorb C_{18} column (compared with, e.g., TEA; see Figures 1B and 1C) and others, including the polymeric Hamilton (see Table IV and Figure 1D). In other words, the masking of reactive silanols was completed, and the effect of the silanol groups on the retention using an adequate concentration is minimum or negligible. On these bases and with data obtained from Figures 2 and 3 for TRI as an example of AES, a relative effectiveness index for amines (REIA) can be suggested, the units of which are percent. This is defined for a given amine concentration by the following formula, which is adequate for gradient elutions:

$$\text{REIA} = 100 \times (t_R)_{HA}/(t_R)_A \qquad \text{Eq 4}$$

For isocratic elutions:

$$\text{REIA} = 100 \times k_{HA}/k_A \qquad \text{Eq 5}$$

where $(t_R)_A$ and k_A or $(t_R)_{HA}$ and k_{HA} are, respectively, the retention time (see Figure 2) and retention factor of TRI obtained for any concentration of a definite amine A or for the most effective amine HA used in excess (e.g., 7.5mM [REIA = 100%]). TRI was selected for this purpose because of the higher variability in retention times observed using different amines. In order to calculate REIA for other columns, the HA concentration used in excess should be evaluated. This is illustrated in Table III, which reports REIA values found for amines at concentration values of 4.5 and 7.5mM along with other useful characteristics (type, NCA) for comparing REIA. As can be observed, REIA values increased with amine concentration up to a maximum of 100%. Constant retention times for all compounds could be achieved using amine concentrations higher than 7.0mM.

Table VII. Typical Nitrogen Groups Containing DIU, PRO, and ATN Not Showing Silanophilic Interactions in RPHPLC Using C_{18} Columns

Group	Compounds
A: $CO–NH_2$ or CO–NHR	ACT, CLR, CLP, IND, ATN, XIP, AML, TRS
B: $SO_2–NH_2$	ACT, CT, HCT, HFM, DCP, CLR, TCM, FRS, ALT, CLP, IND, BNZ, POL, BND, XIP, BUM, PIR
C: $SO_2–NR_1R_2$ or SO_2NHR_2	CT, HCT, HFM, TCM, ALT, BNZ, POL, BND, PRO, TRS
D: $NR_1R_2R_3$; NHR_1R_2; $N{=}R_1R_2$; $N–NR_1R_2$ R_1: aromatic ring	CT, HCT, HFM, TCM, FRS, ALT, IND, BNZ, POL, BND, BUM, PIR, TRS

Structure–retention of AES and ANES solutes

Despite the fact that most of these solutes are molecules containing nitrogen atoms (exceptions are made for ETA, CAN, and SPL) only AES showed silanophilic interactions; then modifying their retention in the presence of amines added to the eluent. Most of the ANES compounds contain an unhindered $–SO_2NH_2$ group that is acidic, and despite the fact that the groups contain $–NH_2$, they cannot interact with the acidic silanols on the silica surface. Other nitrogen atoms in ANES molecules are mostly strongly hindered and also cannot interact with residual silanols on the packing's surface. ANES compounds containing the mentioned groups are summarized in Table VII.

Mechanism evaluation and amine effectiveness in ion-interaction RPHPLC

Available publications have reported that tertiary amines are more effective than secondary or primary and vice versa (25,30). In this regard, amine effectiveness has been studied (16,25,27,30), but the same amines and concentrations were not always used.

The role of amine type, concentration, and column have been widely discussed herein. Nevertheless, amine effectiveness is also in close relation with amine structure. The stabilization of the formed silanol–amine complex (ion pair) will depend significantly on the hydrophobic interaction between the hydrocarbonaceous ligates of the stationary phase and the alkyl chain of the amine forming the ion pair. In this way, similar solute retention should be expected if the NCA remains constant. However, in practice, this is not so (e.g., when HA is compared with CHA or TEA, as in Figures 2 and 3). Other examples have also been reported (25,30). Thus, hydrophobicity does not explain the results obtained in many cases.

When the steric effect is evaluated, primary amines should be more effective than secondary or tertiary, even though the NCA remains constant. However, this is not the case in practice. Structures such as $R–NH–CH_3$, $R–N(CH_3)_2$ (27), or $R–N(CH_3)_3^+$ (24) (where R is an alkyl chain) gave similar and higher efficacy than expected (24,26,27,29,30). In such cases, the steric hindrance produced by 1–3 $–CH_3$ groups does not

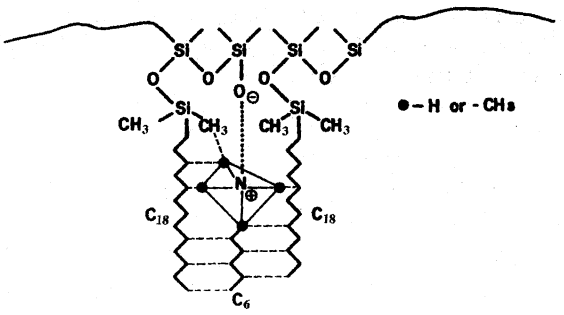


Figure 5. Model showing different interactions in the formation mechanisms of the silanol–hexylamine (or its methyl derivatives) complex. Electrostatic interactions (....) and hydrophobic interactions (——).

seem to be significant with regard to hydrogen. An increase in the alkyl length (R) enhances amine effectiveness.

Conclusion

In conclusion, amine effectiveness is due to a compromise between hydrophobicity and geometrical effects and generally follows the above rules (exception is made for methyl-amines). In Figure 5, the ionic and hydrophobic interactions are illustrated with a model also showing low steric hindrance produced by –H or $-CH_3$ groups. The use of HA is more appropriate than amines such as DMOA and CTA because larger eluent volumes are required to restore columns to the original condition (i.e., when changing to another analytical procedure). Moreover, the crudeness of amines can give rise to undesirable consequences such as loss of column efficiency with larger use of these compounds (14,38).

Amines such as R_1R_2–NH ($R_1 = R_2$ or $R_1 \neq R_2 \neq -CH_3$) can also be proposed (e.g., DBA is very similar to the HA series, as reported by Gill [27]) because its steric effect is compensated by DBA hydrophobicity. Thus, TEA required higher concentrations than HA to be effective (31) (see Table III and Figure 2). Other amines such as R–NR_1R_2, R–NHR_1 (R with exalted NCA and R_1, R_2 with NCA > 2), despite their hydrophobicity, will present high steric hindrance (unfavorable geometry) with a loss of efficacy.

On the other hand, hydrophobic interactions and hydrogen bonding (the main cause of peak asymmetry) is a third mechanism proposed by Kiel et al. (25). This mechanism, based on the poor results obtained in improving peak symmetry using quaternary modifying agents, does not agree with the results and conclusions mentioned above because steric effects were not discussed. The hydrogen bonding proposed for active hydrogen-containing amines with –SiOH is less likely to occur because hydrogen bonds are easier to form with pure methanol (approximately 27M), for example, as an organic modifier (or eluents enriched with solvents forming hydrogen bonds) than with amine modifiers. In addition, because a definite amine concentration was used throughout the work, the resulting information was probably incomplete. Thus, using TMOA acceptable peak symmetry at higher amine concentrations was obtained (29), and using HDTA, a lower concentration was required to practically reduce the silanol effect (24).

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References

1. M. Liter. *Farmacología Experimental y Clínica*, 7th ed. El Ateneo, Buenos Aires, Argentina, 1988.
2. F.T. Delbecke and M.Debackere. The influence of diuretics on the excretion and metabolism of doping agents. *Arzneim-Forsch.* **36:** 1413–16 (1986).
3. *Diuretics.* J.B. Puschett and A. Greenberg, Eds., Elsevier, Amsterdam, The Netherlands, 1984.
4. R.R. Brodie, L.F. Chasseaud, T. Taylor, and L. M. Walmsley. Determination of the diuretic triamterene in the plasma and urine of humans by high-performance liquid chromatography. *J. Chromatogr.* **164:** 527–33 (1979).
5. E. Bechgaard. Sensitive high-performance liquid chromatography determination of amiloride in human plasma by direct injection with pre-column enrichment. *J. Chromatogr.* **490:** 219–33 (1989).
6. H.J. Reeuwijk, V.R. Tjaden, and J. Van der Greef. Simultaneous determination of furosemide and amiloride in plasma using high-performance liquid chromatography with fluorescence detection. *J. Chromatogr.* **575:** 269–74 (1992).
7. F. Varin, J.P. Villeneuve, and Y. Theoret. High-performance liquid chromatographic determination of spironolactone and its metabolites in human biological fluids after solid-phase extraction. *J. Chromatogr.* **574:** 57–63 (1992).
8. T. Sakata, A. Matsuura, and T. Kitagawa. Determination of trichlormethiazide in human plasma and urine by high-performance liquid chromatography. *Chromatographia* **33:** 339–43 (1992).
9. P. Campis-Falcó, R. Herráez-Hernández, and A. Sevillano-Cabeza. On-line enrichment for the determination of ethacrynic acid in urine by liquid chromatography and column-switching. *Anal. Chim. Acta* **284:** 67–71 (1993).
10. R.O. Fullinfaw, R.W. Bury, and R.F. Moulds. Liquid chromatographic screening of diuretics in urine. *J. Chromatogr.* **415:** 347–56 (1987).
11. S. Park, H. Pyo, Y. Kim, M. Kim, and J. Park. Systematic analysis of diuretics agents by HPLC screening and GC/MS confirmation. *J. Anal. Toxicol.* **14:** 84–89 (1990).
12. R. Herráez-Hernández, P. Campis-Falcó, and A. Sevillano-Cabeza. Improved screening procedure for diuretics. *J. Liq. Chromatogr.* **15:** 2205–13 (1992).
13. F. de Croo, W. Van Den Bossche, and P. de Moerloose. Influence of the chain length of chemically bonded phases on the behavior of several thiazide, potassium-sparing and loop diuretics in high-performance liquid chromatography. *J. Chromatogr.* **563:** 301–309 (1991).
14. D. Chan, M.A. Stadalius, J.S. Berus, and L.R. Snyder. Reversed-phase HPLC of basic samples. *LC–GC* **1(5):** 21–30 (1988).
15. R.K. Gilpin, S.S. Yang, and G. Werner. Recent review of secondary mobile phase modifiers used to enhance the chromatographic analysis of pharmaceuticals. *J. Chromatogr. Sci.* **26:** 388 (1988).

16. G. Stoev and D. Uzonov. Influence of the structure of the amine modifiers on the mechanism of the separation of basic compounds by reversed phase high-performance liquid chromatography. *J. Liq. Chromatogr.* **15:** 3097–3114 (1992).
17. L.R. Snyder, J.L. Glajch, and J.J. Kirkland. *Practical HPLC Method Development.* John Wiley & Sons, New York, NY, 1988.
18. E. Bonet, M.J. Medina, G. Ramis, and M.C. García. Evaluation of diuretics in pharmaceuticals by high-performance liquid chromatography with a 0.05 moldm^{-3} sodium dodecylsulfate–3% propanol mobile phase. *Analyst* **117:** 843–51 (1992).
19. E. Bonet, M.J. Medina, G. Ramis, and M.C. García. High-performance liquid chromatographic determination of diuretics in urine by micellar liquid chromatography. *J. Chromatogr.* **582:** 189–94 (1992).
20. F. De Croo, W. Van Den Bossche, and P. De Moerloose. High performance liquid chromatographic behavior of some pharmaceutically important thiazide, loop and potassium sparing diuretics. *J. Chromatogr.* **325:** 395–411 (1985).
21. R.W. Roos and C.A. Lau-Cam. General reversed-phase high-performance liquid chromatographic method for the separation of drugs using triethylamine as competing base. *J. Chromatogr.* **370:** 403–18 (1986).
22. S.F. Cooper, R. Masse, and R. Dugal. Comprehensive screening procedure for diuretics in urine by high-performance liquid chromatography. *J. Chromatogr.* **489:** 65–88 (1989).
23. A. Nahum and C. Horvath. Surface silanols in silica-bonded hydrocarbonaceous stationary phases. *J. Chromatogr.* **203:** 53–60 (1981).
24. K.E. Bij, C. Horvath, W.R. Melander, and A. Nahum. Surface silanols in silica-bonded hydrocarbonaceous stationary phases. II. Irregular retention behavior and effect of silanol masking. *J. Chromatogr.* **203:** 65–84 (1981).
25. J.S. Kiel, S.L. Morgan, and R.K. Abramson. Effects of amine modifiers on retention and peak shape in reversed phase high performance liquid chromatography. *J. Chromatogr.* **320:** 313–23 (1985).
26. Z. Varga-Puchony and G. Vigh. Role of silanophilic retention in reversed-phase chromatography of chloramphenicol intermediates. *J. Chromatogr.* **257:** 380–83 (1983).
27. R. Gill, S.P. Alexander, and A.C. Moffat. Comparison of amine modifiers used to reduce peak tailing of 2-phenylethylamine drugs in reversed-phase high performance liquid chromatography. *J. Chromatogr.* **427:** 39–45 (1982).
28. J.W. Dolan. Changing system conditions. *LC–GC International* **5(11):** 16–17 (1992).
29. P.O. Lagerstrom, I. Marie, and B.A. Persson. Solvent extraction of tricyclic amines from blood plasma and liquid chromatographic determination. *J. Chromatogr.* **273:** 151–60 (1983).
30. A. Sokolowsky and K.G. Wahlund. Peak tailing and retention behavior of tricyclic antidepressant amines and related hydrophobic ammonium compounds in reversed-phase ion-pair liquid chromatography on alkyl-bonded phases. *J. Chromatogr.* **189:** 299–316 (1980).
31. D.W. Hill and A.J. Kind. The effects of type B silica and triethylamine on the retention of drugs in silica-based reversed phase high-performance liquid chromatography. *J. Liq. Chromatogr.* **16:** 3941–64 (1993).
32. J.L. Peral-Fernandez, R. Izquierdo-Hornillos, A. Cabrera Martin, and R. Gallego-Andreu. Determination of mercury in the presence of chloride by means of the ternary complex Hg(II)/Amberlite LA-2 in non-aqueous media. *Talanta* **30:** 179–83 (1983).
33. *The Merck Index,* 10th ed., M. Windholz, Ed., Merck, New York, NY, 1983.
34. *Clarkes's Isolation and Identification of Drugs.* A.C. Moffat, Ed., Pharmaceutical Press, London, UK, 1986.
35. P. Chaminade, A. Baillet, D. Ferrier, B. Bourguignon, and D.L Massart. Efficient determination of the pK values of six chlorinated phenols by reversed-phase liquid chromatography. *Anal. Chim. Acta* **280:** 93–101 (1993).
36. *Phenomenex Columns Catalog.* Phenomenex, Torrance, CA, 1995.
37. R.G. Brownlee and J.W. Higgins. Peak symmetry as a measure of column performance in high-performance liquid chromatography. *Chromatographia* **11:** 567–72 (1978).
38. M. Verzele and C. Dewaele. Stationary-phase characterization in high-performance liquid chromatography. Test for trace metal activity in octadecyl-bonded silica gel. *J. Chromatogr.* **217:** 399–404 (1981).

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