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Study of retention and peak shape in hydrophilic interaction chromatography over a wide pH range

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ABSTRACT

Retention factor and column efficiency measurements were made for 14 test compounds comprising acids, bases and neutrals on two pairs of amide and bare silica HILIC columns, each pair obtained from a different manufacturer. The columns were tested with up to 6 different mobile phases with acetonitrile–water containing formic (FA), trifluoroacetic (TFA), heptafluorobutyric acids (HFBA) and ammonium salt buffers at $_{\mathrm{w}}^{\mathrm{w}}$pH 3, 6 and 9. Measurements of mobile phase pH in water ($_{\mathrm{w}}^{\mathrm{w}}$pH) and in the aqueous–organic mixture ($_{\mathrm{w}}^{\mathrm{s}}$pH) were performed, and calculations of ionic strength made, in order to aid interpretation of the chromatographic results. Stronger acids like TFA produced very different selectivity compared with ammonium formate buffers at similar aqueous pH. On a given column using TFA as additive, the retention of strongly acidic solutes was considerably increased relative to that of bases. Some bases even showed exclusion on both amide, and on a hybrid silica column. Conversely, in ammonium formate buffers of similar aqueous pH, bases had increased retention compared with acids, particularly on the bare silica columns. This result can be attributed to the higher pH of the salt buffers when measured in the aqueous–organic phase and interaction with negatively charged silanols. It is possible that the silica surface becomes positively charged at the low pH of TFA, leading to anion exchange properties that become competitive with the cation exchange properties normally attributed to silanol dissociation, although other explanations of these results are possible. Very marked selectivity differences were obtained by use of TFA in the mobile phase. Useful selectivity differences may also be obtained with salt buffers at different pH if the use of TFA is not desired due to its relatively unfavourable properties in mass spectrometry.

1. Introduction

Hydrophilic interaction chromatography (HILIC) has over the last 10 years become accepted as a complimentary LC separation mechanism to reversed-phase (RP), especially for the analysis of polar and ionised solutes that may be poorly retained by the latter technique. Many compounds of biomedical and clinical significance are amenable to analysis by HILIC [1–4]. The mechanism of the separation is complex, but recent studies have thrown more light on its detailed nature. Retention is thought to occur principally through partition of the solute between a water layer held on the surface of the polar stationary phase and the bulk mobile phase, which contains typically a high concentration of acetonitrile (ACN). However, ionic, adsorptive and even RP interactions can also occur, which are dependent on the nature of the solute, stationary and mobile phases [5,6]. The relative contributions of partition and adsorption to retention is likely to depend on the thickness of the water layer on the column surface, with adsorption contributing more when the water layer is limited. Using the Karl Fischer (KF) procedure, direct measurements of the water concentration inside the pores of different HILIC stationary phases have been made [7]. Similar investigations have been performed by using frontal analysis in combination with the KF method [8]. These studies have shown that water absorption is much greater on zwitterionic or amide-based phases, which can be attributed partially to the polymeric bonding of popular commercial phases of these types. In contrast, the water layer is much more limited on bare silica phases. Molecular dynamics studies have pointed to the existence of a tightly bound water layer close to the silica surface, followed by a more diffuse layer which gradually attains the composition of the bulk mobile phase with increasing distance from the surface [9]. Recent studies have confirmed that the mechanism of retention may be solute dependent. For example, the reduced b coefficient in the van Deemter equation determined by peak parking was appreciably smaller for cytosine compared with nortriptyline, the former

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solute being considerably more hydrophilic [10]. This result was caused by slower effective diffusion relative to bulk diffusion for cytosine, suggesting that it is held in the (more viscous) water layer whereas nortriptyline is held in the adjacent (less viscous) diffuse water layer containing a higher acetonitrile concentration. Broadly similar results were obtained in another study [11], suggesting nortriptyline might be retained more by a partition-like and cytosine by a more adsorption-like mechanism, at least on the bridged ethyl hybrid silica (BEH) used in that study. The complexity of the mechanism, however, is illustrated by the finding that the retention of nortriptyline at least on conventional bare silica (rather than BEH silica) is dominated by ionic interactions between the protonated base and ionised silanol groups on the stationary phase [5].

The effect of mobile phase composition in HILIC has also been investigated in more detail recently. Aqueous-acetonitrile solvents containing soluble buffer salts such as ammonium formate (AF) or ammonium acetate (AA) are recommended over solutions containing formic (FA) or acetic acids, as they produce better peak shapes, especially for ionogenic solutes [12]. Improved results may be due to the higher ionic strength of solutions containing salts compared with simple formic or acetic acid solutions and the competing effect of buffer cations with solute ions for ionised silanols on the stationary phase. Nevertheless, it was shown previously that stronger acids such as trifluoroacetic acid (TFA) could give acceptable peak shapes for acidic and basic compounds, which could be attributed to the higher ionic strength of such solutions (at least compared with FA), and/or suppression of the ionisation of silanol groups at the lower pH attained [1]. For a bare silica column, changes in selectivity were observed using TFA. With ammonium formate buffers (${}_{w}{}^{w}$pH 3.0) in acetonitrile, long retention times of bases were observed, whereas acids eluted near the void volume of the column. Conversely, in 0.1% TFA the order of elution was reversed, with strong acids having much longer retention times than the bases. These results might partially be attributed to the greatly decreased ionic interactions of protonated bases with ionised silanols at the lower pH of TFA. However, use of stronger acids like TFA has hardly been studied in HILIC, apart from some work in the analysis of peptides [13]. The previous study was confined to a single column, and no detailed rationalisation of the results was attempted. In this study, we first investigated in more detail the acidic properties of a number of potential HILIC mobile phases containing TFA, heptafluorobutyric acid (HFBA), phosphoric acid (PA) and formic acid (FA) in order to assist with interpreting the different results they produce. We then investigated retention and peak shape in a wide variety of mobile phases at different pH, using two pairs of silica and amide-bonded HILIC phases, with each pair coming from a different manufacturer. Only a few papers have discussed HILIC at high pH, when applied to the separation of peptides [14,15]. A selection of neutral, acidic and basic solutes was used as probes for the present study.

2. Experimental

Experiments with 4.6 mm ID columns were performed with a 1100 binary solvent mixing system optimised for low extra column volume, equipped with a UV detector (1 μL flow cell), and with a 1290 binary high pressure mixing instrument with photodiode array detector (0.6 μL flow cell) for 2.1 mm columns (Agilent, Waldbronn, Germany). 5 μL injections were used for the former, and 1 μL injections for the latter system. The columns used were Atlantis silica (5 μm particle size, pore Size 100 Å, surface area 360 m^2/g), 25 cm × 0.46 cm ID; XBridge BEH Amide (3.5 μm particle size, pore size 140 Å, surface area 190 m^2/g), 15 cm × 0.46 cm ID; XBridge HILIC, (3.5 μm particle size, pore size 136 Å, surface area 183 m^2/g), 15 cm × 0.46 cm ID, all from Waters (Milford, USA); Poroshell HILIC (2.7 μm particle size, pore size 120 Å, surface area 130 m^2/g) and AdvanceBio Glycan Map-an amide phase (2.7 μm particle size, pore size and surface area unavailable) both 10 cm × 0.21 cm ID (Agilent). Flow rates were 1.0 mL/min for the 0.46 cm ID and 0.25 mL/min for the 0.21 cm ID columns. A higher linear flow velocity than the geometrically scaled value was used in the narrow bore columns as the optimum flow velocity increases with decrease in particle size. Temperature was maintained at 30 °C using the Agilent column compartments. Acetonitrile (far UV grade), ammonium formate (AF), ammonium acetate (AA), trifluoroacetic (TFA) and orthophosphoric acid were obtained from Fisher (Loughborough U.K.). AF and AA buffers were prepared by adjusting 5 mM aqueous solutions of the salt to pH 3.0 and pH 6.0 with formic (FA) and acetic (AA) acids, respectively. Ammonium bicarbonate (AB) buffer was prepared by adjusting the 5 mM aqueous solution to pH 9.0 with ammonia. Note that preparation of the buffers in this way results in a different concentration of acid anion at different pH. For example, 5 mM AF ${}_{w}{}^{w}$pH 3 requires >30 mM/L of added FA to reach the desired pH, whereas 5 mM AA ${}_{w}{}^{w}$pH 6 requires <0.3 mM/L of added acetic acid. Nevertheless, according to the results (see below) the added formic acid is largely undissociated in high concentrations of ACN, and thus less likely to give major effects on the retention of ionised solutes. The overall concentration of ammonium ions potentially involved in ion exchange with dissociated silanol groups remains constant at 5 mM, and the ionic strength at least 5 mM due to the salt. This would not be the case if alternatively, the buffers had been made up using the same starting concentration of acid (rather than salt) and adjusting the pH with ammonia solution. Standards were prepared at a concentration of 20 mg/L and made up in the exact mobile phase. The pH values of the mobile phase quoted are those either in the aqueous portion of the buffer (${}_{w}{}^{w}$pH), as measured in the organic-aqueous combination with the electrode calibrated in aqueous buffers (${}_{w}{}^{s}$pH) or as the true thermodynamic pH, equivalent to that measured in the organic–aqueous solution with the electrode calibrated in organic–aqueous buffers (${}_{s}{}^{s}$pH). pH was measured using a Metrohm 827 meter equipped with Unitrode electrode. All test solutes were obtained from Sigma–Aldrich (Poole, U.K.). Log *D* and log *P* values were calculated as the average from 3 different programmes: ACD version 12.0 (ACD labs, Toronto, Canada), Marvin (ChemAxon, Budapest, Hungary) and MedChem Designer (Simulations Plus, Lancaster, California, USA). Column efficiency was measured from the first (M_1) and second (M_2) statistical moments according to the relationship:

$$N = \frac{M_1^2}{M_2} \qquad (1)$$

3. Results and discussion

3.1. Measurement of ${}_{w}{}^{s}$pH, ${}_{w}{}^{w}$pH and ionic strength of aqueous–organic mobile phases

Fig. 1(a) shows the variation in ${}_{s}{}^{s}$pH with ACN concentration for TFA, FA and HFBA at 13.1 mM concentrations, which is the molar concentration equivalent to 0.1% v/v TFA often used in LC work. Also included are measurements for 0.1% v/v formic acid (26.5 mM) and 0.1% (v/v) of concentrated (85%) phosphoric acid (14.7 mM), which are commonly used in practice. (Note that phosphoric acid is completely soluble at acetonitrile concentrations up to at least 90% v/v in water at this concentration. It was used as a chromatographic mobile phase without evidence of precipitation in a previous study [12].) The true thermodynamic ${}_{s}{}^{s}$pH can be derived from the

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