Advantages and Disadvantages of HILIC; a Brief Overview

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Hydrophilic interaction chromatography (HILIC) is fast becoming the preferred technique when encountered with polar and/or basic solutes. In comparison to reversed-phase, HILIC affords several advantages making the technique in such cases. In particular, the kinetic advantages yield much higher solute diffusivity, increased sensitivity with ESI-MS and highly symmetrical peak shapes. Heavy reliance on the aprotic solvent acetonitrile can be viewed as the major disadvantage of HILIC predominantly from a sourcing perspective. Furthermore, in light of the highly valued and well understood method development associated with reversed-phase liquid chromatography it could be perceived that HILIC is a less flexible technique. Therefore while the HILIC mode of LC is being taken up on a more widespread basis, especially in the arena of drug and biological substance analysis, it is worthwhile to consider its basis, ad as well as some advantages and disadvantages of the technique, particularly in the arena of drug and biological substance analysis are presented.

Basis of HILIC

Reversed-phase chromatography is the widely adopted retention mechanism for the majority of separations. However with respect to the analysis of small polar analytes this technique becomes difficult to apply. Martin and Synge [1] applied their normal phase technique to the separation of amino acids. In their work the silica used was saturated with water and the mobile phase was a mixture of chloroform and alcohol. This separation technique is regarded as a liquid-liquid partitioning separation mechanism due to the water bound onto the silica acting as an immobilised stationary phase. The utility of normal-phase systems in modern laboratories is seen as environmentally unfriendly and expensive, in particular disposing of potentially toxic eluents. Moreover, the use of alkanes and apolar (e.g. chloroform, ethyl acetate) solvents can result in poor analyte solubility with hydrophilic compounds.

As an alternative to using normal-phase, Alpert [2] investigated a technique he christened hydrophilic interaction chromatography. This work involved using a polar stationary phase, much like a normal phase system, except the mobile phase was an aqueous organic mixture, containing mainly higher proportions (>60%) of acetonitrile. Due to the more polar nature of the eluent, solubility issues of polar analytes associated with normal phase could be solved. It is commonly believed the HILIC retention mechanism works on the basis that water adsorbs onto the stationary phase surface and becomes immobilised such that analyte partitioning takes place between this and the bulk mobile phase. In reality the retention mechanism is more complex and many processes take place. Of course, this depends on the exact nature of the sorbent used and the analytes involved. The simplest stationary phase for HILIC is bare silica, where the underivatised silanol groups act as the functional group and are themselves both acidic and hydrophilic in nature. These groups are able to interact with, for example, basic analytes through hydrogen bonding and electrostatic interactions (ion-exchange). In Figure 1, the hypothetical retention mechanism for the basic and hydrophilic solute phenylephrine on bare silica is shown. The extent of the tightly bound aqueous layer was investigated by McCalley et al. [3] indicating that significant proportions of the pore volume are occupied by molecules of water, as inferred by the exclusion of benzene.

Kinetic advantages of HILIC

There are several advantages to using HILIC versus reversed or normal-phase methodologies. Particularly, the perceived kinetic performance gains are due to the inherently organic rich mobile phase, affording increased analyte diffusivity. This is illustrated in the following equation [4, 5]:

$$D_{m,T} = D_{m,303} \frac{\eta_{303}}{\eta_T} \frac{T}{303}$$

where η303 and ηT are the mobile phase viscosities at 2 different temperatures, Dm,303 and Dm,T are the diffusion coefficients at different temperatures (T in Kelvin). Typically, this allows for twice as fast

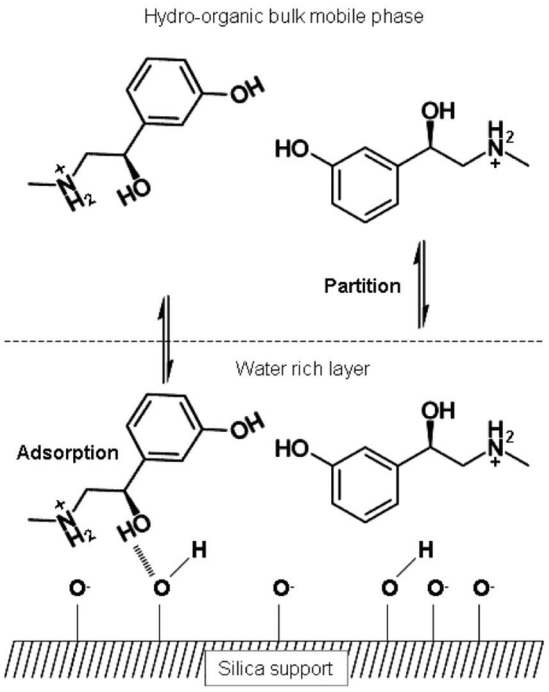

Figure 1. Hypothetical retention mechanism of phenylephrine on silica.

diffusion in comparison to reversed-phase eluents thus enhancing mass transfer. By lowering the C-term contribution to the van Deemter curve, as shown by McCalley [5], column performance at high flow is less affected than with reversed-phase eluents. This principle was applied to developing a separation of ephedrine-like substances using the HILIC approach [6]. The separation of these substances had been previously reported using a high pH reversed-phase approach [7]. This method entailed using a mostly aqueous mobile phase, hence it seemed appropriate to use elevated temperatures. Moreover, using temperatures > 60°C would not be ideal at pH 10 for extended time periods. By manipulation of temperature, the resolution of the diastereoisomers ephedrine-pseudoephedrine and cathine-phenylpropanolamine was greatly improved using bare BEH silica as the stationary phase. The reversed-phase method reported previously was then used as a means to gauge the performance of the HILIC approach. It was shown that HILIC is a highly favourable alternative to reversed-phase for the separation of these substances, both kinetically and in achieving superior peak shapes. The separation of these substances is shown in Figure 2 and their structures are shown in Figure 3. In addition, owing to the low viscosity of the HILIC mobile phase, longer columns could potentially be used and or increased flow rates accommodated for higher throughput. Interestingly, it could be envisaged that the HILIC separation be used on conventional LC equipment. However, the system volume would have to be optimised to accommodate the 2.1mm ID column used in this work.

Appelblad [8] showed that due to the effect of eluent viscosity on permeability extremely fast separations could be achieved, as well as the retention of very polar solutes using HILIC. This study further highlighted the kinetic advantages of using HILIC by means of kinetic plots, showing that much lower separation impedances are realised. Using the low viscosity mobile phases associated with HILIC the serial coupling of bare silica columns has been investigated by several workers. McCalley [9] showed that by serially coupling three 15cm long 2.7µm superficially porous (bare silica) packed columns, approximately 100,000 plates in around 15 minutes were obtained, using conventional instrumentation. Although not applied directly, this article demonstrates that powerful separations can be performed

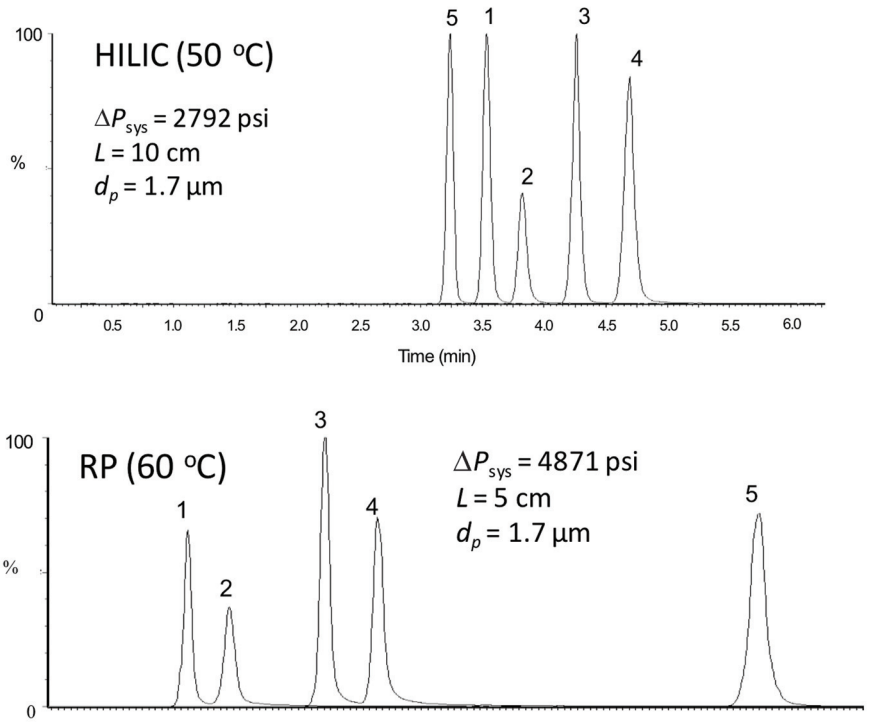

Figure 2. Separation of ephedrine substances (1) phenylpropanolamine (2) cathine (3) pseudoephedrine (4) ephedrine and (5) methylephedrine. Both columns were 2.1mm ID and operated at 500µL / min. The mobile phase for the HILIC separation was 95:5 (v/v) CH3CN:200 mM ammonium acetate pH 5 whereas the revered-phase was 90:10 (v/v) 10mM ammonium bicarbonate pH 10:CH3CN.Readers are referred to Heaton et al. [6] for further details.

using conventional instrumentation taking advantage of the low viscosity eluent. Sandra et al. [10] generated around 130,000 plates by combining six 25cm long 5µm packed (bare silica) columns using < 350 bar back pressure in around 30 minutes. This was subsequently applied to polar genotoxic impurity profiling in a pharmaceutical sample. Grumbach et al. [11] showed that 1.7µm BEH versus 3µm Atlantis silica produced more efficient separations yielding sharper peak shapes increasing sensitivity for the former. Another advantage is the comparable loading capabilities for basic solutes over reversed-phase as shown by McCalley [5]. However since the injection solvent contains mainly acetonitrile, problems with solubility can be encountered with certain analytes and compromises must be made. Veuthey et al. [12] investigated the effect of injection solvent on peak shape in HILIC. They concluded that injection solvent polarity could be attenuated by displacing the water content with alcohols where lower concentrations of acetonitrile are necessary to enable the solubility of the analyte.

Selected applications of HILIC

The main advantage of HILIC lies in the application of retaining polar solutes, particularly in the analysis of drug compounds and their metabolites as reviewed by Hseih [13]. Often drug

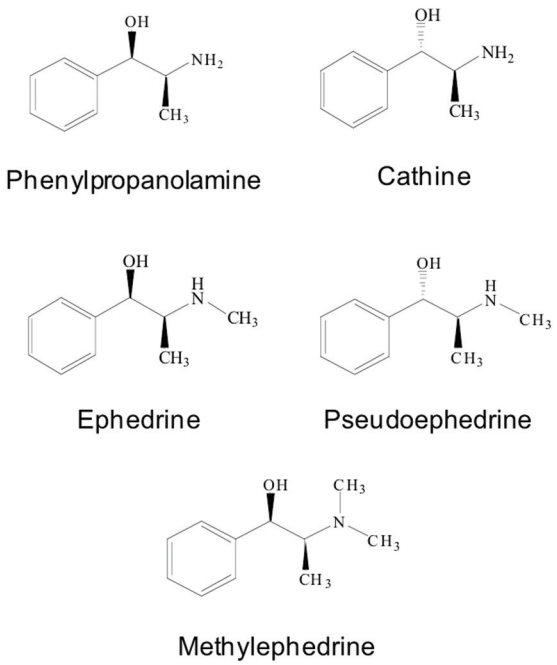

Figure 3. Structures of ephedrine substances investigated in references [6, 7].

metabolites are more hydrophilic than the parent compound, especially if conjugation to the glucuronide has taken place. Several applications have been developed using HILIC, in particular for the analysis of morphine and its major glucuronidated metabolites. Kolmonen et al. [14] developed a HILIC method for this application reporting that direct injection of the collected SPE eluent without requiring evaporation, hydrolysis or reconstitution could be performed. A reversed-phase method was published [15] for this application. However the workers had to use a neutralising solvent to prevent degradation of the glucuronide due to the high pH used during extraction and chromatographed using a phase designed to offer retention of polar solutes. Nucleosides are extremely hydrophilic as they are sugar-conjugates of nucleobases and are extremely difficult to retain and separate by typical reversed-phase methodologies. Jansen et al. [16] reviewed the analysis of these conjugates intimating that HILIC should be considered, having particular advantages over ion-pairing techniques particularly if detection by electrospray mass spectrometry is required. Johnsen et al. [17] utilised HILIC for the separation of eight deoxynucleoside triphosphates which would have proved challenging using typical reversed-phase methodology. McCalley et al. [18] showed that superior peak shapes and efficiencies could be achieved for the separation of catecholamines to those obtained using reversed-phase using a ZIC-HILIC phase. Olsen [19] showed that HILIC was highly effective for the analysis of purine, pyrimidine and low-molecular weight amide substances in a pharmaceutical setting, comparing bare silica and amino phases for this application. Lurie et al. [20] showed that HILIC was an entirely suitable technique for the analysis of seized narcotics which are both polar and basic. Using HILIC in the nano-bore scale for the separation of sympathomimetic drug substances, Fanali et al. [21] showed this approach was an effective alternative to reversed-phase.

Another advantage of the low viscosity HILIC eluent is the enhanced desolvation properties encountered with electrospray ionisation (and other aerosol based detectors – see later). This feature of HILIC was illustrated by Mitchell et al. [22] in that up to 10 times greater signal was observed in HILIC mode versus reversed-phase. This affords greater sensitivity in comparison to more aqueous rich-mobile phases, enhancing ion transfer from solution into the gas phase. This corresponds well with the use of ammonium formate/acetate based volatile buffers which are soluble in acetonitrile rich mobile phases. The use of such buffers was shown by McCalley [5] to be essential for achieving optimum peak shape, also illustrating that simply using carboxylic additives (i.e. acetic and formic acids) alone was not suitable. In contrast, it was also reported was that trifluoroacetic acid (TFA) yielded good peak shape, however this was for small sample loads on column. This was also reported by Liu et al. [23, 24] in that good peak shapes were obtained using TFA and or buffered mobiles for very polar and basic analytes.

Disadvantages of HILIC

The main disadvantage in adopting HILIC is the reliance on acetonitrile, as outlined by Sandra et al. [25] during times of shortage of this solvent. They postulated using per aqueous retention which entails using water rich mobile phases (< 5% organic) on polar stationary phases to obtain retention, the opposite of conventional HILIC operation. Instead of acetonitrile, ethanol was adopted and shown to be useful for the separation of amino acids and biogenic amines. Acetone has many similar properties to acetonitrile in terms of its relative polarity, solubility in water and aproticity making it an ideal solvent for HILIC based separations. An obvious disadvantage would be the incompatibility of UV detection systems at low wavelengths with acetone based separations. Fountain et al. [26] investigated using acetone as a direct replacement for acetonitrile in HILIC. They reported losses in signal when using acetone for certain analytes; moreover only generic electrospray voltages were applied in their study which may have not been optimal. Fritz et al. [27] compared acetone, acetonitrile and methanol for peptide analysis using reversed-phase chromatography. They observed sharper peaks when using acetone, most likely due to enhanced diffusivity as a function of reduced viscosity, shorter run times and overall superior separations with this organic modifier. There was no mention of electrospray performance or self-condensation products of acetone in this article. Keppel et al. [28] also evaluated acetone for RPLC-MS for a similar application involving peptide analysis. This time the authors discussed chemical noise and ionisation efficiency as well as column performance features of acetone, concluding in some cases this solvent to be inferior to acetonitrile. Based on the observations of previous workers, it was [29] decided to make further investigations on the use of acetone for HILIC-ESI-MS. By interrogating the source parameters which might affect the signal-to-noise, such as the capillary voltage and cone/declustering potential, it was found again that acetone is certainly problematic in electrospray positive ionisation. It appears that the formation of acetone condensation products in source contributes significantly to noise, which was much worse at elevated pH. These effects could not be negated even by extensive attenuation of the source parameters. Nevertheless, acetone should certainly be considered for HILIC hyphenated to alternative detection systems such as charged aerosol (CAD) and evaporative light scattering (ELSD) detectors. This was explored by Haddad et al. [30] for the analysis of carbohydrates by HILIC-CAD showing the favourable use of acetone as an alternative to acetonitrile.

McCalley [31] also investigated the chemical selectivity of several bonded HILIC phases. Guo et al. [32] reviewed extensively the diverse retention and selectivity possible when using HILIC. Clearly, an understanding of the physicochemical properties of a substance is essential for selecting the appropriate HILIC phase to work with. This is particularly so as a diverse range of bonded chemistries are available commercially. Such diverse phases are important for method development and afford advantage were mobile phase solvent selectivity is limited. Several commercial and novel stationary phases were compared by Linder et al. [33] concluding that different degrees of partitioning and adsorption are dependent on the ligand and analyte present. The use of sulfobetaine zwitterionic phases has gained much interest, particularly as it is charged across the entire pH range. An investigation by Greco et al. [34] aimed to address the retention mechanisms of ZIC-HILIC (SeQuant/Merck) phases. They concluded the phase behaves negatively charged and has strong retention of the water bound layer, the thickness of which is manipulated by changing acetonitrile concentration. Rodriguez-Gonzalo et al. [35] used ZIC-HILIC for the analysis of modified nucleosides and nucleobases. Amide functionalised silicas have been used in HILIC for analytes rich in hydroxyl groups. Hernandez et al. [36] showed that for oligosaccharide separations, the use of BEH-Amide at high pH was more suitable than ZIC-HILIC. The best resolution was found using the former by eliminating split peaks due to the interconversion of

anomers observed in low pH studies. Nevertheless, HILIC is not as straight forward a technique as reversed-phase. There may be a perceived reluctance to adopt such a technique even when the advantages are distinct. This is understandable as many of the well identified method development approaches of reversed-phase LC are highly valued and characterised.

Conclusions

HILIC is certainly becoming an established technique for the analysis of polar and or basic substances. In comparison to reversed-phase many advantages have been highlighted. This technique should be evaluated where appropriate as several key features allow for faster and more efficient analyses to be carried out. The low viscosity of the eluents utilised with HILIC are highly desirable especially in terms of analytical throughput. Such low viscosities are only achievable with reversed-phase systems by means of applying elevated temperatures. Furthermore, the application of elevated temperature in RP-LC is mainly restricted by the availability of thermally stable stationary phases. Of the few disadvantages, the reliance on acetonitrile only seems to be of concern during times of shortage. Solubility issues may certainly be an ongoing challenge with some analytes. However this slight inflexibility can be addressed using fine adjustment of the injection diluent and mobile phase composition.

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