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Systematic comparison of different functionality columns for a classical pharmaceutical problem

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Abstract

The performance of five reversed-phase columns which included a standard C_{18} phase, a polar embedded phase (amide group), a polyethyleneglycol phase, a cyano phase, and a perfluorinated phase, all coming from the same manufacturer, have been studied. They were systematically compared with a test mixture containing basic, neutral and acidic compounds of very different polarities, as well as different functional groups (acetaminophen, phenylephrine hydrochloride or phenylpropanolamine hydrochloride, chlorpheniramine maleate, 4-aminophenol, 4-chloracetanilide and 4-nitrophenol) at three pH levels (2.5, 4.6 and 7.0) and three proportions of buffer/acetonitrile (80:20, 50:50 and 20:80, v/v). The results obtained have permitted our group to develop unique applications with these columns, as these compounds are not only a test mixture, due to their chemical characteristics, but they are also usually contained in pharmaceutical formulations for the relief of common cold symptoms and have been selected as a real-life case study.

Moreover, after observing the reversed-phase and normal-phase-like characteristics for certain analytes on the perfluorinated phase, a systematic study was developed in this column to understand the chromatographic behaviour of these compounds at three pH (2.5, 4.6 and 7.0) and seven different organic proportions from 20 to 80% acetonitrile. The predominant electrostatic interactions observed on the perfluorinated phase could explain its special behaviour and the high retention at higher percentages of organic solvent, especially for amine compounds, which makes this column very advantageous in working with LC/MS. Different applications with volatile buffers, such as TFA at pH 2.5 and ammonium acetate at pH 4.6 and 7.0, were also considered. Some results have been related to parameters frequently employed for column description.

Keywords: Acetaminophen; Phenylephrine; Phenylpropanolamine; Chlorpheniramine maleate; 4-Aminophenol; 4-Chloracetanilide; 4-Nitrophenol; Stationary phases

1. Introduction

In recent years, significant improvements have been made in the quality of bonded silica particles used in HPLC. An increased understanding of the chemical nature of the silica surface and the manufacture of purer silicas have led to a tightening of physical specifications. Additional improvements have also been made in bonding and column packing techniques. Consequently, lately there has been a dramatic increase in the number of improved reversed phase columns available to the chromatographer, including different functionalities. Recently, a work by Euerby and Petersson [1] has characterised 135 commercially available reversed-phase columns in terms of their surface coverage, hydrophobic selectivity, shape selectivity, hydrogen-bonding capacity and ion-exchange capacity at pH 2.7 and 7.6.

These research efforts within the synthesis and characterisation of novel LC stationary phases with enhanced selectivity are providing new tools to solve classical problems in pharmaceutical analysis. One of these problems is the analysis of pharmaceutical formulations against the com-

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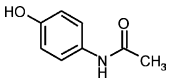
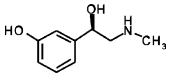
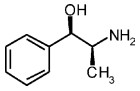
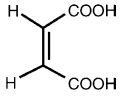
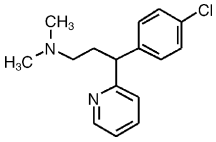
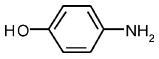
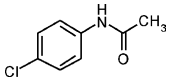
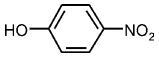
Table 1
Molecular and structural forms, molecular weights, pK_a and log P of the assayed compounds

Compound	Molecular form	MW	Estructural form	pK_a	Reference	ACD pK_a	ACD log P
Actives							
Acetaminophen	$C_8H_9NO_2$	151.17		9.5	[7]	15.32 ± 0.70 (NH), 9.86 ± 0.13 (OH), 1.72 ± 0.50 (NH_2^+)	0.339
Phenylephrine	$C_9H_{13}NO_2$	167.21		8.9 and 10.1	[8]	14.32 ± 0.20 (OH), 9.76 ± 0.10 (OH phenol), 9.22 ± 0.20 (NH_2^+)	−0.03
Phenylpropanolamine	$C_9H_{13}NO$	151.21		9.44	[9,10]	12.07 ± 0.45 (OH), 8.47 ± 0.10 ($NH3^+$)	0.808
Maleate	$C_4H_4O_4$	116.07		1.93 and 6.58	[11]	4.79 ± 0.10, 3.15 ± 0.10	−0.008
Chlorpheniramine	$C_{16}H_{19}ClN_2$	274.8		9.1	[9,10]	9.33 ± 0.28 (NH^+), 3.77 ± 0.19 (NH^+ pyridine)	3.389
Impurities							
4-Aminophenol	C_6H_7NO	109.13		10.30 and 5.29	[12]	10.17 ± 0.13 (OH), 5.28 ± 0.10 (NH_3^+)	−0.287
4-Chloracetanilide	C_8H_8ClNO	169.61				14.25 ± 0.70 (NH), -0.11 ± 0.50 (NH_2^+)	2.05
4-Nitrophenol	$C_6H_5NO_3$	139.11		7.15	[13]	7.23 ± 0.13 (OH)	1.57

mon cold. They use to contain acetaminophen, phenylephrine hydrochloride or phenylpropanolamine hydrochloride, chlorpheniramine maleate, 4-aminophenol, 4-chloracetanilide and 4-nitrophenol (the last three commercially available impurities of acetaminophen) plus different excipients. In our previous works, we developed and validated different HPLC and LC/MS methods for the analysis of these compounds in different preparations [2–6]. These compounds present an analytical problem because of diverse characteristics inherent in their formulation, such as their different chromatographic behaviour, and the important imbalance between the different actives in the dosage forms. Moreover, formulations such as sachets usually contain flavouring agents among the excipients and more recently, sucrose, a non-UV absorbing compound, has been replaced by saccharine in formulations developed for diabetic or dieting individuals. All these characteristics make this combination of compounds very useful as a test mixture to check column performance with real samples. It can also be tested how the stationary phases fit into the prevision, because the mixture contains not only acidic, neutral and basic compounds with very different polarities, but also different functional groups as can be observed in Table 1. Therefore, we have made a comparison among a group of columns containing a perfluorinated phase, a cyano phase, a polar embedded phase (amide group), a polyethyleneglycol phase and a standard C_{18} phase. Fig. 1 shows the structures of the different stationary phases. All of them come from the same manufacturer to avoid differences in the silica nature or packing procedure and with the same geometry. Some of these phases are claimed by the manufacturers to give superior peak shape, and also to offer a selectivity different to conventional phases, although little comparative data has been published for columns using the same silica [14]. Furthermore, after observing some special behaviour with interesting applications in the pentafluorophenyl phase, a systematic study was developed with this column.

2. Experimental

2.1. Chemicals

Sodium hydroxide was from Panreac (Barcelona, Spain); phosphoric acid; ammonium acetate; acetic acid and acetonitrile (HPLC) from Merck (Darmstadt, Germany); trifluoroacetic acid was from Sigma–Aldrich (Steinheim,

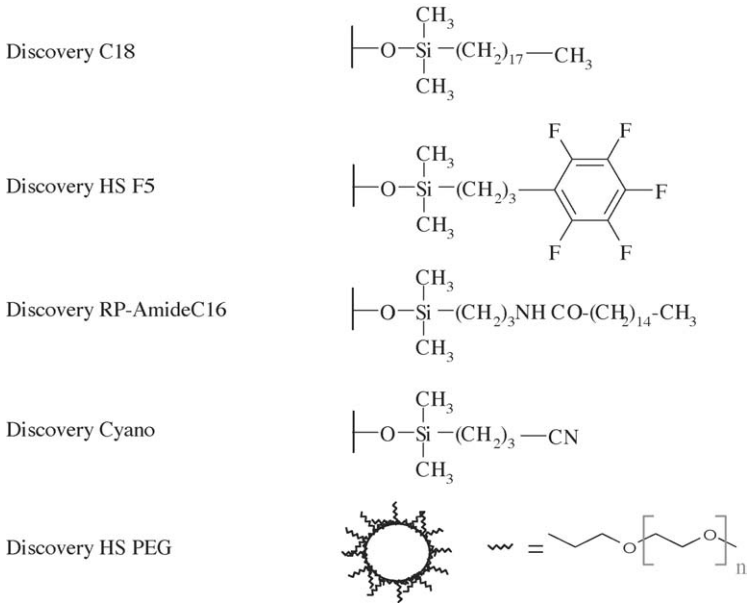


Fig. 1. Structures of the different stationary phases.

Germany); and water was purified with a Milli-Q plus system from Millipore (Bedford, MA, USA).

Standards of actives (acetaminophen; phenylephrine hydrochloride; phenylpropanolamine hydrochloride and chlorpheniramine maleate) and impurities (4-aminophenol, 4-chloracetanilide and 4-nitrophenol) were kindly provided by CINFA, S.A. (Pamplona, Spain). Individual solutions of all the compounds were prepared in a mixture of CH_3CN:H_2O 80:20 at 1 mg/ml. The molecular and structural forms, the molecular weight, pK_a and log P are included in Table 1. Predictions of pK_a and log P were calculated using Advanced Chemistry software programmes (Toronto, Canada).

2.2. HPLC analysis

The HPLC system consisted of an Agilent Technologies 1100 liquid chromatograph (Agilent Technologies, Waldbronn, Germany) with a quaternary pump, an automatic injector, a diode-array detector and a column oven. The columns

Table 2
Physical and chemical properties of the particles of the Discovery columns [15] and others characteristics of the stationary phases [1]

Discovery Phase	C18	CN	RP-Amide C16	HS F5	HS PEG
USP code	L1	L10	(Pending L57)	L43	
Bonded phase	Octadecyl-silsilane	Cyano-propil	Palmitamido-propylsilane	Pentafluoro-phenylpropyl	Polyethylene-glycol
Endcapping	Yes	Yes	Yes	Yes	No
Particle platform	Silica	Silica	Silica	Silica	Silica
Particle shape	Spherical	Spherical	Spherical	Spherical	Spherical
Particle purity	<10 ppm metals	<10 ppm metals	<10 ppm metals	<10 ppm metals	<10 ppm metals
Particle size (μm)	5	5	5	5	5
Pore size (Å)	180	180	180	120	120
Surface area (m^2/g)	200	200	200	300	300
Packing density (g/mL)	0.58	0.58	0.58	0.58	0.58
% C	12	4.5	11	12	12
Coverage (μmol/m^2)	3	3.5	2.6	4	3.8
pH Range	2–8	2–8	2–8	2–8	2–8
Temperature range (°C)	≤70	≤70	≤70	≤70	≤70
K_{PB}	3.32	0.29	1.65	1.70	0.23
α_{CH_2}	1.48	1.00	1.35	1.26	1.06
$\alpha_{T/O}$	1.51	1.00	1.81	2.55	2.57
$\alpha_{C/P}$	0.39	1.00	0.49	0.68	0.02
$\alpha_{B/P\ pH\ 7.6}$	0.28	1.60	0.44	0.85	0.07
$\alpha_{B/P\ pH\ 2.7}$	0.10	0.55	0.19	0.34	−0.04

K_{PB}, retention factor for pentylbenzene; α_{CH_2}, hydrophobicity or hydrophobic selectivity; $\alpha_{T/O}$, shape selectivity; $\alpha_{C/P}$, hydrogen bonding capacity; $\alpha_{B/P\ pH\ 7.6}$, total ion-exchange capacity; $\alpha_{B/P\ pH\ 2.7}$, acidic ion-exchange capacity.

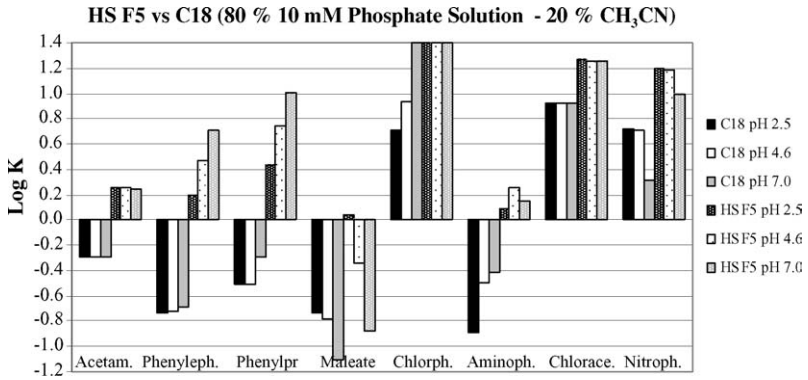


Fig. 2. Comparison of retention between the HS F5 column and the C18 column at pH 2.5, 4.6 and 7.0. Mobile phase: 80%, 10 mM phosphate solution/20% acetonitrile. Compounds: acetaminophen, phenylephrine HCl, phenylpropanolamine HCl, maleate, chlorpheniramine, 4-aminophenol, 4-chloracetanilide and 4-nitrophenol.

used were Discovery C18, Discovery Cyano, Discovery RP-Amide C16, Discovery HS F5 and Discovery HS PEG from Supelco (Tres Cantos, Madrid, Spain). Physical and chemical properties of the particles can be found in Table 2. In all cases, column dimensions were 5 μm particle and 15 cm × 0.46 cm. All of them were kept at 35 °C during the analysis. The flow rate was 1 ml/min and the injection volume was 2 μL. UV detection was performed at 215 and 254 nm. All results were the mean of duplicate injections. Solutes were injected separately at a concentration of 1 mg/ml. Column void volume was estimated with the first disturbance of the baseline on the injection of methanol.

Phosphate solutions were prepared from 10 mM phosphoric acid and made up the corresponding pH with NaOH. Solutions of ammonium acetate were prepared from the salt, making up the corresponding pH with acetic acid. The different percentages in the mobile phase were fixed with the pump.

3. Results and discussion

The five columns were assayed at three pH levels (2.5, 4.6 and 7.0) and three proportions of aqueous phase/acetonitrile (80:20, 50:50 and 20:80, v/v). The condition that provided higher selectivity was 80:20, which was employed for the comparison. Results are shown in Figs. 2–5.

In Discovery C_{18} the elution order was consistent with ordinary RP expectations based on solute hydrophobicity and it gave excellent peak shape for basic and acidic compounds. Increasing the pH did not affect neutral compounds, such as acetaminophen or chloracetanilide. However, it increased the retention of amino compounds and decreased the retention of compounds with acidic groups.

Regarding the nature of the compounds, their performance was as expected: the substitution of the hydroxyl group in acetaminophen by a chloro in chloracetanilide greatly increases the hydrophobicity (log *P*) and therefore, the retention. The same effect takes place when an amino in 4-aminophenol is changed for a nitro in 4-nitrophenol, but it depends on the pH. At pH between 5.3 and 10.2, 4-aminophenol is as a zwitterion and, therefore, its retention maximizes at or near the isoelectric point. Meanwhile, for 4-nitrophenol it increases around pH 7.2 or higher. Chlorpheniramine retention clearly increases with increasing pH, and just the opposite occurs with the ionisation degree. Chlorpheniramine has a very large retention dependence on pH in

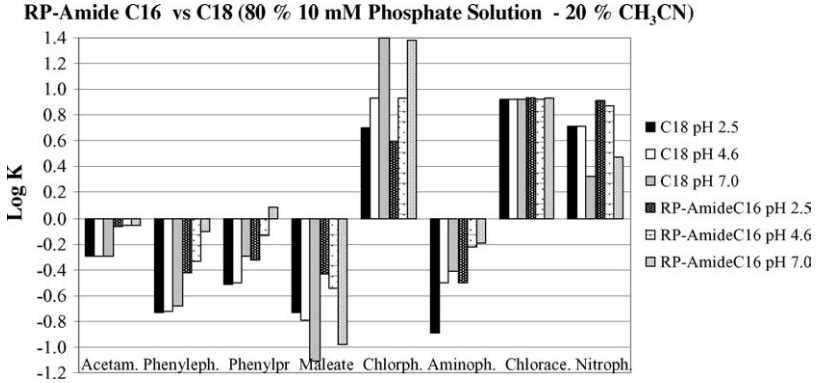


Fig. 3. Comparison of retention between the RP Amide C16 column and the C18 column at pH 2.5, 4.6 and 7.0. Mobile phase: 80% 10 mM phosphate solution/20% acetonitrile. Compounds: acetaminophen, phenylephrine HCl, phenylpropanolamine HCl, maleate, chlorpheniramine, 4-aminophenol, 4-chloracetanilide and 4-nitrophenol.

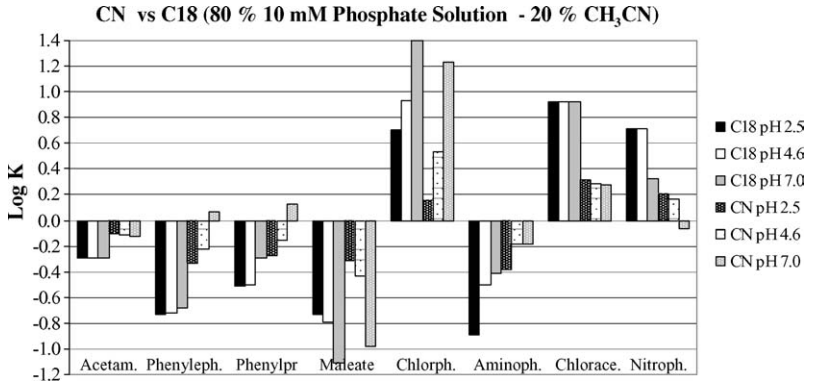


Fig. 4. Comparison of retention between the CN column and the C18 column at pH 2.5, 4.6 and 7.0. Mobile phase: 80% 10 mM phosphate solution/20% acetonitrile. Compounds: acetaminophen, phenylephrine HCl, phenylpropanolamine HCl, maleate, chlorpheniramine, 4-aminophenol, 4-chloracetanilide and 4-nitrophenol.

the range of about $2.5 < pH < 5.5$ due to changes in ionisation of the pyridine group with pH. In this case, the retention changes are not dominated by silanol effects, although an additional mechanism must be considered to explain the change in retention with amino containing compounds with pK_a higher than 9. These compounds are fully ionized at all these pH values and the retention difference with pH may be due to ion exchange on the silanols. At pH 7.0 the chlorpheniramine charge is about the same as at 4.6, the silanols are fully ionized, and any increase in retention compared to pH 4.6 is predominantly due to increased ion exchange. In 4-nitrophenol, a clear change of retention with pH occurs at pH 7.0, which is near its pK_a (7.15). Maleate retention generally increases with decreasing pH, with the corresponding ionisation decrease.

Perfluorinated stationary phases have shown novel selectivity for several compound classes and in many instances have proven useful as an alternative to traditional C8 and C18 phases [16–18]. The unusual selectivity of these fluoro columns compared to alkyl-silica columns can be rationalized by large differences in bonded-phase polarisability (as related to ligand refractive index), leading to differential dispersion interactions of solute and column [19]. When comparing the C18 and HS F5 columns at the different pHs and 80:20 aqueous phase/acetonitrile (Fig. 2), it can be observed that all the compounds, except maleate, show higher retention at every pH in the HS F5, especially amine compounds in which the difference is even higher. This permits very polar compounds eluting too close to the void volume on C_{18} to be sufficiently retained by HS F5. This effect might be related to the interaction with the activated phenyl moiety or with the amino group. As expected, the pH has no practical effect on neutral analytes (acetaminophen, 4- and chloracetanilide), while the retention of amines is proportionally increased at higher pH in the HS F5 column. A decrease in the retention of amino compounds in the F5 column from pH 4.6–2.5, even when they were already fully protonated, makes one think about a decrease in the ion exchange mechanism. 4-Nitrophenol (pK_a 7.15), which becomes negatively charged around pH 7, is less retained with this buffer. Meanwhile, 4-aminophenol shows higher retention at pH 4.6 in the F5 column, which is the nearest value to its first pK_a, and therefore, where the charge density must be lower. Moreover, silanols cannot contribute much to ion-exchange retention at pH 2.5, but it contributes greatly for this hydrophilic solute at pH 4.6. After considering the parameters for describing these columns included

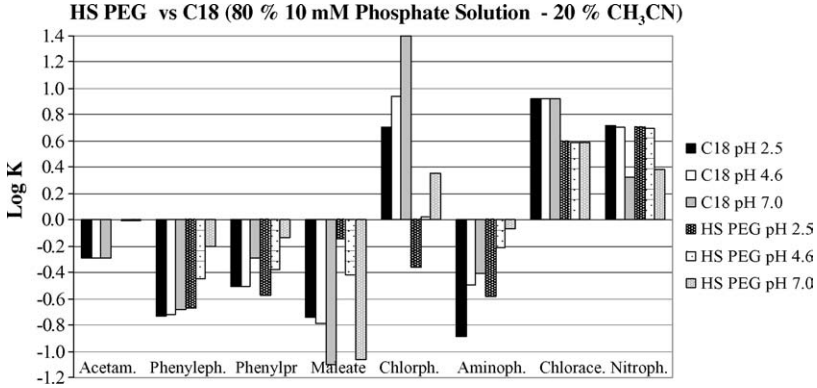


Fig. 5. Comparison of retention between the HS PEG column and the C18 column at pH 2.5, 4.6 and 7.0. Mobile phase: 80% 10 mM phosphate solution/20% Acetonitrile. Compounds: acetaminophen, phenylephrine HCl, phenylpropanolamine HCl, maleate, chlorpheniramine, 4-aminophenol, 4-chloracetanilide and 4-nitrophenol.

in Table 2, $\alpha_{T/O}$ (shape selectivity) = 2.55 and $\alpha_{B/P\ pH\ 7.6}$ (total ion-exchange capacity) = 0.85 could explain this performance.

The Discovery RP-Amide C16 presents unique retention and selectivity compared to C18 (Fig. 3). The polar-embedded groups, in particular the amide, were originally chosen for their ability to deactivate silanol interactions with basic analytes [20]. Subsequently, polar-embedded phases have found application as stable phases for highly aqueous mobile phases [21] and have exhibited novel properties for polar analytes [22]. Many of the amide and carbamate phases exhibit lower hydrophobicity and methylene selectivity [23,24] when compared with conventionally bonded C8 and C18 stationary phases. Results of Fig. 3 show that the Discovery RP-Amide C16 provides slightly higher retention for more polar compounds than C18, while clearly lower retention for less polar compounds. Due to its lower hidrophobicity, it can provide faster analysis and can help to avoid gradients in many cases. The lower K_{PB} (retention factor for pentylbenzene), which is 1.65 for RP-amide column versus 3.32 for C18, could be associated with this performance.

The cyano column (Fig. 4) shows this effect at an even higher extent, which could affect selectivity at some instances, because differences in retention of less polar compounds are very small. For less polar compounds, the CN phase presents very low retention compared with C_{18} because it is one of the less hydrophobic columns. In the case of chlorpheniramine at pH 7, the analysis time is reduced from over 40 min in C_{18} to 25 min in the CN phase. This important decrease in the retention of chlorpheniramine, 4-chloracetanilide and 4-nitrophenol in the CN column is concordant with the low values of K_{PB} (retention factor for pentylbenzene) 0.29 (3.32 in C18) and α_{CH_2} (hydrophobicity) 1.00 (1.48 in C18), in comparison with C_{18}. Following Table 2, this is the column with the highest $\alpha_{C/P}$ (hydrogen bonding capacity), $\alpha_{B/P\ pH\ 7.6}$ (total ion-exchange capacity) and $\alpha_{B/P\ pH\ 2.7}$ (acidic ion-exchange capacity) in the group.

The HS PEG (polyethylene glycol) column provides reversed-phase separation very different to the C_{18} column (Fig. 5). This phase has ether groups that can attract other polar analytes. It again shows a performance similar to the cyano column, but with higher differences among the less polar compounds, which makes it useful for the analysis of this group of compounds, as it is more rapid. For this reason, this column is a good election when trying to avoid gradients in the separation of compounds with very different polarities. Considering the stationary phase characteristics described in Table 2, this column shows the lowest value for K_{PB} and the highest for $\alpha_{T/O}$.

Chromatograms in Fig. 6 show the different separations obtained in real samples containing acetaminophen, phenylephrine hydrochloride and chlorpheniramine maleate into three stationary phases out of the group studied. The chromatograms selected corresponded to the columns that provided the shortest run times with a good resolution for the separation of the three actives.

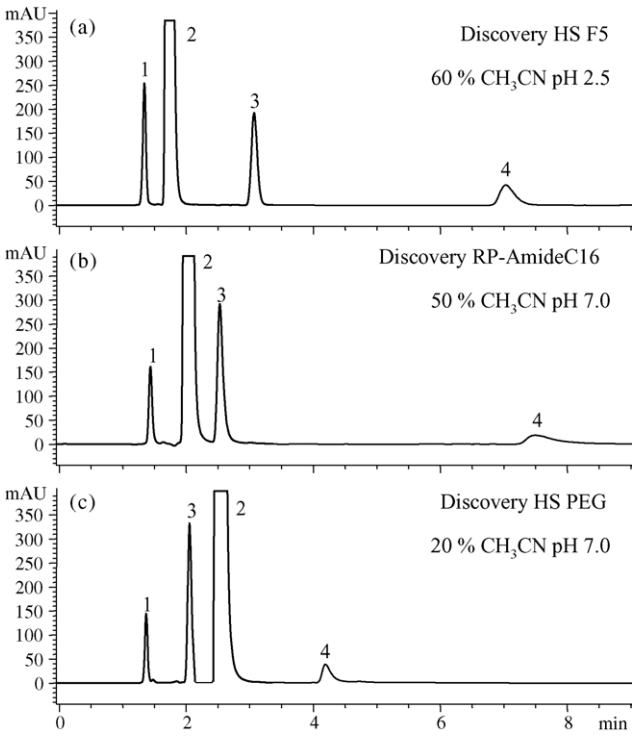


Fig. 6. Chromatograms of real samples (capsules containing acetaminophen, phenylephrine hydrochloride and chlorpheniramine maleate) under different chromatographic conditions. (a) Column Discovery HS F5 (5 μm, 4.6 mm × 150 mm), mobile phase: 40% 10 mM phosphate buffer pH 2.5–60% CH_3CN. (b) Column Discovery RP-Amide C16 (5 μm, 4.6 mm × 150 mm), mobile phase: 50% 10 mM phosphate buffer pH 7.0–50% CH_3CN. (c) Column Discovery HS PEG (polyethyleneglycol) (5 μm, 4.6 mm × 150 mm), 80% 10 mM phosphate buffer pH 7.0–20% CH_3CN. Flow rate, 1 ml/min; λ, 215 nm; temperature, 35 °C. Actives compounds: 1, maleate; 2, acetaminophen (10 mg ml^{-1}); 3, phenylephrine (0.2 mg ml^{-1}); 4, chlorpheniramine (0.08 mg ml^{-1}).

The results described here have permitted our group to develop unique applications, such as the purification and identification of a degradation compound in preparations for the relief of common cold symptoms, using an HS F5 column [4], or the development of an isocratic separation in less than 5 min for actives with very different polarities in capsules as pharmaceutical forms after their dissolution test [25], employing an HS PEG column. Using this column, it was also possible to develop an isocratic and rapid HPLC method for the simultaneous determination of these actives, including the separation of impurities and excipients in capsules as pharmaceutical formulations [5] and for sugar-free sachets of cough-cold products, using a CN column [2].

3.1. Pentafluorophenyl column

During the assays, while checking different proportions of organic solvent, a special performance of an HS F5 column, already described by Needham et al. [26–28] was observed. They found that the pentafluorophenylpropyl (PFPP) modi-

Table 3
Retention times (min) of the assayed compounds on the HS F5 column at three pH (2.5, 4.6 and 7.0) and seven different organic percentages from 20 to 80% acetonitrile

	% CH_3CN						
	20%	30%	40%	50%	60%	70%	80%
10 mM phosphate buffer (pH 2.5)							
Acetaminophen	2.800	2.222	1.963	1.816	1.721	1.683	1.680
Phenylephrine	2.573	2.440	2.503	2.682	3.028	3.668	5.276
Phenylpropanolamine	3.683	3.086	3.023	3.175	3.521	4.187	5.928
Maleate	2.084	1.688	1.486	1.369	1.302	1.291	1.286
Chlorpheniramine	30.867	12.907	8.636	7.226	6.719	6.989	8.921
4-Aminophenol	2.221	2.229	2.327	2.491	2.767	2.930	2.978
4-Chloracetanilide	19.720	8.044	4.567	3.213	2.532	2.202	2.031
4-Nitrophenol	16.809	7.671	4.534	3.195	2.486	2.135	1.933
10 mM phosphate solution (pH 4.6)							
Acetaminophen	2.789	2.221	1.973	1.817	1.730	1.680	1.679
Phenylephrine	3.987	3.843	4.020	4.378	4.905	5.871	8.501
Phenylpropanolamine	6.519	5.482	5.417	5.672	6.131	7.069	9.689
Maleate	1.455	1.277	1.194	1.144	1.117	1.106	1.093
Chlorpheniramine	>50	>50	23.924	17.933	15.180	14.484	17.116
4-Aminophenol	2.783	2.517	2.287	2.085	1.932	1.818	1.744
4-Chloracetanilide	19.233	7.916	4.516	3.177	2.525	2.184	2.019
4-Nitrophenol	16.364	7.565	4.477	3.166	2.490	2.137	1.947
10 mM phosphate buffer (pH 7.0)							
Acetaminophen	2.756	2.199	1.942	1.799	1.717	1.685	2.259
Phenylephrine	6.104	5.618	5.662	5.819	6.092	6.451	13.088
Phenylpropanolamine	11.272	8.866	8.036	7.546	7.077	6.507	12.778
Maleate	1.133	1.100	1.075	1.058	1.057	1.062	1.641
Chlorpheniramine	>80	>80	69.108	43.231	34.300	21.500	>30
4-Aminophenol	2.415	2.111	1.938	1.860	1.752	1.720	3.583
4-Chloracetanilide	18.966	7.805	4.440	3.126	2.482	2.174	2.668
4-Nitrophenol	10.906	5.289	3.365	2.563	2.199	2.056	2.634

Note: Boxes show organic percentages in which reversed-phase behaviour exists. Gray shows organic percentages in which normal-phase behaviour exists. Data in the box shows the percentage in which the behaviour of the column changes from reversed to normal phase.

fied silica columns gave good retention of several kinds of basic drugs with a mobile phase containing 90% acetonitrile, whereas, with C_{18} columns, a mobile phase containing less than 40% acetonitrile should be used to achieve good retention of basic drugs [27]. However, in our experiment, both reversed-phase and normal-phase-like characteristics for certain analytes were observed in the Discovery HS F5. Therefore, in order to understand this effect in depth, we decided to study the chromatographic behaviour of these compounds at three pH (2.5, 4.6 and 7.0) and 7 different organic proportions from 20 to 80% acetonitrile. Results are shown in Table 3. At pH 2.5, all the compounds containing an amino group are protonated and show a performance, which depends on their polarity (log *P*). The higher the polarity, the wider the normal phase-like performance interval the compound shows and that is true with positively as well as negatively charged substances. For bases, this occurs when the positive charge increases and the pH is lower and for acids or compounds with a negative charge, when this charge increases and the pH is higher. The situation is even clearer at increasing pH, because, as can be observed, at pH 4.6, 4-aminophenol is very close to its first pK_a (5.3) and the normal phase behaviour disappears. For chlorpheniramine, the range of normal phase-like behaviour decreases from pH 2.5 to 4.6, because the positive charge of the pyridine group decreases as the pH is higher than its pK_{a_1} (3.77). At pH 7.0, phenylpropanolamine normal phase-like performance range decreases because the ionisation grade of the amine group (pK_{a_1} 8.5) decreases, but curiously, all the compounds showed this effect at 80% of organic solvent at a certain extent, probably associated with a higher participation of the ionic exchange mechanism in the stationary phase. Our results suggest two different types of behaviours. On one hand, when the compound is in its neutral state or presents a small degree of ionisation, the HS F5 predominantly follows a reversed-phase behaviour at different organic percentages and the retention decreases with increasing organic percentage. On the other hand, when the compounds have either a positive or negative charge, the reversed-phase behaviour predominates at low organic percentage. Interestingly, at a higher percent of organic solvent, retention increases with increasing organic percentage, following normal-phase behaviour. This is probably due to the electrostatic interactions between the negative charge density of fluoro atoms with the positive charge of amino groups at low pH and between the positive charge density of the highly deactivated phenyl in the stationary phase with the negatively charged compounds. In many compounds these electrostatic interactions are real hydrogen-

bond between the pentafluoro-stationary phase, and acidic protons in the analytes (protonated amines, alcohols, acids). Moreover, at higher pH, the silanol negative charge can also act. Because the interaction between fluoro and amine groups is stronger, amines present the normal-phase behaviour in a broad extension from low percentages of organic mobile phase to 100%. Therefore, the parameters previously established are not sufficiently valid to characterise this types of stationary phases.

Different authors have established that stationary phases, i.e., pentafluorophenyl (PFP) bonded to silica, separate compounds based upon selective stationary phase interactions, such as steric recognition, charge transfer or π–π interactions [29]. Moreover, Yamamoto and Rokushika [30] found that silanol group interactions can dominate the reaction mechanism of many solutes, in particular those of basic compounds, and concluded that the alkyl fluorinated phase may display some shape/size selectivity. On the other hand, Sadek and Carr [29] studied the retention properties of the PFP and concluded that the PFP phase could be useful for the separation of molecules containing aromatic groups, but unfortunately, they could not rule out π–π interactions to provide any firm retention mechanism for explaining this behaviour. Sadek and Carr were cautious about proposing retention mechanisms for polar hydrogen-bond acceptor or donor solutes used in their study, because of the possibility of silanophilic interactions. Przybyciel and Santangelo [31] found that the retention behaviour of the PFP phase for separation of the nitronaphthalenes might suggest some π–π interactions as well as other mechanisms, including charge-transfer or electrostatic modes.

Nevertheless, the retention characteristics of alkyl- and aryl-fluorinated phases are complex and a clear understanding of these interactions is pending [31,32].

3.2. Applications of the pentafluorophenyl column

The higher retention of the Discovery HS F5, especially for basic compounds, is very advantageous in obtaining separations with a higher percentage of organic solvent, because it could permit an increase of the ionisation when working with LC/MS or facilitate the evaporation during the purification of compounds after fraction collection.

Once the usefulness of this column for LC/MS was established, the performance of this stationary phase with volatile solutions, such as TFA 0.05% for pH 2.5 and 10 mM ammonium acetate for pH 4.6 and 7.0, was compared with 10 mM phosphate solutions at the same pH values. The chromatographic behaviour of these compounds was compared with the volatile and non-volatile solutions at three pH (2.5, 4.6 and 7.0) and seven different organic proportions from 20 to 80% acetonitrile. In general terms, the performance of neutral compounds was very similar both in volatile and phosphate solutions (data not shown). Nevertheless, slight differences appeared with ionisable, mainly basic, compounds (Figs. 7–9). In general, at pH 2.5 (Fig. 7) basic compounds

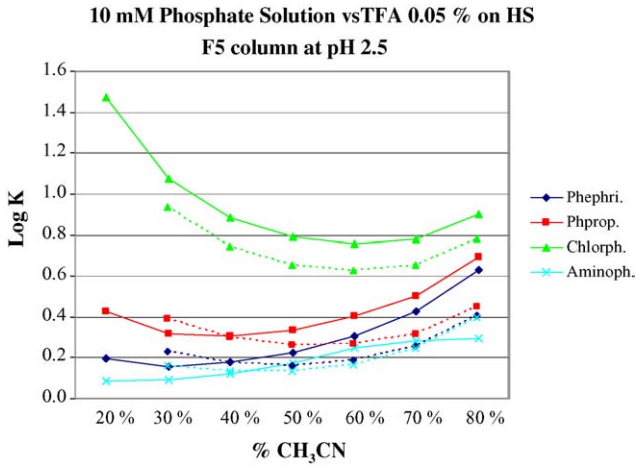


Fig. 7. Comparison of retention on the Discovery HS F5 column employing 10 mM phosphate solution (continuous line) and TFA 0.05% (discontinuous line) at pH 2.5. Compounds: phenylephrine HCl, phenylpropanolamine HCl, chlorpheniramine and 4-aminophenol.

were more retained with 10 mM phosphate buffer than with 0.05% TFA. At pH 4.6 (Fig. 8), the retention was higher with the ammonium acetate than with the phosphate solution and the differences increased with the organic percentage. At pH 7 (Fig. 9), the retention was higher with the ammonium acetate in all the range, but it tended to make equal at 80% of acetonitrile for phenylephrine and phenylpropanolamine. 4-Aminophenol presented the same retention in both media at pH 2.5, 4.6 and 7.0 from 20 to 70% of acetonitrile and only differences are found at 80% at pH 2.5, where the retention was higher with TFA 0.05% and at pH 7.0, where the retention was higher with the phosphate buffer. The differences found between both media at the different pH assayed are probably related to differences in ionic strength, to the

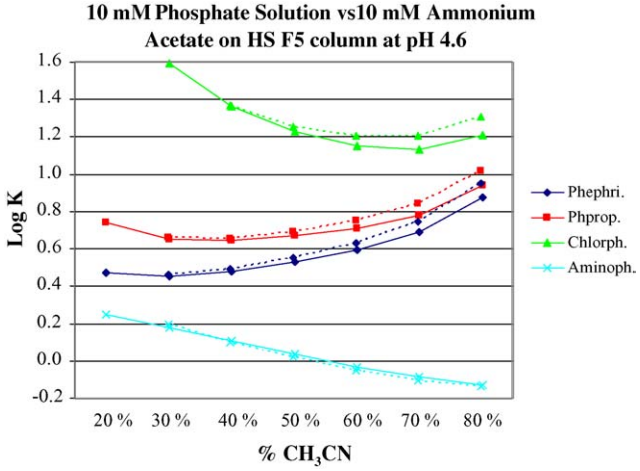


Fig. 8. Comparison of retention on the Discovery HS F5 column employing 10 mM phosphate solution (continuous line) and 10 mM ammonium acetate (discontinuous line) at pH 4.6. Compounds: phenylephrine HCl, phenylpropanolamine HCl, chlorpheniramine and 4-aminophenol.

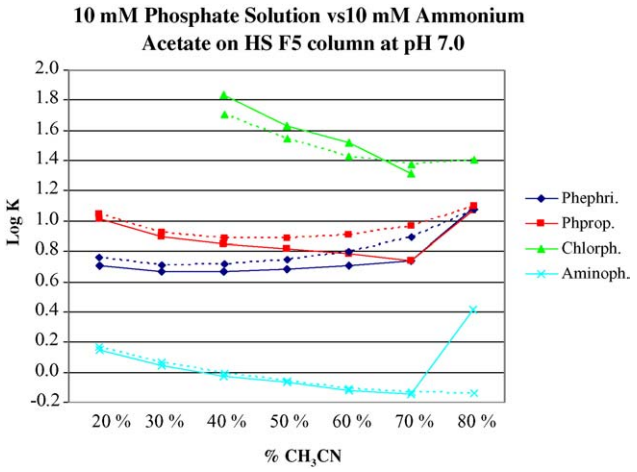


Fig. 9. Comparison of retention on the Discovery HS F5 column employing 10 mM phosphate solution (continuous line) and 10 mM ammonium acetate (discontinuous line) at pH 7.0. Compounds: phenylephrine HCl, phenylpropanolamine HCl, chlorpheniramine and 4-aminophenol.

effective pH in the mobile phases and to changes in the ionisation degree. Again the electrostatic interactions seem to play an essential role in explaining this effect, because in previous studies with the same analytes and different columns, no important changes were found in selectivity between ammonium acetate and phosphate buffer [3].

4. Concluding remarks

After a systematic study with five columns, which included a standard C18 phase, a polar embedded phase (amide group), a polyethyleneglycol phase, a cyano phase, and a perfluorinated phase from the same manufacturer with the same geometric characteristics, some interesting points have arisen, mainly, about the pentafluorophenyl phase employed in a wide range of organic solvent percentages. Results point to a higher participation of dipolar or electrostatic interactions in this stationary phase than in the other four columns. This leads to a normal phase-like performance with ionisable compounds when the amount of water present in the mobile phase is low, as it cannot compete with dipolar interactions between the analytes and the stationary phase. These data could help both to advance the understanding of the mechanisms of retention and to develop new methods with different selectivity.

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