Hydrophilic Interaction Liquid Chromatography-an Update.

David V. McCalley

Centre for Research in Biosciences, University of the West of England, Frenchay, Bristol BS16 1QY, UK

david.mccalley@uwe.ac.uk

Abstract.

This update on the technique of hydrophilic interaction liquid chromatography (HILIC) covers recent ideas on the mechanism of separation, and how it may be manipulated to suit the separation of particular sample types. The advantages of HILIC are discussed, but also the actual and perceived disadvantages of the technique, and how the latter can be overcome. Some new applications of HILIC in the characterisation of biopharmaceuticals, where it can be applied even to the separation of intact proteins, and to applications in metabolomics, are discussed.

1 Introduction

It is now almost 30 years since the publication of Alpert's landmark paper which named the technique of hydrophilic interaction liquid chromatography (HILIC) and discussed its mechanism and applications [1]. Alpert clearly recognised that separations were influenced by the partition of solutes between a water layer held on the surface of a polar column and the bulk mobile phase rich in an organic solvent such as acetonitrile. Additional mechanisms such as ionic retention and adsorption can be superimposed on this process. Retention increases with increasing polarity of the solute, broadly opposite to that in reversed-phase (RP). Nevertheless, there are considerable differences in the selectivity of the techniques, indicating their complimentary nature and orthogonality, an advantage also for 2D separations. Polar solutes are retained in HILIC that have little or no retention in RP, For instance, uracil can show good retention in HILIC whereas it is used as a void volume marker in RP.

There are many advantages of HILIC over RP; indeed, for some polar neutral solutes, there is hardly an alternative to HILIC in order to achieve sufficient retention for separation. However, there are real and perceived disadvantages of HILIC that can provide a barrier to more widespread uptake of the technique. The aim of this paper is to provide an update on the mechanism of separation of HILIC, to discuss the manipulation of its selectivity, its advantages and limitations (and how they may be overcome) together with some new applications of the technique.

2 Which solutes are suitable for HILIC?

Polar and ionised solutes which are hydrophilic are likely to be retained in HILIC; as a guide, the log of the solute distribution ratio between octanol and water can be considered where

Log D_{ow} = log {[neutral + ionised solute]$_{octanol}$] / [neutral + ionised solute]$_{water}$}

Log D_{ow} values can be measured experimentally, or obtained from a number of commercial simulation software packages. Hydrophobic solutes have a high positive log D value i.e. they will prefer to partition into the relatively non-polar (octanol) in a shake flask experiment (simulates the bulk mobile phase in HILIC), thus they are less retained. Conversely, very hydrophilic solutes have a large negative value (prefer to partition into water) and thus are more retained. Typically, solutes with a value of log D > ~+1 will show low retention in HILIC. However, ionic effects can contribute to retention e.g. the protonated base nortriptyline (log D ~+1) gives high retention on a silica column using aqueous acetonitrile buffered at $_{w}^{w}$ pH 3 due mainly to interaction with negatively charged silanols [2]. Fig. 1 indicates a rough correlation on three HILIC stationary phases between log D_{ow} and retention for some acidic, basic and neutral solutes. The correlation is best for TSK amide (r = 0.83), which has a thick neutral polymer layer that shields stationary phase silanol groups (negatively charged) from ionic retention of protonated bases or repulsion of charged acids. The bare silica (Atlantis) column shows no such shielding from ionic effects, resulting in a much poorer correlation (r = 0.42). Clearly, the retention of neutrals by partition should depend on the extent of the water-rich layer on the surface, while ionic retention/repulsion and adsorption effects will also depend on the particular stationary phase used. These factors contribute to the substantial selectivity obtainable by changing the stationary phase in HILIC (see below).

Whereas HILIC has classically been applied to small molecules, it has recently been applied successfully to the separation of peptides and even intact proteins (see below).

3 The mobile phase in HILIC.

3.1 Organic solvent concentration. The ratio of organic solvent to water in the mobile phase is a crucial factor in controlling overall retention in HILIC. Reducing the water (the strong solvent) concentration increases retention, which is the opposite effect to that in RP. Retention can increase exponentially at high levels of ACN (e.g 80-95%) [3]. Although other water miscible polar organic solvents have also been used (e.g methanol) , ACN is by far the most commonly employed, in the concentration range ~ 60-95 %. As the water concentration increases in the mobile phase, polar solutes increasingly partition into it, reducing retention.

3.2 Buffers and mobile phase pH.

3.2.1 Approaches to pH measurement.

Buffers are necessary to stabilise the charge both on the solute and the stationary phase. The choice of buffer is relatively restricted, due to solubility problems in high concentrations of ACN; ammonium formate, (AF, ${}_{w}^{w}$pK_a = 3.75) and ammonium acetate (AA, ${}_{w}^{w}$pK_a 4.75) are commonly used. In addition to favourable solubility, these buffer salts are volatile and thus useful for MS detection. High concentrations of ACN unfortunately influence pH measurement. Typically, pH is measured in the aqueous component of the mobile phase (${}_{w}^{w}$pH) but is more informatively measured after addition of the organic solvent, directly in the final mixture (${}_{w}^{s}$pH). This measurement (by use of published delta "correction" factors) can be related to the true thermodynamic pH of the solution (${}_{s}^{s}$pH).

$${}_{s}^{s}\text{pH} = {}_{w}^{s}\text{pH} - \delta$$

The ${}_{s}^{s}$pH ultimately determines the retention properties of the solute in the system. Correspondingly, the ionisation of buffers is also governed by their ${}_{s}^{s}$pK_a values. Unfortunately, these values are rarely available, leading to the widespread adherence to the (less correct and less informative) ${}_{w}^{w}$pH and ${}_{w}^{w}$pK_a values. This difficulty can be

considered as a disadvantage of the HILIC technique, as it complicates interpretation and prediction of retention.

3.2.2 Effect of buffer pH.

McCalley studied the use of volatile AF, AA and ammonium bicarbonate buffers at $_{w}^{w}$pH 3.0, 4.4, 6.0 and 9.0 for analysis of acidic, neutral and basic solutes on a relatively inert amide column, containing a low concentration of acidic silanols and a neutral bonded ligand (see Fig. 2). The neutral solutes thiourea (peak 3) and uracil (peak 4) showed little variation in *k* over the entire pH range. The bases procainamide and nortriptyline (peaks 5 and 6) gave increased retention as the pH was increased from 3 to 6. It is possible that increased silanol ionisation over this range produces increased ionic retention of these basic solutes. At pH 9 however, their retention drops, probably due to suppression of solute ionisation and thus diminution of ionic attraction. The strong sulfonic acids (peaks 1 and 2) showed little retention that further decreased as the pH was raised from 3 to 9, attributable to increased solute repulsion of these fully ionised acids from increasingly ionised silanols as the pH is raised. The weak acid 4-hydroxybenzoic acid (peak 7) showed increased retention as pH is raised from 3-6. The increase in pH causes increased solute hydrophilicity, reflected in a decreased log D value as the solute is increasingly ionised. It may also encourage hydrogen bonding between ionised acidic solute and neutral polar column groups. However, at pH 6 the acid is mostly ionised, and repulsion effects from increasingly ionised silanols dominate causing reduced solute retention at pH 9. The retention of many weak acids on silica-based columns can be explained by a balance of the opposing effects of increased ionisation/hydrophilicity against increased repulsion as pH is raised [4] .

Changing the mobile phase pH is a powerful means of adjusting the selectivity in a HILIC separation even when restricting the choice to volatile “mass spectrometer friendly” buffer systems.

3.2.3. Effect of buffer concentration.

An increase in volatile buffer concentration of AA or AF tends to increase the retention of neutral compounds. This result may be due to the salt increasing the volume of water in the stationary phase layer, a selective process that may depend on the nature of the salt. For basic compounds, retention decreases with increasing buffer concentration on silica based columns, due to the competitive interaction of the buffer cation with ionised stationary phase silanols. Conversely, the insulating effect of buffer ions on charged silanols results in reduced repulsion from the column and higher retention of acidic solutes. Clearly, ionic interaction effects (both attractive and repulsive) could be minimised or in some cases even eliminated by the use of high buffer concentrations, although their deliberate suppression is questionable, as they can give rise to useful selectivity effects. In addition, high buffer concentrations are undesirable for use in LC-MS, as they can suppress solute ionisation and thus reduce detection sensitivity [3] .

3.2.3. Alternative buffers to AA and AF.

Simple organic acid additives such as 0.1 % formic and acetic acid have been employed instead of salt buffers as they give reduced ion suppression effects in MS; unfortunately they can give poor peak shapes for some acidic and basic solutes. The inferior peak shapes may be due to the very low ionic strength of simple acids, especially in high concentrations of ACN, causing overloading effects even at relatively low solute concentrations [5]. These effects may be in addition to the lack of deactivating effect of the ammonium ion (present in the usual salt buffers) on ionised silanols. Stronger acids (e.g 0.1% TFA) give access to lower $_{w}^{s}$ pH values while retaining sufficient ionic strength in high

concentrations of ACN, to yield satisfactory peak shapes [6]. TFA may also improve peak shape through ion pairing effects. TFA gives unusually increased retention of strongly acidic solutes on silica based columns due partially to reduction of silanol repulsion. In addition, the column surface may become positively charged, attracting anionic solutes (see Fig. 3).

Alpert explored a much more diverse series of buffer compounds, including non-volatile salts that are not suitable for mass spectrometry [7] . In particular, he aimed to study the effects on retention of kosmotropic ions such as sulfate, which are well hydrated, and chaotropic ions such as perchlorate which are poorly hydrated. The former were found to promote partitioning of charged solutes into the immobilised aqueous layer. The effects on neutral solutes were more modest; retention times were unchanged or increased slightly with an increase in concentration of any salt. Concentrations of salt ranged from 5-120 mM at $_{w}^{w}$ pH values of both 3 and 6.

4 The stationary phase.

Stationary phases can be divided broadly into those that have an essentially neutral surface e.g. amide or diol; those which have an acidic surface e.g. poly(2-sulfoethyl) which possesses sulfonic acid groups; and basic e.g. those with alkyl amino groups. There are also other categories such as zwitterionic columns e.g. sulfobetaine which can behave in a somewhat similar way to neutral columns due to the proximity of negatively and positively charged ligands (but with some superimposed ionic retention behaviour). Bare silica columns are another distinct group that show acidic properties due to the presence of negatively charged silanol groups, particularly at higher pH. Principal components analysis [8] or correlation analysis considering retention data for different probe solutes has demonstrated that columns in these various categories show distinct retention behaviour.

The retention of neutral solutes is influenced by partitioning into the water layer on the column surface, and it is therefore hardly surprising that their retention is affected by its extent, which varies on different columns. Clearly, polymeric zwitterionic phases like ZIC HILIC and ZIC-cHILIC have an extensive water layer, which may result from their thick layers of bonded polymeric stationary phase ligands. In comparison, diol phases and silica hydride (Cogent C) have thinner water layers. Adsorption may be a more important mechanism here, especially on the latter phase. Neutral solute retention is affected by water layer thickness and can be assessed by measuring the hydrophilic selectivity of the phase α_{OH} [9]

$\alpha_{OH} = k_{uridine}/k_{deoxyuridine}$

Dexyuridine has one less –OH group than uridine, resulting in less hydrophilicity and reduced retention compared with uridine. Measurements of this parameter show a broad correlation with estimates of water layer thickness [2]. For instance using 5 mM AF pH 3.0 in 95 % ACN gave α_{OH} as 1.6, 2.3 and 3.0 for a bare silica, amide and zwitterionic phase respectively. Clearly, the zwitterionic phase should give the greatest retention of neutral solutes.

The propensity of a phase to retain cationic solutes over neutral solutes can be assessed by measuring its cationic selectivity α_{CXT}

$\alpha_{CXT} = k_{TMPAC}/k_{uridine}$

where TMPAC is trimethylphenylammonium chloride, a quaternary salt which is ionised under all conditions. Using 5 mM AF in 85 % ACN, silica, zwitterionic, diol and amide columns gave values of 7.1, 1.0, 0.6 and 0.5 respectively for this parameter, demonstrating considerable preferential retention of cationic solutes on bare silica. Similarly, the anionic selectivity of columns can be measured from:

$\alpha_{AS} = k_{BSA}/k_{uridine}$

where BSA is the strong acid benzenesulfonic acid. Using again 5 mM AF in 85 % ACN, amino and amide columns gave values of 6.6 and 0.33 respectively, demonstrating the considerably increased selectivity for acids of the amino phase [4].

Changing the stationary phase is probably the most effective way to change the selectivity of the separation [2] as shown in Fig. 4 for neutral uridine, the acid 4 – hydroxybenzoic acid and the base nortriptyline [10]. Both bare silicas (Cortecs and BEH) show preferential retention of nortriptyline, due to attractive interactions with negatively charged silanols. The low pore occupancy of water in silica columns (7-9% in 89 % ACN, [11]) and their poor hydrophilic selectivity explains their low retention of uridine. Bonded phases have fewer silanols, which may in addition be partially shielded, resulting in low retention of nortriptyline on the zwitterionic and amide phases. Reduced silanol interactions and good hydrophilic selectivity on these phases may also explain their improved retention of neutral and acidic probes. A “toolbox” of phases of different selectivity might first contain the neutral and quasi-neutral amide and zwitterionic columns, which are good “general purpose” phases. A bare silica column or phase containing bonded acidic groups (e.g. sulfonic acid) should be included to give good retention/separation of bases and an amino column could be used for the separation of acids. The substantial variation in selectivity between different columns in HILIC is an advantage over RP methods, where interchange of stationary phases has a relatively smaller effect on selectivity.

5 Advantages of HILIC.

Besides its high retention of polar and ionised compounds, its complimentary nature to RP and the beneficial changes in selectivity of different stationary phases, HILIC demonstrates a number of other advantages over RP.

5.1 Low viscosity of the mobile phase.

Many of the advantages of HILIC stem from its use of high concentrations of ACN (typically 60-95 %) in the mobile phase, giving low viscosity, allowing use of long columns, or fast flow with conventional columns. A long 45x 0.46 cm column packed with 2.7 μm shell particles operated at a flow of 2.0 mls/min. generated over 100,000 theoretical plates for 4 basic drugs in a reasonable analysis time (<15.0) minutes [12]. For solutes amenable to either HILIC or RP (such as moderately hydrophobic bases like nortriptyline), the organic rich mobile phase used in the former technique provides increased solute diffusion and thus flatter van Deemter curves in the C term region (mass transfer). However, if the increase in solute diffusion is factored out using reduced plots, RP shows a slight advantage in the C term region at fast flow rates. This means that somewhat improved performance at high flow rates is obtained for hydrophobic solutes using RP than hydrophilic solutes using HILIC [13]. However, HILIC is clearly favoured in many cases by the possibility of use of longer columns (at normal or reduced flow) to generate high efficiency.

5.2 Peak shape of ionogenic compounds.

Peak shapes of basic compounds can be surprisingly good in HILIC. The basic drugs diphenhydramine, procainamide and nortriptyline gave excellent peak shapes using a bare silica column (Atlantis) with a simple AF buffer pH 3 in ACN. This column has a high cationic selectivity; in RP chromatography, strong ionic interactions can be associated with poor peak shape, which is not always true in HILIC. Good peak shapes may be associated with higher sample capacity in HILIC, with column efficiency being maintained at much higher solute mass than in RP [14] .

5.3 Compatibility with ESI mass spectrometry and other evaporative detectors.

The low viscosity, high volatility and low surface tension of high concentrations of ACN are conducive to higher sensitivity in ESI-MS [15].

Fig. 5 compares the relative signal/noise ratio for 6 basic and 4 acidic solutes by flow injection analysis-ESI MS using a modern triple quadrupole MS (Agilent 6460) and multiple reaction monitoring (MRM) conditions that were optimised for each solute. On average, a sensitivity increase of ~3 times for the HILIC conditions (90 % ACN : 10 % 5 mM AF pH 3.0) compared with RP conditions (10 % ACN : 90 % 5 mM AF pH 3.0) was obtained. Use of FIA avoids introduction of the samples into a chromatographic surface which potentially might confound the results with adsorption effects. Gradient elution of mixtures of solutes using an appropriate column can also be used to assess difference in detection sensitivity. While possible column adsorption effects may indeed confound the results, as well as possible elution in different mobile phase composition (dependent on the column) this method is less laborious than single injections required for FIA. It also arguably presents a more realistic simulation of normal experimental conditions. The average gain in sensitivity was reported as 7-10 times [16], but improved designs of modern interface showed more modest gains [17] . Similar beneficial increases in sensitivity can be obtained with other mobile phase evaporation detectors such as the charged aerosol detector [18] .

6 Disadvantages of HILIC and how they may be overcome.

6.1 Sample injection.

Using injection solvents of higher eluotropic strength than the mobile phase (i.e. increased water concentration) gives increasing deterioration in peak shape [19]. This effect can be problematic if the sample is not soluble in high concentrations of ACN (i.e. in appropriately “weak” mobile phases). The effect can be moderated by injecting small volumes. Alternatively, for small MW compounds, isopropyl alcohol (IPA) or a mixture of 50:50 ACN:IPA has been recommended. For drug discovery applications, dimethylsulfoxide in at

least 80 % ACN can be employed, whereas for peptide analysis, pure ethanol or IPA is possible [20] .

Use of the mobile phase or a weaker solvent as an injection solvent to avoid peak distortion is not normally possible with biopharmaceuticals due to limited solubility in high concentrations of ACN and possible protein denaturation/precipitation. Thus, in one study, an aqueous sample was injected followed by a fast gradient ramp incorporating a high % of ACN at the beginning of the method, in addition to a small injection volume [21], which produced good results.

6.2 Long equilibration times.

Full isocratic equilibrium of the column (where retention times stabilised to 99-101 % of the value at “infinite” equilibration time) with buffered ACN mobile phase, can require more than 20 min. (> 40 column volumes) when purging the 10x 0.21 cm columns at 0.5 mL/min [10]. In isocratic analysis, full equilibration is necessary, as the selectivity of the separation can change with equilibration time. Full equilibration was found to depend on the nature of the HILIC stationary phase, the purging flow rate and the original or “storage” solvent. These long equilibration times are not however a barrier to the use of gradient elution in HILIC. In gradient elution, a repeatable partial equilibrium was demonstrated [10] in an equilibration time of as little as 5 min., implying that HILIC can be reliably used under these conditions. Selectivity changes can occur in the separation dependent on the particular equilibration time between gradient runs. Therefore, this parameter must be kept constant in a series of analyses; however, this does not appear to be a problem when using modern HPLC instruments.

6.3 Retention time instability and drift.

HILIC has sometimes been found to suffer from irreproducible or drifting retention times. Undoubtedly, this problem is often associated with insufficient equilibration of the column (see above) especially in isocratic applications. However, one study found retention time irreproducibility was associated with storage of the (organic rich) mobile phase while connected to the instrument, rather than in tightly sealed bottles, which alternatively gave excellent day to day repeatability of retention [22].

Extra care may be necessary in mobile phase preparation. The commonly used buffer salts AF and AA are hygroscopic, and should be stored in a dessicator at room temperature in order to improve reproducibility of buffer preparation. For isocratic analysis using high concentrations of ACN (low concentrations of water) some consideration should be given to preparing the mobile phase by premixing aqueous and organic liquids by weight, taking into consideration the density of the liquids. Errors might otherwise result if metering relatively small volumes of aqueous phase using the HPLC instrument.

In gradient analysis, especially with high pressure mixing systems that use a separate pump for each solvent flow, the use of one channel delivering small volumes (difficult to achieve reproducibly and accurately) throughout the gradient run should be avoided. This is especially true when the total flow is relatively low as is necessary with a small diameter column, and when a relatively shallow gradient is employed, as is often the case with HILIC. Thus a gradient from 90 to 80 % ACN is best not devised with Bottle A containing 90% ACN and Bottle B 0% ACN at a total flow of 0.4 mLs/min. This would result in pump B delivering only ~0.045 mLs/min. even at its maximum flow at the end of the gradient.

7 Some new applications of HILIC.

7.1 Analysis of glycans-“bottom up” methods.

HILIC has established itself as an essential technique for monitoring glycans in monoclonal antibody (mAb) drugs designed to target specific antigens. mAbs have MW ~150,000 of which about 5 % by weight can be glycans. Much analytical work needs to be performed in the characterisation of mAbs or their biosimilars that have similar efficacy and safety to the original drug, or biobetters, which are improved products. Many of the original biopharmaceuticals are coming off patent, giving scope for the development of these substitute drugs. Glycosylation is one of the important causes of microheterogeneity caused by post-translational modification (PTM) that can occur e.g. during production and storage; thus its characterisation is of major importance to enable potential differences in the products to be assessed.

About 10 sugars are commonly found as constituents of glycans, which may be attached to the F_c fragment of the mAb. A glycan is a mono-, poly- or oligosaccharide, but typically contains ~ 10 monosaccharides. N-glycans can be cleaved from the mAb by use of an enzyme such as PNG ase-F. The resulting free N-glycans can be analysed in their native state or reacted e.g. with 2-amino benzamide (2-AB) or procainamide to give sensitive fluorescent derivatives. However, 2-AB derivatives are difficult to detect by ESI-MS due to poor ionisation efficiency. An alternative derivative after PNGase-F deglycosylation, which apparently gives both good fluorescent and ESI-MS sensitivity (Waters RapiGest SF reagent) has recently been proposed [23]. In addition to releasing N-glycans from IGg F_c domains, the proposed approach also produced complete release of F_{ab} domain N-glycans. Gradient elution analysis on a wide pore (300 Å) amide column was used for their analysis. Apparently, complete release of N-glycans from F_{ab} domains was obtained using this procedure with HILIC-MS, showing a peak of mass 148.4 kDa before deglycosylation and 145.3 kDa after, implying the loss of two N-Glycans.

7.2 Characterisation of intact/large fragment of protein biopharmaceuticals and mAbs.

Guillarme and co-workers [24] employed a wide pore sub 2μm amide HILIC column successfully to characterise intact and digested (25-100 kDa fragment) protein biopharmaceuticals using gradients of 65-80 % ACN and 0.1 % TFA. The 300 Å pore size packing allowed the accommodation of large biomolecules and fragments without resulting in restricted diffusion. The separations were reported to be highly orthogonal to RPLC, while the kinetic performance remained comparable. The authors stressed as advantages of HILIC i) the compatibility with MS, ii) a reduced requirement of high temperatures that are necessary in RPLC to limit undesirably strong adsorption and iii) the possibility of coupling columns in series to gain extra resolving power. Applications were shown to the analysis of 6 different insulins (one of the oldest biopharmaceuticals, RMM~6000); RPLC and HILIC were shown to be complimentary e.g. with better separation of insulin and insulin glulisine by HILIC, but superior separation of insulin and insulin lispro by RP. High efficiencies were obtained in both (isocratic) analyses. The authors compared characterisation of trastuzumab by HILIC, RP and ion exchange, even gaining some results with intact proteins, although reduction of disulphide bonds prior to chromatography, or partial digestion of the mAb, typically yields better results in terms of both chromatographic and mass spectrometry characterisation. Trastuzumab (Herceptin) is a mAb widely used for treatment of some types of breast cancer. Whether these large molecules are denatured during separation (which should still allow for their analytical, if not their preparative separation, retaining their biological activity) remains to be confirmed.

HILIC-MS was used [21] to compare originator and biosimilar therapeutic mAbs at the intact and the so called “middle-up” level of analysis, again using a wide-pore 300 Å amide column. While “bottom up” analysis (where the sample is processed to give the simplest building blocks e.g. liberated peptides or glycopeptides) is most often used due to facilitation of the subsequent analytical procedures, it loses structural information on these complex molecules. In the HILIC analysis, 0.1 % TFA again was used as mobile phase

additive, even though it can cause some ion suppression in MS detection. It was preferred due to its solubilising effect on proteins and its low pH suppression of silanol ionisation. The intact mAb was digested, then reduced to give Fd', light chain (LC) and Fc/2 subunits (2 of each) of about 25 kDa, containing attached intact glycans, and subjected to RP and HILIC analysis. The two techniques were complimentary as shown in Fig. 6 showing middle-up separations of Trastuzumab (Herceptin) and its biosimilar Trastuzumab B. RP gave a separation of the three main fragments, but offered information about the glycosylation pattern only after examination of the MS data. In contrast, HILIC-MS on the same sample allowed for a direct and immediate comparison of the glycosylation profiles. Furthermore, Guillarme [25] demonstrated the use of HILIC coupled with MS to characterise the antibody-drug conjugate (ADC) Brentuximab vedotin, used in the treatment of Hodgkin's Lymphoma. ADCs enable the delivery of cytotoxic drugs (present in this case with an average drug to antibody ratio of 4) to therapeutic targets with an antibody directed mechanism. As with mAbs, these materials need to be characterised due to structural complexity and heterogeneity. A middle-up approach with fragment (~25 kDa) analysis using a 300 Å amide column and gradient 85-73 % ACN with 0.08 % TFA and 0.02 % formic acid was used. It was found that HILIC analysis offered a completely complimentary and orthogonal set of information to RP; elution order was essentially the opposite to that observed in RP. Only one HILIC-MS run was necessary to obtain highly important information on structural microheterogeneity.

7.3 HILIC in metabolomics

The human metabolome consists of small molecules, generally accepted as having a MW <1500 Da, that is dictated by the genes of the individual but also the individual's environment-thus it consists of a mixture of endogenous and exogenous compounds. Whereas nuclear magnetic resonance spectroscopy provides standard analytical

methodology which is common to most samples and does not require analytical development, it suffers from spectral overlap and low sensitivity. Thus, mass spectrometry, which is considerably more sensitive, coupled with separation techniques such as GC and LC provide valuable alternatives to NMR. LC clearly has broader application possibilities than GC, in that it is amenable also to non-volatile, highly polar and thermally labile sample without derivatisation. The complimentary use of HILIC and RP coupled with MS expands the number of detected analytes and provides considerably more comprehensive analyte coverage [26] . Important classes of compound that have been analysed by HILIC include phospholipids, which are the main constituents of biological membranes. They are important signalling molecules and potential biomarkers for ovarian cancer, diabetes mellitus and other conditions. They consist of a polar phosphate head group, two fatty acid chains and a glycerol group. HILIC eluents are more compatible with ESI-MS than normal phase chromatography eluents, traditionally used for these solutes. Typical mobile phases are acetonitrile containing AA or AF buffers, in conjunction with bare silica or diol columns. Organic acids, sugars, amino acids, nucleosides and nucleotides, which can be biomarkers of inherited metabolic disease, are other examples of metabolites amenable to HILIC. A comparative study of the analysis of 764 metabolites using either HILIC or RP showed that HILIC methods markedly improved the coverage of polar metabolite groups sucha as phosphates or carbohydrates and therefore represented a worthy alternative to RP separations [27]. Zwitterionic sorbents such as those containing sulfobetaine groups had a particular broad application range. Additionally, selectivity was highly diverse among the HILIC methods investigated (which used different stationary phases). For example, an amide column gave good retention of nucleosides, whereas a phophorylcholine sorbent was most appropriate for the separation of carbohydrates.

A further advantage of HILIC over RP in metabolic studies appears to be that glycerophospholipids, generally observed in cell and plasma samples tend to appear in

narrow retention time ranges instead of covering major parts of the retention window as often found for RP separations [28]. These compounds give extensive ion suppression in ESI-MS. For analysis of a broad range of metabolites, the risk of suppression increases with increased spreading of the retention window of the interferents. In the same work, considerable differences in selectivity for metabolites and matrix interferents were shown between bare silica, zwitterionic and amide HILIC stationary phases. Thus a particular HILIC column may be the optimum for each individual application.

8 Conclusions.

HILIC has become an indispensable technique for the analysis of polar and ionised solutes, which are poorly retained by traditional RP methods. For samples amenable to both HILC and RP, the techniques show a complimentary nature. In fact, for such samples, the use of HILIC may be advantageous due to favourable coupling with MS and other evaporative detectors, the low viscosity of the mobile phase allowing use of long columns and good peak shapes for some basic pharmaceuticals. Its mechanism of separation however, appears complex, which can pose a barrier to the more widespread adoption of the technique. Nevertheless, a greater understanding of the effect of some simple parameters can lead to more facile method development. Problems like longer equilibration times are shown not to be a barrier to the use of gradient methods. Some new applications e.g. to the characterisation of biopharmaceuticals, and use in metabolomics, indicate good potential for the use of the technique in these areas, where it is complimentary to RP methodology.

References

[1] A.J. Alpert, J. Chromatogr., 499, 177-196 (1990).
[2] A. Kumar, J.C. Heaton, D.V. McCalley, J. Chromatogr. A, 1276, 33-46 (2013).
[3] D.V. McCalley, J. Chromatogr. A, 1523, 49-71 (2017).
[4] D.V. McCalley, J. Chromatogr. A, 1534, 64-74 (2018).
[5] J.C. Heaton, J.J. Russell, T. Underwood, R. Boughtflower, D.V. McCalley, J. Chromatogr. A, 1347, 39-48 (2014).
[6] D.V. McCalley, J. Chromatogr. A, 1411, 41-49 (2015).
[7] A.J. Alpert, J. Chromatogr. A, 1538, 45-53 (2018).
[8] N.P. Dinh, T. Jonsson, K. Irgum, J. Chromatogr. A, 1218, 5880-5891. (2011).
. Kawachi, T. Ikegami, H. Takubo, Y. Ikegami, M. Miyamoto, N. Tanaka, J. Chromatogr. A, 1218,5903-5919 (2011).
[10] D.V. McCalley, J. Chromatogr. A, 1554, 61-70 (2018).
[11] N.P. Dinh, T. Jonsson, K. Irgum, J. Chromatogr. A, 1320, 33-47 (2013).
[12] D.V. McCalley, J. Chromatogr. A, 1193, 85-91 (2008).
[13] J.C. Heaton, X. Wang, W.E. Barber, S.M. Buckenmaier, D.V. McCalley, J. Chromatogr. A, 1328, 7-15 (2014).
[14] D.V. McCalley,J. Chromatogr. A, 1171, 46-55 (2007).
[15] S.R Needham, P.R. Brown, K. Duff, D. Bell, J.Chromatogr. A 869, 159-170, (2000).
[16] A. Periat, J. Boccard, J.L. Veuthey, S. Rudaz, D. Guillarme, J. Chromatogr A, 1312, 49-57 (2013).
[17] A. Periat, I. Kohler, A. Bugey, S. Bieri, F. Versace, C. Staub, D. Guillarme, J. Chromatogr. A, 1356, 211-220 (2014).
[18] J.J. Russell, J.C. Heaton, T. Underwood, R. Boughtflower, D.V. McCalley, J. Chromatogr. A, 1405 72-84 (2015).
[19] J.C. Heaton, D.V. McCalley, J. Chromatogr. A, 1427,37-44 (2016).
[20] J. Ruta, S. Rudaz, D.V. McCalley, J.L. Veuthey, D. Guillarme, J. Chromatogr. A, 1217, 8230-8240 (2010).
[21] V. D'Atri, S. Fekete, A. Beck, M. Lauber, D. Guillarme, Anal. Chem., 89,2086-2092 (2017).
[22] N. Gray, J. Heaton, A. Musenga, D.A. Cowan, R.S. Plumb, N.W. Smith, J. Chromatogr. A, 1289, 37-46 (2013).
[23] M.A. Lauber, Y.Q. Yu, D.W. Brousmiche, Z. Hua, S.M. Koza, P. Magnelli, E. Guthrie, C.H. Taron, K.J. Fountain, Anal. Chem., 87, 5401-5409 (2015).
[24] A. Periat, S. Fekete, A. Cusumano, J.L. Veuthey, A. Beck, M. Lauber, D. Guillarme, , J. Chromatogr. A, 1448,81-92 (2016).
[25] V. D'Atri, S. Fekete, D. Stoll, M. Lauber, A. Beck, D. Guillarme, J. Chromatogr. B Analyt Technol Biomed Life Sci, 37-41 (2018).
[26] D.Q. Tang, L. Zou, X.X. Yin, C.N. Ong, Mass. Spectrom. Rev., 35 574-600 (2016).
[27] S. Wernisch, A. Pennathur, Anal. Bioanal. Chem 408 6079-6091 (2016).
[28] A. Elmsjo, J. Haglof, M.K. Engskog, I. Erngren, M. Nestor, T. Arvardsson, C. Pettersson, J. Chromatogr. A, 1568, 49-56 (2018).

Legend to Figures

Fig. 1 Plots of log k vs average log D (from 3 calculation programs) on 3 stationary phases. Mobile phase 5 mM ammonium formate pH 3.0 in 85 % ACN. Blue circles = basic solutes; black squares = acidic solutes; red triangles = neutrals. Based on [2]

Fig. 2 Chromatogram of a mixture of acidic basic and neutral test compounds on a BEH amide column using 95% ACN containing 5 mM ammonium formate buffers w/w? pH 3.0, 4.4, ammonium acetate buffer pH 6.0 and ammonium bicarbonate buffer pH 9.0. Peak identities as Fig. 3. From reference [4].

Fig. 3 Chromatograms of acidic, basic, and neutral solutes on Agilent glycan (amide shell column) using 95 % ACN containing 0.1 % TFA or 5 mM ammonium formate pH 3.0. Peak identities: 1= p-xylenesulfonic acid; 2= 2-naphthalene-2-sulfonic acid; 3= thiourea; 4 = uracil; 5 = nortriptyline; 6 = procainamide; 7 = 4-hydroxybenzoic acid; 8 = cytosine. From reference [6]

Fig. 4 Selectivity of different columns using mobile phase 5 mM ammonium formate pH 4.4 in 95% ACN. Peak identities : 1 = uridine; 2= 4-OH benzoic acid; 3 = nortriptyline. From reference [10].

Fig. 5 Signal:noise ratios using flow injection analysis coupled with optimised MRM triple quadrupole mass spectrometry for 10 solutes with a HILIC mobile phase (5 mM AF pH 3.0 in 90 % ACN) and a RP (5 mM AF pH 3.0 in 10% ACN).

Fig. 6 Middle up analysis of Trastuzumab and Trastuzumab B (biosimilar). Total ion chromatograms using i) RPLC-MS and ii) HILIC MS. From reference [21].