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The hydrophilic interaction like properties of some reversed phase high performance liquid chromatography columns in the analysis of basic compounds

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ABSTRACT

The hydrophilic interaction chromatography (HILIC) like properties of an ACE cyano (CN) HPLC column was studied for the separation of some basic compounds. Good separation of a test mix of basic compounds was obtained with a mobile phase consisting of acetonitrile/water (95:5) containing 3.25 mM ammonium acetate. The retention times of the basic compounds decreased with increased ionic strength or with increased water content in the mobile phase. When Trishydroxymethyl aminomethane (Tris) (pK_a 8.0), which is a weaker amine than ammonia (pK_a 9.3), was used as an additive in the mobile phase retention of the basic compounds was increased. The ACE CN column gave excellent peak shapes for all the basic compounds. The utility of the column for impurity profiling of two basic drugs was tested and some impurities in oxprenolol were characterised by interfacing with Fourier transform mass spectrometry. It was also observed that ACE butyl and ACE phenyl columns retained basic compounds when the columns were eluted with a mobile phase consisting of acetonitrile/water (95:5) containing 3.25 mM ammonium acetate.

1. Introduction

The term hydrophilic interaction chromatography was coined by Alpert in 1990 [1]. In the pharmaceutical industry the analysis of basic compounds can still present problems with regard to peak shape and column overloading [2]. The requirement for good peak symmetry in the analysis of basic compounds has driven the development of high purity silica gel phases free from metallic impurities for use in the preparation of reversed phases. In addition processes such as end-capping have been developed which are designed to reduce the interaction of basic compounds with silanol groups. Another problem which has arisen in the development of reversed phases is the need to adequately retain very polar basic compounds which have limited affinity for reversed phases and hence polar embedded phases have been developed which can be used with high water content in the mobile phase without the alkyl stationary phase coating collapsing. Another method for the HPLC analysis of basic compounds is HILIC and there have been a number of studies in this area. Bare silica gel and modified silica phases have been used for the separation of basic compounds using conditions with high organic modifier content in the mobile phase and a low level of aqueous content. In a recent paper McCalley studied the retention of some model basic compounds on bare silica gel, a zwitterionic phase and a silica bonded diol phase [3]. Of the phases studied silica was the most retentive and where the silica gel surface was masked, for instance with diol groups, the retentivity of the phase was reduced. The studies were carried out at pH 3.0 and increased ionic strength of the mobile phase and increased water content of the mobile phase reduced the retention times of the test probes [3]. In our earlier work we studied a cyanopropyl (CN) column with regard to its ability to produce dipole–dipole interactions and also the tendency of the ligand coating of the column to be unstable [4] and it was of interest to extend this work to study the HILIC properties of the CN column. The use of CN columns in HILIC mode has not been extensive and as recently as 2008 the CN column was presented as being suitable for normal phase separations where a mixture of hydrocarbon based solvent and a polar modifier was used in the chromatography of basic compounds [5]. In addition, the CN column has been used as an alternative reversed phase column [6–8]. However, it has also been used successfully in HILIC mode with the authors of the papers perhaps not appreciating that that was the mechanism they were exploiting [9,10]. In addition, there are some examples where a CN column was used explicitly in HILIC mode. A recent paper tested a number of HILIC columns with regard to their ability to retain catecholamines and their acidic metabolites [11]. The methodology exploited the ion exchange interactions of the amines with ionised silanol groups in the phase as well as a purely HILIC mechanism to retain acidic compounds. A CN column was among the columns tested and it was found that retention of

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the amines increased as the pH of the mobile phase was increased which was attributed to increased ionisation of the silanol groups in the stationary phase promoting increased interaction with the amine functionalities of the analytes [11]. A cyanoethyl column was investigated for its ability to retain strongly basic alcohol denaturants in HILIC mode using acetonitrile as the organic modifier [12]. Successful chromatography was only achieved when perchlorate or trifluoroacetate was used to form strong ion pairs with the analytes in order to reduce their interaction with the silanol groups of the stationary phase. By varying the % of acetonitrile it was possible to resolve all three analytes of interest and the optimum resolution was achieved at 60% acetonitrile. A sensitive and quantitative method was developed for the analysis of piperazine in HILIC mode using a cyanopropyl column [13]. The method worked best when strongly acidic conditions were used with either nitric acid or trifluoroacetic acid being used to suppress the ionisation of the silanol groups. Several phases were tested for their ability to retain folates in order to develop a tandem MS method for the analysis of these compounds in plasma [14]. Most of the columns tested were used in reversed phase mode but among the columns tested was a cyanopropyl column which was eluted with acetonitrile/methanol (60:40) containing ammonium acetate or ammonium formate, thus under HILIC conditions. The CN column gave broad tailing peaks and in the end a bare silica column was used for the analysis in HILIC mode. A CN column was tested for its ability to retain a series of peptides under aqueous normal phase conditions (HILIC conditions), however the peptides were found elute at the void volume of the column [15]. The applications of CN columns and other columns in HILIC mode have been recently reviewed [16].

From the review above it can be seen that although CN columns have been used in HILIC mode there has been no detailed evaluation their performance in this mode. In the current paper we have studied the mechanism of retention of basic compounds on an ACE CN column in HILIC mode and compared the retention of a base test mix under the same conditions on two other reversed phases prepared from the same base silica gel.

2. Materials and methods

2.1. Chemicals

Ammonium acetate, Tris free base, HPLC grade acetic acid, HPLC grade methanol and HPLC grade acetonitrile were obtained from Fisher Scientific, Loughborough, UK. HPLC water was prepared in house using a Milli Q purification system. The basic compounds used in the running tests were from either Sigma Aldrich, Dorset, UK, were European Pharmacopoeia Standards or were part of an in house stock donated by companies over the years. Anisole and 1,2 dinitrobenzene (1,2 DNB) were obtained from Sigma Aldrich, Dorset, UK.

2.2. Mobile phase preparation

3.25 mM and 6.5 mM ammonium acetate mobile phases were prepared by dissolving respectively 125 mg and 250 mg of ammonium acetate in 500 ml of the required ratio of acetonitrile/water. Where pH adjustment was carried out 65 mM ammonium acetate or Tris buffers were prepared at pH 5.0, 6.0 or pH 7.0 by adjusting with ammonia or acetic acid and then 25 ml of buffer was mixed with 475 ml of acetonitrile.

2.3. HPLC columns

ACE CN, ACE phenyl (Ph), ACE butyl (C4), ACE octadecyl (C18) and ACE silica gel (Si) columns (150 mm × 3 mm i.d. × 3 μM particle size) were obtained from HiChrom Ltd., Reading, UK. The alkyl columns and CN column are all endcapped.

2.4. HPLC instrumentation

HPLC analysis was carried out on a ThermoFinnigan HPLC system consisting of a P2000 pump, P200 UV dual wavelength detector and an AS2000 autosampler. The mobile phase compositions were mixed off-line in the proportions required and the HPLC system was used in isocratic mode, 10 μl of sample was injected. The flow rate was 0.8 ml/min and the column was run at room temperature. The UV detector was set to monitor 220 and 280 nm. The continued stability of the CN column was monitored by injecting a mixture of anisole and 1,2 DNB with a mobile phase composition consisting of methanol/water (30:70). The void volume of the columns was determined according to the minor disturbance peak produced by injecting 10 μl of pure methanol.

2.5. LC–MS instrumentation

LC–MS was carried out on an Orbitrap Exactive instrument (ThermoFisher, Hemel Hempstead, UK). The instrument was interfaced with a Dionex HPLC system (Dionex, Camberly, UK). The flow rate was 0.4 ml/min, the gas flows in the ESI source were sheath gas 60 (arbitrary units) and auxiliary gas 25 (arbitrary units), the capillary temperature was 275 °C and the needle voltage was 4.5 kV. The instrument was freshly tuned with the manufacturer's standard tuning solution with the addition of pyridine to calibrate the lower mass range. The residual caffeine peak from the tuning solution was used as a lock mass.

2.6. Sample preparation

Samples were prepared as stock solutions of 1 mg/ml in methanol and diluted to a concentration of 0.1 mg/ml in mobile phase. The sample of oxprenolol for impurity profiling was dissolved at a concentration of 2 mg/ml in mobile phase.

3. Results and discussion

3.1. Factors governing the retention properties of a test mix of basic compounds under HILIC-like conditions

Fig. 1A shows a chromatogram of a test mixture containing five basic compounds: propranolol, chlorpromazine, salbutamol, nortriptyline and benzalkonium chloride (Fig. 2) run on an ACE CN column with using 3.25 mM ammonium acetate in the mobile phase without pH adjustment. The peak shapes obtained for the basic compounds were excellent with theoretical plates being >60,000 plates/m. There is no immediately clear mechanism for the retention of the compounds except that the completely ionised quaternary ammonium compound is most strongly retained. The three secondary amines propranolol, salbutamol and nortriptyline, which have similar pK_a values, are retained to a differing extent. Fig. 1B shows the effect when the concentration of the ammonium acetate in the mobile phase is increased to 6.5 mM, which results in the retention of the basic compounds being reduced by nearly 50%. This supports a proposal that much of the retention mechanism is due to ion exchange with increased ionic strength reducing the interaction of the basic compounds with the stationary phase. Although the phase is endcapped, as is the case with all endcapped phases, many free silanol groups remain within the phase. Fig. 1C shows the effect of decreasing the water content of the mobile phase from 5% to 3% which produces a marked increase in retention times. The effect of changing the aqueous content of the mobile phase is complex since there are several

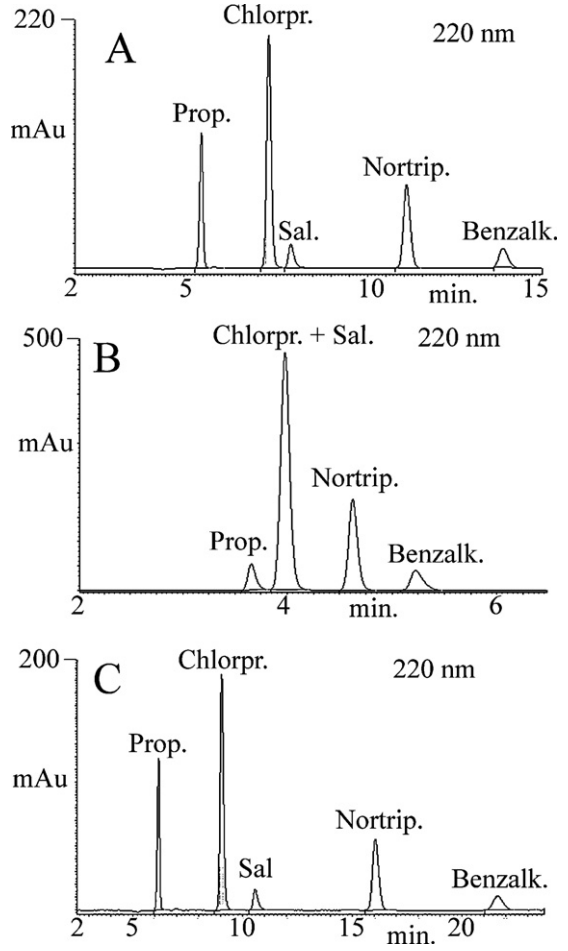


Fig. 1. (A) Separation of test mixture of basic compounds (propranolol, chlorpromazine, salbutamol, nortriptyline, benzalkonium chloride) on an ACE CN column (150 mm × 3 mm) in acetonitrile/water (95:5) containing 3.25 mM ammonium acetate, flow rate 0.8 ml/min. (B) Separation of test mixture of basic compounds on an ACE CN column (150 mm × 3 mm) in acetonitrile/water (95:5) containing 6.5 mM ammonium acetate, flow rate 0.8 ml/min. (C) Separation of test mixture of basic compounds on an ACE CN column (150 mm × 3 mm) in acetonitrile/water (97:3) containing 3.25 mM ammonium acetate, flow rate 0.8 ml/min.

interacting effects. Log k plots for the basic compounds in the test mixture, were non linear. This reflects the observations of previous workers where the ion-exchange properties of four different HILIC columns were investigated using neutral, acidic and basic test probes [17]. It was found that log k plots against % organic were non-linear thus pointing to complex retention mechanisms in contrast to the linear log k plots which would be obtained for straightforward reversed partition chromatography where the single retention mechanism of hydrophobic interaction predominates [18]. In the current example there are three sets of pK_a values to consider: the pK_a values of the basic compounds in the test mixture, the pK_a value of the ammonium ion and the pK_a value of the silanol groups in the stationary phase. All of these values change with the % of organic modifier used in the mobile phase thus affect retention and contribute to a complex retention mechanism. Table 1 shows the retention factors for a series of basic compounds on the ACE CN column. In Table 1 it can be seen that the very weak basic compounds itraconozole, papaverine, lidocaine and bupivacaine elute at the void volume of the column. Thus the important factor governing retention might be the pK_a of the analyte in comparison with the pK_a of the basic counter ion present in the mobile phase.

Table 1
Retention factors (t_0 = 1.3 min) of basic compounds on an ACE CN column (150 mm × 3 mm × 3 μm particle size) in 3.25 mM ammonium acetate in acetonitrile/water (95:5), flow rate 0.8 ml/min. p, primary amine; s, secondary amine; t, tertiary amine; q, quaternary ammonium salt. pK_a values taken from Clark's Analysis of Drugs and Poisons Third Edition. na, not available.

Base	k	pK_a	Base	k	pK_a
Itraconozole (s)	−0.1	3.7	Chlorpromazine (t)	4.6	9.0
Lidocaine (t)	−0.1	7.9	Salbutamol (s)	5.1	9.5
Bupivacaine (t)	0.2	8.1	Acebutolol (s)	5.1	9.4
Papaverine (t)	−0.2	6.4	Atenonol (s)	5.5	9.6
Ketamine (s)	−0.2	7.5	Methoxytryptamine (p)	5.9	na
Cyclizine (t)	1.3	7.7	Chlorpheniramine (t)	6.1	9.2
Propranolol (s)	3.2	9.7	Synephrine (s)	6.6	8.9
Dosulepin (t)	3.2	na	Nortriptyline (s)	7.5	9.7
Diphenhydramine (t)	3.6	9.0	Pseudoephedrine (s)	6.8	9.8
Oxprenolol (s)	4.1	9.5	Homoatropine (t)	7.5	9.9
Dopamine (p)	4.3	8.8	Benzalkonium Cl (q)	9.6	
Benzylamine (p)	4.5	9.7			

3.2. The effect of changing the counterion in the mobile phase

In order to test the hypothesis that the pK_a of the basic counterion governs elution 3.25 mM Tris acetate buffers at pH 6.0 and pH 7.0 were prepared and compared with ammonium acetate buffers at pH 6.0 and pH 7.0. The pK_a of Tris is 8.0 compared with ammonia which has a pK_a of 9.2, and in comparison it will be less charged than most of the basic compounds listed in Table 1 at any pH. Table 2 shows the retention times obtained for some of the basic compounds listed in Table 1 when Tris acetate buffer and ammonium acetate buffers at pH values 6.0 and 7.0 were used in the mobile phase in combination with acetonitrile in a 5:95 ratio at a concentration of 3.25 mM. Using Tris as the buffer increased the retention times of all the basic compounds and the weak base bupivacaine (pK_a value 8.1) was also weakly retained when Tris acetate at pH 6.0 was used to provide a counter ion, however, lidocaine (pK_a 7.9) was not. Thus it would appear that the retention times of basic compounds can manipulated in accordance with the pK_a of the base used to buffer the mobile phase.

3.3. Separation of salbutamol and its manufacturing impurities

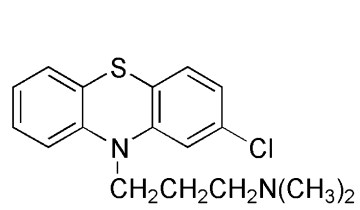
Regardless of the exact retention mechanism the CN column performs well for the separation of basic compounds. Fig. 3 shows the separation of salbutamol (1 mg/ml) from three of its manufacturing impurities listed in the European Pharmacopoeia (Fig. 2) spiked at 1 μg/ml and although the differences in structure of the impurities from salbutamol are quite minor the column exhibits both good selectivity for the mixture and a wide dynamic range with low level impurities being efficiently eluted and detected. The RSD values (n = 6) for the areas and retention times of the impurity peaks at the 1 μg/ml level were salbutamol ketone (area ± 3.4%, retention time ± 0.4%), salbutamol aldehyde (area ± 0.6%, retention time ± 0.4%) and desoxysalbutamol (area ±1.2%, retention time ± 0.5%). The impurities are well resolved from the salbutamol peak and, advantageously all elute before it so that there is no interference from the tail of the overloaded salbutamol peak. The method in the European Pharmacopoeia uses a less convenient ion pairing method with a run time of 50 min [19].

3.4. Impurity profiling of oxprenolol

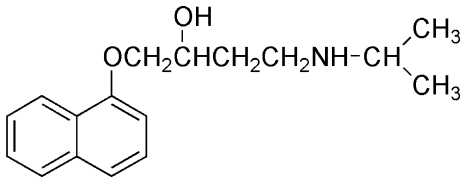
Another example of the usefulness of the column for impurity profiling is provided by the analysis of impurities in oxprenolol. Fig. 4 shows the analysis of a 2 mg/ml solution of oxprenolol where impurities at a level <0.05% of the height of the main peak can be observed. Although there are no impurities listed in the European

Table 2
Retention factors (t_0 = 1.3 min.) of basic compounds run on an ACE CN column (150 mm x 3 mm) in: acetonitrile/water (95:5) containing 0.35 mM ammonium acetate buffer pH 6.0; acetonitrile/water (95:5) containing 0.35 mM Tris acetate buffer pH 6.0; acetonitrile/water (95:5) containing 0.35 mM ammonium acetate buffer pH 7.0 or acetonitrile/water (95:5) containing 0.35 mM Tris acetate buffer pH 7.0. Flow rate 0.8 ml/min. RSD based on n = 3.

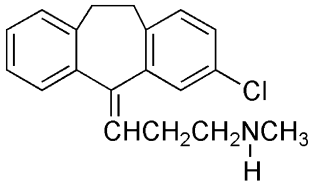
Basic compound	k NH_4AcpH 6	RSD ± %	k Tris AcpH 6	RSD ± %	k NH_4AcpH 7	RSD ± %	k Tris AcpH 7	RSD ± %
Propranolol	2.4	0.6	3.7	0.3	3.1	0.6	4.0	1.91
Chlorpromazine	3.5	1.6	6.2	0.2	5.2	0.3	7.4	1.06
Salbutamol	4.2	2.4	8.0	0.1	7.0	0.2	11.9	0.08
Nortriptyline	6.90	2.2	11.9	0.1	11.5	0.2	15.4	0.13
Benzalkonium	9.5	2.7	15.6	0.2	17.2	0.2	22.0	0.48
Bupivacaine	−0.1	0.1	1.7	0.1	1.0	0.1	1.0	0.11
Lidocaine	−0.2	0.1	−0.2	0.1	−0.2	0.1	−0.2	0.12



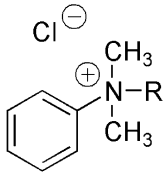
chlorpromazine pKa 9.0



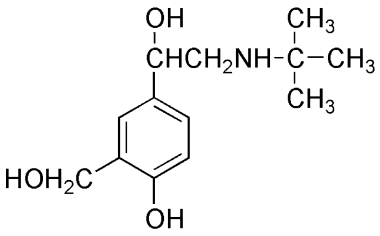
propranolol pKa 9.5



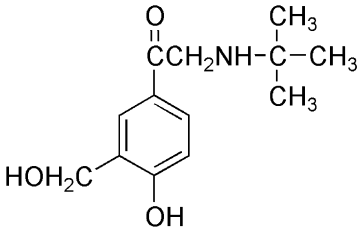
nortriptyline pKa 10.3



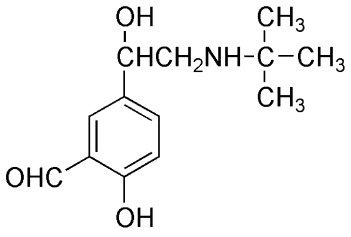
benzalkonium chloride R= C_8H_{17}-$C_{18}H_{37}$



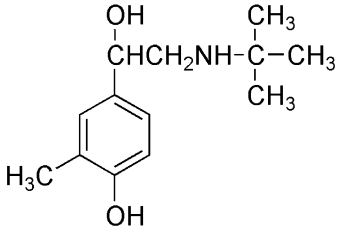
salbutamol pKa 9.5



salbutamol ketone



salbutamol aldehyde



desoxysalbutamol

Fig. 2. Structures of the basic compounds included in the test mixture and manufacturing impurities in salbutamol.

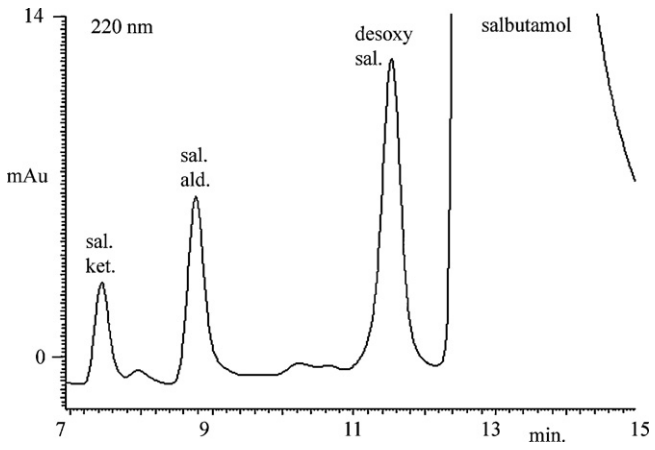


Fig. 3. Separation of salbutamol and its manufacturing impurities on an ACE CN column (150 mm × 3 mm) in acetonitrile/water (95:5) containing 3.25 mM ammonium acetate pH 5.0, flow rate 0.8 ml/min.

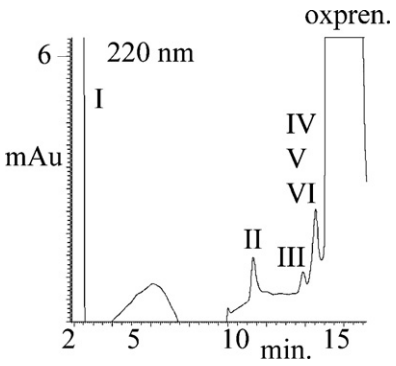


Fig. 4. Separation of oxprenolol and its manufacturing impurities on an ACE CN column (150 mm × 3 mm) in acetonitrile/water (95:5) containing 3.25 mM ammonium acetate, flow rate 0.4 ml/min.

Pharmacopoeia for oxprenolol it is possible to propose identities for the impurities on the basis of the synthetic route to the compound and by analogy with impurities listed for other β-adrenergic blocking agents such as atenolol or propranolol in the EP. Fig. 5 shows the structures proposed for the impurities on the basis of their elemental composition determined by FT-MS (Table 3) and the synthetic route to the drug. This analysis indicates that the retention mechanism on the CN column is also influenced by molecular shape for instance absence of the propenyl side chain causes impurity II to elute 4 min earlier than oxprenolol and the bulky product I which is formed from the reaction of oxprenolol with an epoxide intermediate is very early eluting.

Table 3
Impurities in oxprenolol determined by LC–MS on an Orbitrap Exactive instrument using an ACE CN column in 3.25 mM ammonium acetate in acetonitrile/water (95:5) flow rate 0.4 ml/min. The mass deviations from the exact mass of the proposed elemental composition are shown in ppm (10^6 × mass deviation/exact mass).

Elemental comp.	Deviation (ppm)	*k*
I $C_{27}H_{38}NO_6$	+0.46	1.2
II $C_{12}H_{20}NO_3$	−0.22	7.8
III $C_{15}H_{25}NClO_4$	+0.24	9.7
IV $C_{24}H_{34}NO_6$	+0.50	10.2
V $C_{15}H_{26}NO_3$	−0.11	10.3
VI $C_{12}H_{20}NO_2$	0.21	10.4
Oxprenolol $C_{15}H_{24}NO_3$	−0.11	10.7

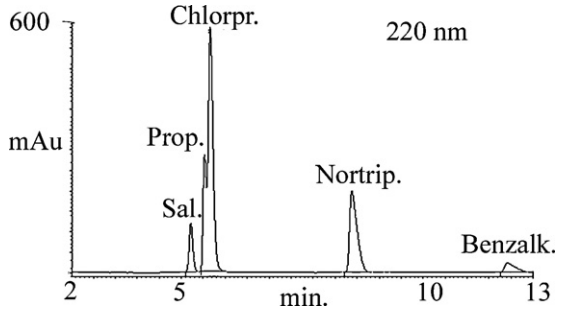


Fig. 6. Separation of the basic compound test mixture on an ACE Ph column (150 mm × 3 mm) in acetonitrile/water (95:5) containing 3.25 mM ammonium acetate, flow rate 0.8 ml/min.

3.5. HILIC-like properties of other alkyl phases

The ion exchange effect of course can occur on other silica based columns Fig. 6 shows the basic test mixture run on an ACE phenyl

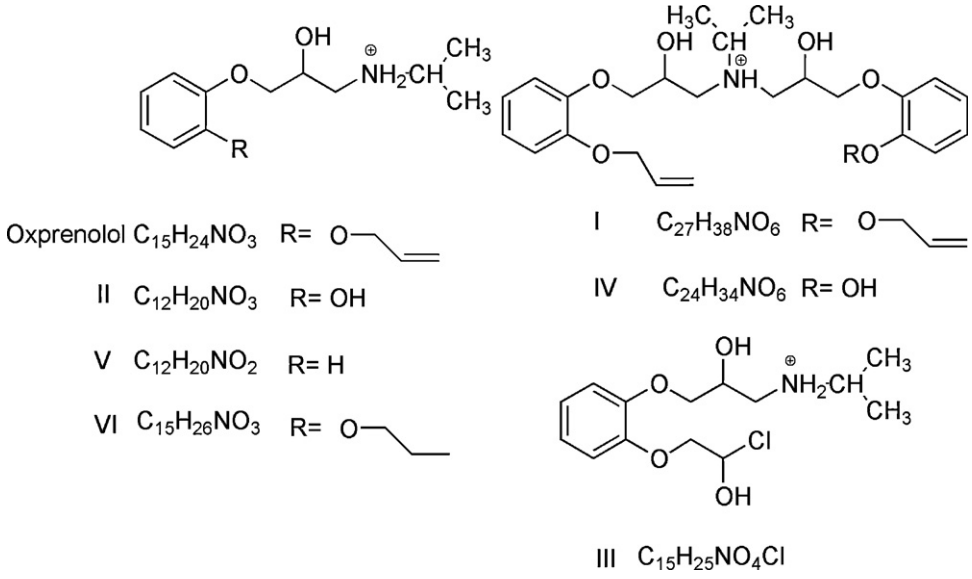


Fig. 5. Manufacturing impurities (as the protonated basic compounds) in a sample of oxprenolol proposed from elemental composition data. ppm = 10^6 × mass deviation/exact mass.

Table 4
Comparison of retention factors of basic compounds on ACE CN, ACE Ph, ACE C4 and ACE Si columns (all 150 mm × 3 mm × 3 μm particle size) in 3.25 mM ammonium acetate in acetonitrile/water (95:5) flow rate 0.8 ml/min.

Base	*k* CN	*k* Ph	*k* C4	*kSi*
Chlorpromazine	3.2	2.9	2.7	6.6
Propranolol	4.6	3.0	3.2	10.5
Salbutamol	5.1	2.7	2.6	3.4[a]
Nortriptyline	7.5	5.5	5.3	13.0[a]
Benzalkonium Cl	9.6	8.3	7.8	9.5, 10.0, 10.4[b]

[a] Wide peak.
[b] Compound has a range of alkyl chain lengths.

column in acetonitrile/water (95:5) containing 3.25 mM ammonium acetate. It should be possible to optimise separations of basic compounds on this column and get different selectivity from the CN column, however, the initial impression under the test conditions is that the CN column has better selectivity and better mass transfer characteristics. Table 4 compares the retention times of the basic compounds in the test mixture on ACE CN, C4, Ph and Si columns. The selectivity of these columns is rather different from that of the CN column and on both the C4 and the Ph columns salbutamol elutes earliest suggesting that the ligand attached the surface of the column may affect the ability of different analytes to interact with the silanol groups. On the silica gel column the retention times of the basic compounds in the test mix are very different from those obtained on the CN column.

3.6. Column stability

The ACE CN column was found to produce good peak shape and the retention times for the base test mix were stable over 200 h of running time indicating its stability in HILIC mode and thus pointing to stability of the base silica under these conditions. With regard to the stationary phase coating, CN columns have been found to be relatively unstable and the cyanopropyl ligand is more likely to hydrolyse than purely alkyl ligands [4]. In order to evaluate the stability of the cyanopropyl ligand the column was monitored with two test probes anisole and 1,2-dinitrobenzene (1,2-DNB) both run in methanol/water (30:70). The lipophilicity of the ACE CN column is quite low and the anisole was not strongly retained under the test conditions, however, it does give an indication of any changes in the lipophilicity of the column which is due to both the cyanopropyl ligand and the endcapping groups. The 1,2-DNB undergoes dipole–dipole interaction [4] with the CN group in the stationary phase ligand and is a sensitive indicator of the loading of the cyanopropyl ligand. The retention times of the anisole (2.7 min) and the 1,2-DNB (5.5 min) had not changed after 6000 column volumes of mobile phase has passed through the column. To preserve the column life it is important to ensure that the ammonium acetate is flushed out of the column when it is to be stored for any length of time.

4. Conclusion

From Fig. 6 and Table 4 it can be seen that the alkyl ligand attached to the silica gel surface affects the selectivity of the column for basic compounds. The structure of the bound ligand might well govern the ability of a basic compound to interact with the residual silanol groups in the phase. The nature of these interactions remains to be explored. Working with high organic content in the mobile phase also offers improved mass transfer properties and the efficiencies obtained on the CN column studied in this paper are very high. The low backpressures obtained with high organic content mean that longer 3 μm columns could be used and it there was a need to economise on acetonitrile long narrower bore columns should work well with the high acetonitrile mobile phases which generate only relatively low backpressures.

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