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Considerations on HILIC and Polar Organic Solvent Based Separations: Use of Cyclodextrin and Macrocyclic Glycopeptide Stationary Phases

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Abstract

There is a natural tendency in science to prefer straightforward, logical classification systems. The use of mobile phase-stationary phase combinations that do not fit neatly into the standard “normal phase” or “reversed-phase” categories has been going on for over 50 years. The term “hydrophilic interaction chromatography” (HILIC) is sometimes being used as a general category for these “other” separations. In some cases it may be appropriate and in others, not. Indeed the mechanistic constraints used to define the method seem to be varying with time. Given the name HILIC, it is assumed that water is not only present in the mobile phase, but also plays an essential role in the retention mechanism. However, there is residual water present in all organic solvents. Regardless, the number of reported separations in this alternative mode has increased tremendously in the last two decades. This is due to the advent of new stationary phases and an emphasis on polar, biologically important molecules. We discuss the relationships between HILIC and other chromatographic modes. We then examine two classes of stationary phases that have played a major role in these separations. These particular stationary phases can be used to provide appreciable mechanistic information as well.

Keywords

Cyclodextrins; Hydrophilic interaction chromatography (HILIC); Macrocyclic Glycopeptides; Polar organic solvents

1 Introduction

Liquid chromatography (LC) separations that use polar stationary phases and nonpolar mobile phases are referred to as normal phase liquid chromatography (NPLC). Solutes have a characteristic elution order in NPLC, i.e., from less polar to more polar analytes. Reversed-phase LC (RPLC) operates with mainly aqueous based mobile phases and hydrophobic or nonpolar stationary phases, and the retention order of solutes separated by this approach is opposite to that of NPLC, i.e., from more polar analytes to less polar ones. RPLC is much more widely used today because of its broad selectivity, reproducibility, compatibility with

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biological samples, and its suitability for MS detection. As early as the 1950s, a special mode of LC was developed to assay polar analytes using polar stationary phases (often ion exchange types). In these cases, the mobile phases contained some water and a higher percentage of a water miscible organic solvent [1]. Despite the use of common reverse phase mobile phase components, this specific mode was referred to as the aqueous normal phase mode because retention increased with increasing polarity of the analytes, which was analogous to normal phase retention behavior [2]. From the 1970s to 1990, many papers appeared that separated polar analytes (e.g., carbohydrates, carboxylates, amino acids, etc) using polar stationary phases and polar mobile phases (often mixture of a polar organic solvent with some water) [3–14]. Subsequently Alpert referred to this LC separation approach as hydrophilic interaction chromatography (HILIC) to emphasize the presence of water in the mobile phase and the partition retention mechanism involved [15].

The exact retention mechanism for HILIC, however, is still open to considerable debate. The partitioning mechanism arises from the preferential adsorption of water on the polar stationary phase, which results in a relative higher water content in the stagnant liquid phase of the stationary phase support than in the mobile phase eluent [16–22]. Others reported that the separation in the HILIC mode was mainly governed by polar-polar interactions (i.e., hydrogen bonding, dipole-dipole, charge-dipole interactions) because of the strong dependence of the elution order on the number of polar functional groups presented [10, 12, 14, 23–28]. Berthod *et al.* systematically studied the retention behavior of oligosaccharides on a Cyclobond I column (β -cyclodextrin, Astec, Whippany, NJ), and found that both partitioning and hydrogen bonding were present [29]. In a more recent review [30], Hemstrom and Irgum gathered the HILIC retention data from literature and found that they fit better with Snyder-Soczewinski adsorption model [31] than the partitioning model [32].

Acetonitrile-water mixtures are the most commonly used mobile phases for HILIC separations. The water content for the optimized separation depends on the polarity of both the stationary phase and the analytes to be separated. In general, the more polar the stationary phase and the analyte, the higher water content is needed for the separation. For example, the water content needs to be doubled for a triamino stationary phase in order to achieve similar retention and separation of saccharides as that on a monoamino stationary phase [20]. Also, on an aminoalkyl-bonded silica phase, the optimized mobile phase for the separation of mono-, di-, and oligo-saccharides contained 15%, 20%, and 25–35% water in acetonitrile respectively [4]. In other words, water content must be low enough to achieve a separation, but be high enough for the mobile phase to effectively dissolve the analytes and elute them in a reasonable time.

In the paper where the term HILIC was coined, one of the questions addressed was “how hydrophobic a stationary phase can be and still be used in the HILIC mode if one is prepared to use levels of organic solvent approaching 100%” [15]. As mentioned previously, in addition to the hydrophobicity of the stationary phase, the polarity of the analyte can play a major role in determining the mobile phase water content for a HILIC separation. To put it in another way, elution would be practically impossible for extremely polar analytes (e.g., saccharides [29]) with many neat organic solvents because of their weak dissolving power and low elution strength for those analytes. However, for other polar analytes (e.g., native

amino acids [33], tetrahydropapaveroline hydrochloride salt [34]) which are sufficiently soluble in neat polar organic solvents (like acetonitrile, and short chain alcohols) separations were successfully carried out using only the neat polar organic solvents (the polar organic mode) [33–40]. The polar organic mode (POM) may be considered as a bridge between the HILIC and the NPLC: both NPLC and the POM use neat organic solvents; both HILIC and the POM provide better dissolving power for polar solutes and are MS compatible; the polarity of analytes that are separated increases from NPLC to the POM, and to HILIC, largely due to the solubility of solutes in the corresponding mobile phases. On the other hand, these three modes share the same retention behavior (elution from less to more polar analytes). This analogy in retention behavior allows us to speculate: would a separation analogous to HILIC occur if that nonaqueous mobile phase solvent could dissolve the extremely polar analytes? In other words, is the partitioning of water to the polar stationary phase a necessary phenomenon or a determining factor for the HILIC separation? In most of the cases, the added water is deleterious to the separation: the retention and selectivity decreases with the increasing water content in high polar organic mobile phases [5, 9, 10, 16, 24, 41–43]. In the practice of enantiomeric separations, the partitioning of achiral water on the chiral selector surface is not likely to enhance the chiral environment for discriminating solute enantiomeric pairs. Instead, the adsorbed water is more likely to shield the chiral selector from solutes. Nevertheless, water can be indispensable for the elution of very polar analytes in the so-called HILIC mode.

Despite the varied meanings and mechanisms that are included under the HILIC banner, these applications for polar analytes have increased greatly during the past several years [43–50]. In this paper, we discuss the separation of polar analytes on polar stationary phases using polar (water miscible) organic solvents both with and without small percentages of water. Specifically, the early applications of cyclodextrin based and later macrocyclic glycopeptide based stationary phases for the separation of saccharides, amino acids and peptides are reviewed.

2 Cyclodextrin Stationary Phases

Cyclodextrins are cyclic oligomers containing 6 to 12 D-(+)-glucopyranose units bonded via α -(1, 4)-linkages. They are chiral, toroidal-shaped molecules with a relatively hydrophobic cavity and hydrophilic rims lined with hydroxyl groups. The most commonly used α -, β -, and γ -cyclodextrins consist of six, seven, and eight glucopyranose units respectively. Armstrong and DeMond first anchored cyclodextrins to a silica gel support with stable chemical linkages via primary 6-hydroxyls at the narrower rim of the cyclodextrins [51]. Stable cyclodextrin bonded phases are widely used in the separation of enantiomers. There are over 300 articles published and over 1000 chiral compounds separated on cyclodextrin and derivatized cyclodextrin based stationary phases [52]. While they are more often used for enantiomeric separations, the versatility of the cyclodextrin stationary phases lies in their wide application to more routine achiral reversed-phase separation due to their hydrophobic cavity [53], as well as in the normal phase mode separation where their selectivity parallels and is often better than that of the diol or polyol columns [54].

2.1 Separation of saccharides on cyclodextrin stationary phases

So-called HILIC separations of saccharides may date back to the 1950s when Samuelson and co-workers reported the separation of saccharides using low water-high ethanol solvents on a bisulfate saturated anion-exchange resin [1]. The selectivity was postulated to arise from partitioning between mobile phase and the water enriched stagnant liquid layer in the pores of the matrix [16–18]. Such separations using ion-exchange stationary phases were carried out at elevated temperatures, and usually were time consuming [55]. High performance separation of saccharides was first carried out on amino bonded silica stationary phases, using acetonitrile-water as the mobile phase in the HILIC mode [3–6]. However, there are two major drawbacks of amino bonded phases [56–58]: (1) they complex with reducing saccharides to form Schiff bases, which negates their application for quantitative analysis; (2) these stationary phases have short lifetimes due to hydrolysis. Diol- and polyol-bonded phases were developed to replace the amino-bonded phases [7, 8]. Bonded cyclodextrin phases were thought of and used as polyol-bonded stationary phases. They were shown to be highly effective for the separation of saccharides of all types using either an acetonitrile or acetone mobile phase containing smaller amounts of water [9–11, 29, 59–61].

The separation of a variety of saccharides has been reported on native cyclodextrin phases: mono-, di-, tri-, and tetra-saccharides [10]; deoxy sugars and sugar alcohols [10]; sugar anomers [11]; cyclodextrins [9]; oligosaccharides of various linkages with degree of polymerization (DP) up to 25 [29, 59, 60]; sugar acids and sugar lactones [61]. These separations are all done in what is often referred to as the HILIC mode, with mobile phases containing low amounts of water and higher volume percentages of polar organic solvents.

2.1.1 Mobile phase selection—Aqueous acetonitrile is the most commonly used mobile phase solvent. Retention of polar saccharides increases with increasing of organic content in the mobile phase [9]. This trend is the opposite to that found for RPLC, where hydrophobic partitioning governs the retention. For an optimized separation, the water content in the mobile phase increases with the increase in the DP of the saccharides (or more exactly, the number of available polar functionalities). Approximately, 10%–15% water (note that all percentages are given as volume %) is used for the separation of mono- and disaccharides [10], and 25%–35% water is used for larger saccharides with β -cyclodextrin columns. Gradient elution from low to high water content is recommended for the separation of saccharide mixtures of widely varying molecular weights. To achieve similar retention and separation on the α -cyclodextrin stationary phase, 5% more water is needed in the mobile phase compared to that for the β -cyclodextrin phase [10]. The effectiveness of replacing acetonitrile with methanol and acetone was investigated [10]: the use of methanol resulted in decreased retention and selectivity, which occurs because methanol is a competing hydrogen bonding solvent in high concentration (compared to the hydrogen bonding solutes). Hence it preferentially interacts with the stationary phase. Acetone-water mobile phases also were able to produce excellent saccharides separations. No mobile phase additives are needed for neutral saccharides, and sodium phosphate buffer at certain pHs may be used when analytes are charged [61].

2.1.2 Retention behavior of saccharides—Host-guest complexation between analytes and the hydrophobic cavity of cyclodextrin is the most important interaction for separating large numbers of analytes [62]. In the HILIC and POM, however, the relative high concentration of organic solvents occupies the relatively hydrophobic cavity. Thus the retention and selectivity are mainly due to the polar hydroxyl groups at the rims of cyclodextrins (derivatized cyclodextrins may also offer polar interaction sites via their added functional moieties) [35]. Armstrong and coworkers proposed that the analyte may form a “lid” over the “mouth” of the cavity (Figure 1) based on different enantioselectivities observed on different size cyclodextrins when using polar organic solvents as mobile phases [35]. Polar interactions (i.e., hydrogen bonding, dipolar) are responsible for the retention and selectivity of different saccharides on cyclodextrin stationary phases as well. Roughly, the retention increases with the number of available hydroxyl groups in the saccharide: mono- < di- < tri- < tetra-saccharides [10], deoxy sugar < sugar < sugar alcohols [10], cyclodextrins < linear oligosaccharides of the same DP [29]. In addition to the total number of hydroxyl groups per molecule, the orientation and distribution of these groups, the conformation, and the solubility of the whole molecule also affects their retention. As a result, sugars having the same number of hydroxyl groups also were well separated such as: ribose, lyxose and xylose; sucrose, cellobiose, lactose, and melibiose; melezitose and raffinose [10]. The separation of these saccharides with the same number of hydroxyl groups indicates that specific polar interactions are playing a more important role in retention than hydrophilic partitioning (even though the water in the mobile phase is essential for elution). It should be noted that the separation mechanism for these solutes is not likely to be any different on diol, polyol or alkylamine types of stationary phases with similar mobile phases.

2.1.3 Oligosaccharide separations—Retention of oligosaccharides follows the same trends discussed above. Retention increases with increasing DP for homologous series of oligosaccharides [29, 59, 60]. In addition, Berthod *et al.* found good linearity (with $R > 0.992$) between the log of retention factors and the DPs of homologous series of oligosaccharides [29], and this may be used to predict the retention of oligosaccharides of known DPs. Separations of malto- and isomalto-oligosaccharides with DPs up to 25 and 15 respectively were reported [59]. In cases where baseline separation was difficult to achieve, MS detection could be used as the second dimension method to provide separation between oligosaccharides of different DPs [60]. Simms *et al.* examined the possibility of separating oligosaccharides with the same base unit and DP, but with different linkages [59]. These oligosaccharides have similar polarities, and thus would be less likely to be separated by simply hydrophilic partitioning to a small water “pool” than adsorbing directly to the stationary phase. The results showed that the higher the DP, the easier it was to separate analogous oligosaccharides of different linkages. In this study, malto- and isomaltosaccharides above DP 3 were separated using acetonitrile/water 75/25 on the β -cyclodextrin stationary phase (Figure 2). In these cases, polar interactions must be responsible for the separation of oligosaccharides of similar polarities and/or similar numbers of available hydroxyl groups. Once again, the separation mechanism of these oligosaccharides on diol, polyol or alkylamine stationary phases would be similar when using the same mobile phases.

2.1.4 Anomers—Anomers are a pair of diastereoisomers, which can sometimes interchange in solution from one form to another via the process of mutarotation. The mutarotation always results in band broadening and peak doubling, and thus complicates the chromatography and the quantitative study of saccharides. There are also cases where the separation of anomers is needed. Both coelution and separation of anomers can be manipulated on cyclodextrin stationary phases [11]. An ethyl acetate/methanol/water mobile phase produced short retention times while maintaining high resolution of saccharides, and was used for anomer analysis [11]. Suppression of anomer separations were achieved at high temperatures with medium flow rates, while separation of anomers was obtained at low temperatures and high flow rates (Figure 3) [11]. The application of the β -cyclodextrin phase to monitor the 6-deoxy-D-galactose mutarotation process was demonstrated [11].

2.1.5 Detection—The detection of carbohydrates can be achieved via low wavelength UV, refractive index (RI) detector or evaporative light scattering detectors (ELSD) [9, 10]. A LC-ESI-MS method was developed by Liu *et al.* for the separation of oligosaccharides [60]: acidic additives (e.g., formic acid or acetic acid at 0.1% v/v) were found to improve sensitivity by over 10 times. Using lower flow rates and columns of smaller internal diameters also greatly increased the detection sensitivity. Using the optimized LC-ESI-MS method, the LOD as low as 50 pg was achieved on a Cyclobond I (β -cyclodextrin) column by monitoring sodium-saccharide adducts [60].

2.1.6 Advantages of cyclodextrin stationary phases—Cyclodextrin based stationary phases produce comparable selectivity to alkylamine stationary phases and with shorter analysis times for saccharides [51, 52]. The efficiency on cyclodextrin phases is higher than those reported for many other stationary phases used to separate saccharides [29]. More importantly, cyclodextrin stationary phases do not form Schiff bases with saccharides, and are better suited for quantitative analysis. In addition, cyclodextrin stationary phases have been known for their exceptional durability and reproducibility for these separations. In one stability test, a one-year old column, after about 3000 standard injections, produced an almost identical separation with a new column [10].

2.2 Separation of polar enantiomers on cyclodextrin stationary phases

In addition to their application in carbohydrate separations, cyclodextrin CSPs are widely used for enantiomeric separations in the HILIC and POM, using low water or nonaqueous polar organic mobile phases [13, 35, 37, 63–68]. Enantiomers having polar functionalities near their stereogenic centers tend to be separated because polar interactions are greatly accentuated in these modes. In general, an analyte must have a minimum of two hydrogen bonding groups, with at least one of them near the stereogenic center. On derivatized cyclodextrin CSPs, other polar substituent groups that provide additional polar interactions can substitute for one hydrogen bonding group. Examples of polar analytes separated in the POM or HILIC on cyclodextrin and derivatized cyclodextrin phases are: nicotine and nicotine analogues [37], derivatized-amino acids [13, 63, 64], derivatized di-/tri-peptides [64–66], β -adrenergic blocking agents [35, 67], and other pharmaceutical compounds [35, 43, 68].

Binary mobile phases are often used in these separations on cyclodextrin bonded phases. A very high percentage of acetonitrile (up to 100%) is used to promote stereoselective hydrogen bonding between analytes and the stationary phases. Methanol (and sometimes water) disrupts the hydrogen bonding interactions, and is added as a stronger solvent to adjust (decrease) retention times. However, the added methanol also decreases the enantioselectivity. Approximately 0–0.1% of acetic acid (HOAc) and triethylamine (TEA) are used to adjust retention and selectivity. Basically these two additives control the ionization state of the analyte. Retention decreases with the increase of these additives. Enantioselectivity can be fine tuned by adjusting the ratio of the added HOAc and TEA. For extremely polar analytes, water is used to replace methanol to increase the analytes' solubility in the mobile phase [13]. Figure 4 shows the separation of dansyl-butyrac acid enantiomers on a β -cyclodextrin column in the "HILIC" mode. With the increase of the water content, the efficiency of the peaks improved greatly, whereas the retention and enantioselectivity decreased gradually. The baseline separation achieved at 95/5 acetonitrile/water was completely negated when the water content of the mobile phase was increased to 20%. A recent comprehensive review by Mitchell and Armstrong [52] is available for detailed optimization guidelines for cyclodextrin stationary phases in both the polar organic solvent mode and in the more traditional RPLC/NPLC modes for enantiomeric separations.

3 Macrocyclic Glycopeptide Stationary Phases

Macrocyclic glycopeptides were first introduced as chiral selectors for enantiomeric separations in 1994 [38]. Currently, the four most successful stationary phases are the Chirobiotic V, R, T and TAG (Astec, Whippany NJ), which are based on vancomycin, ristocetin A, teicoplanin and teicoplanin aglycon respectively [34, 38–40]. These glycopeptide antibiotics share some common structure features: they all have three or four fused macrocyclic rings, which form "C-shaped" aglycon "baskets"; they have various saccharide moieties attached to the aglycon; they all have numerous stereogenic centers and functionalities allowing a variety of enantioselective interactions with chiral compounds (e.g., electrostatic, hydrogen bonding, steric, dipole-dipole, π - π interaction, as well as hydrophobic interactions). These unique structure features give them broad selectivity for a wide range of compounds (anionic, neutral, and cationic) [69], and enable their application in all separation modes (NP, RP, POM and HILIC). Moreover, the macrocyclic glycopeptides family is still growing, and more successful CSPs will be added in the near future.

3.1 Separation of amino acids and peptides on macrocyclic glycopeptide phases

Teicoplanin, teicoplanin aglycon and ristocetin A are the most widely used CSPs for amino acid and peptide separations. Typically alcohol/water mobile phases were used for the separation of: enantiomers of protein amino acids [33, 39, 40, 70–73], other unusual amino acids [70, 72, 74–81], N-blocked amino acids [33, 34, 39, 72, 73, 82]; stereoisomers (both enantiomers and diastereoisomers) of di- and tri-peptides [39, 70]; and structurally related peptides (diastereomers, and peptides that differ by one or a few amino acid residues) with up to 30 amino acid units [83, 84].

3.1.1 Mobile phase selection—Hydro-organic solvents are commonly used for the separation of amino acids and peptides on macrocyclic glycopeptides phases. Alcohols usually provide better selectivity and higher resolution than acetonitrile [34, 39, 73, 81], and methanol is the most commonly used organic solvent. When using mobile phases containing high percentages of organic solvents, the retention decreases with increasing water content (see Figure 5 for example) [43, 73, 74, 78, 80]. The elution order from roughly more to less hydrophobic compounds was also observed (see Figure 6) [78]. Mobile phases with up to 50% water in methanol were used with the teicoplanin and ristocetin A CSPs [39, 43, 70]. On teicoplanin and teicoplanin aglycon phases, retention of some amino acids was observed to increase with the addition of methanol starting at as high as 80% water in the mobile phase (see Figure 7) [40, 81]. As will be discussed in the following section, macrocyclic glycopeptide phases are able to provide strong electrostatic interactions with amino acids. Even with very high water content in the mobile phase, this polar interaction still governs retention. Despite the strong electrostatic interaction, elution and enantiomeric separation of neutral amino acids (e.g., those in their zwitterions form) can be achieved with a simple methanol/water mobile phase [34, 39, 70]. However, when the **R** group of the amino acid also is ionizable, mobile phase pH usually needs to be adjusted for proper retention and elution. For example, on the Chirobiotic T CSP, the mobile phase needs to be acidified (e.g., pH 3.80) to retain anionic (aspartic and glutamic acids) and to elute the cationic amino acids (lysine, arginine and histidine) [70]. In addition, the pH and buffer of the aqueous content may also affect the selectivity, efficiency and thus the resolution of the separation. In many cases, higher pHs (e.g., 7.0) gave better selectivity and lower pHs (e.g., 4.1) gave better efficiency [34]. As a result, both pH conditions should be tested for all separations. Triethylammonium acetate (TEAA) is the most commonly used additive on macrocyclic glycopeptide stationary phases. On Chirobiotic TAG CSP, the addition of 0.1% TEAA consistently provided decreased retention, yet it increased the selectivity and resolution for cyclic β -amino acids [75]. Opposite effects were observed for the separation of β -amino acids on the Chirobiotic T CSP: selectivities and resolutions were higher when using HILIC mobile phases without TEAA [80]. Buffers showed less predictable effects in many other cases and produced mixed results [73, 75, 85]. Generally, buffers have minor effects on native amino acid separations, but are important for N-blocked amino acids [33, 72]. Neat methanol with a HOAc/TEA additive has also been widely used for enantiomeric separation of amino acids [36, 74–80]. In this case, retention is adjusted by mobile phase ionic strength (total amount of HOAc and TEA added), while resolution is optimized via the ratio of HOAc/TEA. Acetonitrile can also be added to adjust retention and resolution [34, 39].

For the separation of larger peptides, efficiency becomes an increasingly important consideration, and acetonitrile/water mobile phases are preferred due to its lower mobile phase viscosity [83]. The water content, ionic strength and the pH of the mobile phase can be optimized by following the rationale discussed above for amino acids. Reverse gradients can be used when separating mixtures of peptides with a wide range of retention times [84]. As on many other stationary phases, the retention of amino acids and peptides on Chirobiotic phase produces a “U-shape” curve when retention is plotted against the organic content of the mobile phase [83]. This should not be surprising since the Chirobiotic phases can provide multiple types of interactions with analytes. In fact, high water - low organic

solvent solutions also have been used for the separation of both enantiomers of amino acids and different peptides on Chirobiotic CSPs [39, 78, 79, 84].

3.1.2 Retention behavior of amino acids and di-/tri-peptides—The most important primary interaction that leads to enantiomeric separation of amino acids on teicoplanin phase was identified as the electrostatic interaction between the cationic site ($-\text{NH}_3^+$) on the chiral selector and the carboxylate group of the analytes. As a result, the closer the carboxylate group to the stereogenic center, the better is the enantiomeric separation: α -amino acids are better separated than β -amino acids which have a stereogenic center β to the carboxylic acid group; $\text{G-DL-A} > \text{DL-A-G}$, and $\text{G-DL-L} > \text{DL-L-G}$ [70]. In addition, it is known that teicoplanin's biological activity results from its binding to bacterial cell wall peptides terminating in a D-A-D-A sequence [40]. The consequence of this in chromatography is that the D- α -amino acids tend to bind more strongly to teicoplanin and to retain longer than corresponding L- α -amino acids. Also di-/tri-peptides with D-amino acids at the C-terminus position were retained longer than the corresponding L-terminated di-/tri-peptides [70].

The role of the pendant sugar moieties on teicoplanin has been studied by comparing the retention behavior on teicoplanin and teicoplanin aglycon CSPs [40]. The teicoplanin aglycon has had the three pendant sugar moieties removed leaving three -OH groups (two phenolic and one secondary hydroxyl), which previously were the ether linkages to the sugar moieties. The primary analyte-selector electrostatic interaction mechanisms and the elution order of amino acids enantiomers on the teicoplanin aglycon CSP are the same as those on teicoplanin, but the enantioselectivity is usually different. In most of the cases for α -amino acids, first eluting L- α -amino acids have similar retention factors on these two CSPs, while the second eluting D- α -amino acids are much more strongly retained on teicoplanin aglycon phase, which leads to better separation. Nevertheless, baseline separation for most α -amino acids was reported on both CSPs [40]. The lower enantioselectivity for α -amino acids on the Chirobiotic T column is thought to be due to steric factors associated with the sugar moieties [40, 75]. Additional enantioselective interactions between the sugar moieties and analytes may also complicate the separation results.

The elution order of β -amino acids deviates from that observed for α -amino acids and di-/tri-peptides. The elution order is less predictable, and in many cases teicoplanin gives better enantiomeric separations than the teicoplanin aglycon CSP [75, 76, 78, 80]. While the carboxylate group on β -amino acids is still important for retention, it may play a diminished role in enantioselectivity since it is not directly attached to the stereogenic center. As a result, many other comparatively weaker interactions (e.g., dipolar, hydrogen bonding and steric) become comparatively more important. The net result of this is that the separation and elution order is less predictable.

Ristocetin A showed similar characteristics to teicoplanin and teicoplanin aglycon. Electrostatic interactions are important and D-carboxy-terminal enantiomers are usually retained longer than the corresponding L- enantiomers. However, deviations of the elution order were observed in the following three cases [39]: dansyl-amino acids, dipeptides having two stereogenic centers, and for two tripeptides (DL-A-G-G, and DL-L-G-G). It was

intriguing to observe that DL-A-G-G and DL-L-G-G were both baseline separated whereas DL-A-G and DL-L-G showed no enantioselectivity at all on the Chirobiotic R column [39]. Other important interaction sites distant from the cationic site must provide enantioselectivity for these tri-peptides. In fact, ristocetin A has the most complicated structure, and the largest number of stereogenic centers of the chiral selectors in the glycopeptide family. Chirobiotic R has the broadest enantioselectivity of the Chirobiotic series of CSPs, and separations have been observed in all chromatographic modes (NR, RP, POM and HILIC) for native or derivatized amino acids [39]. More importantly, the ristocetin A CSP provides complementary separations to the teicoplanin and teicoplanin aglycon phases [39].

3.1.3 Separation of large peptides of closely related structures—Peptides from the same family can have the same chain length, and differ from one another only in the identity or chirality of an individual or a few amino acid residues in the peptide sequence. Separation of peptides from 14 families (with 5 to 36 amino acid residues) has been reported on the macrocyclic glycopeptide CSPs [83, 84]. Baseline separation was achieved for all tested peptides of the same family with 13 amino acid residues or less, such as pELYENKPRRP*IL (* stands for D-W, D-Y, Y, or F), four peptides from Neurotensin family (12 amino acid residues). However, when separating peptides families of 14 amino acid residues or more, separation of all peptides (4 or more) with a single chromatographic condition becomes more difficult. Nevertheless, baseline separation of specific two peptides or separation of peptide of interest from others was achievable in most of the cases [84].

Plots of peptide retention versus mobile phase composition (acetonitrile/water) always produced a retention minimum for the macrocyclic glycopeptide CSPs [83]. Optimized separations of peptides have been achieved in both the regular RP mode and HILIC mode with traditional gradients and/or reverse gradients respectively [84]. In the HILIC mode, six peptides from Bombesin family (14 amino acid residues) were separated on the Chirobiotic R column and showed completely opposite elution order to that found for a C18 column (Figure 8) [84]. In another example, A C18 column failed to separate three peptides from the Galanin family (30 amino acid residues), while the Chirobiotic T column baseline separated them within 15 min in the HILIC mode with a acetonitrile/water/formic acid 65/35/0.1 mobile phase [84].

The Chirobiotic T was the most successful column for the separation of closely related peptides in the HILIC mode, but Chirobiotic TAG and R may provide better selectivity in certain cases such as: Chirobiotic TAG for α -Bag cell factors and Vasopressins; Chirobiotic R for Neurotensins and Bombesins [83, 84].

3.1.4 Detection—Low wavelength (210–225 nm) UV detection can be used for the detection of amino acids and peptides without chromophores. LC-MS detection does not rely on chromophores and is most useful for the detection of amino acids and peptides. In addition, separation of amino acids and peptides on macrocyclic glycopeptide stationary phases use high or neat polar organic mobile phase with volatile additives. LC-MS is the most desirable detection method for this analyte-mobile phase combination. Moreover, MS can provide a second dimension separation and increased peak capacity for complicate

compound mixtures [71]. Different ionization methods were compared: APCI-MS gave best sensitivity for small analytes under M_r 200; ESI-MS worked best for large peptides with M_r larger than 300; and similar performance between APCI and ESI was observed for M_r 200–300 analytes [71]. The addition of mobile phase additives, ammonium trifluoroacetate and formic acid, could increase the sensitivity by an order of magnitude for amino acids [71]. Detection limits for amino acids were comparable between APCI and ESI, and the lowest reported detection limit was 250 pg [71]. ESI-MS provided at least two orders of magnitude lower detection limit for large peptides than UV 210 nm detection: a LOD of 2 ng was reported for Vasopressin peptides (9 amino acid residues) using the Chirobiotic TAG column [83].

3.2 Separation of other polar enantiomers

Compared with RPLC, the HILIC and POM are commonly used to promote electrostatic, hydrogen bonding, dipolar interactions for enantiomeric separations on macrocyclic glycopeptide phases. As on the cyclodextrin stationary phases, with high or neat polar organic solvents, many enantiomeric pairs with polar functionalities close to stereogenic center can be separated including: chiral amines [86], β -adrenergic blocking agents [34, 40, 87–90], β -lactams [91, 92], sulfur containing molecules [39, 93, 94], heterocyclic compounds (oxazolidinone, hydantoin, imides etc.) [33, 38–40, 94, 95], and other pharmaceutical compounds [85, 95–99]. In addition, macrocyclic glycopeptides differ with cyclodextrins in that cationic and anionic sites are available and able to provide electrostatic interactions on all the glycopeptide stationary phases. As a result, compounds with acidic groups attached to stereogenic centers are easily separated [34, 36, 39, 40, 85, 89], such as α -hydroxy/halogenated acids and substituted aliphatic acids.

The POM and HILIC mobile phases for macrocyclic glycopeptide CSPs are slightly modified from that for cyclodextrin CSPs. Generally, alcohols (methanol, ethanol, or isopropanol) are used as the solvent, with HOAc/TEA as additives to adjust retention and selectivity. The retention is controlled by the total amount of additives, and the selectivity is optimized via the ratio of HOAc and TEA. If not enough retention is obtained, acetonitrile is added to the alcohol solvent to increase retention. On the other hand, water is added to help dissolve and elute extremely polar analytes. A recent comprehensive review by Xiao and Armstrong [69] is also available for detailed optimization guidelines for the macrocyclic glycopeptide stationary phases in both high polar organic solvent mode and traditional RPLC/NPLC mode separations.

4 Conclusions

Most of the HILIC separations reported in the literature are essentially multimodal [29, 30, 47], and there is rarely, and probably would never be, a chromatographic system that operates simply via partition of polar solutes between the mobile phase and a stagnant water enriched stationary phase. For some separations HILIC can be considered a misnomer as water may be deleterious to a separation, but is still needed in limited amounts to solubilize analytes, additives, etc. Despite the likely pluralistic mechanisms, HILIC provides a rationale for selecting chromatographic systems, and sometimes provides enhanced polar

interactions for polar, yet not NPLC compatible, compounds such as saccharides, amino acids, peptides and nucleic acids.

Since water/acetonitrile is commonly used for both RPLC and HILIC, confusion may arise about exactly what water/acetonitrile ratio constitutes one versus the other mode. The nature of both the solutes and the stationary phases, and the properties of the organic solvents will all affect the turning point between RPLC and HILIC mode [14, 34, 37, 39, 40, 54, 75, 100]. In a few cases, 100% organic modifier may still produce what is considered to be a RPLC separation, whereas 80/20 water/acetonitrile may result in an HILIC separation. In cases where separation is the only goal and the relationship between elution order and hydrophobicity of analytes is elusive (e.g., enantiomeric separations), there is no reason to clearly identify whether the separation is in the RPLC or HILIC mode. Actually, many reported RPLC peptide separations are in the HILIC mode. In addition, many separations can be achieved on both the high and low water mobile phase regions on multimodal stationary phases. In cases where both modes can generate satisfactory separations, HILIC separation may provide extra benefits when using MS detection.

Mobile phases of neat organic solvents with or without volatile additives work best for enantiomeric separations of many polar compounds on both cyclodextrin and macrocyclic glycopeptide CSPs. Armstrong and coworkers have ascertained that the analytes must have at least two polar functional groups if they are to be enantiomerically separated with these mobile phase compositions. Clearly, polar interactions provide the primary contributions for enantioselective separations. This mobile phase is more closely akin to NPLC, but provides extra benefits such as better dissolving power of salts and polar analytes, as well as better MS compatibility. When either solubility or elution strength of neat polar organic solvents is not enough for extremely polar compounds, water is added in various amounts to the mobile phase. In these “polar-interaction-enhanced” mobile phases, separations were achieved for saccharides, amino acids, peptides and many other polar enantiomeric pairs on cyclodextrin and/or macrocyclic glycopeptide phases.

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Figure 1.

Interaction between β -cyclodextrin and propranolol in the polar organic mode. Polar organic solvents occupy the cyclodextrin cavity, while the chiral analyte interacts with the hydroxyl groups on the mouth of the cavity. Reprinted from Mitchell and Armstrong [52].

Figure 2.

Graph of retention time versus DP for two different classes of gluco-oligosaccharides [(○) malto- and (□) iso-malto-series] on a Cyclobond I column (250 x 4.6 mm) with a acetonitrile/water 75/25 mobile phase. Reprinted from Simms et al. [59].

Figure 3.

Separation of five pairs of anomers on a 250 x 4.6 mm β -cyclodextrin column. The peaks are 1, α -naphthyl- α -D-galactopyranoside; 2, phenyl- β -D-glucopyranoside; 3, α -naphthyl- β -D-galactopyranoside; 4, phenyl- α -D-glucopyranoside; 5, *p*-nitrophenyl- α -D-mannopyranoside; 6, *p*-nitrophenyl- β -D-mannopyranoside; 7, *p*-aminophenyl- β -D-glucopyranoside; 8, *p*-aminophenyl- α -D-glucopyranoside; 9, *p*-nitrophenyl- β -D-maltoside; 10, *p*-nitrophenyl- α -D-maltoside. Mobile phase, ethyl acetate/methanol/water 96/2/2; flow rate, 1.5 mL/min; detection, UV 254 nm. Reprinted from Armstrong and Jin [11].

Figure 4.

Enantiomeric separation of dansyl butyric acid as a function of the percentage of acetonitrile in the 0.5% triethylammonium acetate (pH 7.1) buffer on a β -cyclodextrin stationary phase (190 cm x 250 μ m). The α values are 0.0, 1.12, and 1.23 for mobile phase compositions of 85/15, 90/10, and 95/5 acetonitrile/buffer respectively. The dead volume is calculated to be ~43.5 min. Reprinted from Han and Armstrong [13].

Figure 5.

Evaporative light scattering chromatograms demonstrating the chiral separation of norvaline by HILIC using a Chirobiotic T packing and isocratic elution by (A) 50, (B) 60, (C) 70, and (D) 80% acetonitrile in 6.5 mM ammonium acetate, pH 5.5. Reprinted from Risley and Strege [43].

Figure 6. Retention plot of β -amino acids with different **R** groups on Chirobiotic T CSP with a methanol/water 90/10 mobile phase. $k'1$, retention of the first eluting enantiomer; $k'2$, retention of the second eluting enantiomer. Plotted from data in reference [78].

Figure 7.

Effects of methanol content in aqueous mobile phases on the retention of amino acids. (a) Phenylalanine on teicoplanin and teicoplanin aglycone stationary phases: ($\blacktriangledown$) k'_1 for teicoplanin; ($\blacksquare$) k'_2 for teicoplanin; ($\triangledown$) k'_1 for aglycone; ($\square$) k'_2 for aglycone. Reprinted from Berthod et al. [40]. (b) 1-Amino-2-hydroxycyclohexanecarboxylic acids on teicoplanin stationary phase: k'_1 for *cis*-(1R, 2S); k'_2 for *trans*-(1R, 2R); k'_3 for *cis*-(1S, 2R); k'_4 for *trans*-(1S, 2S) isomer. Adapted from Schlauch and Frahm [81].

Figure 8.

The separation on bombesin peptides on (a) Chirobiotic R (ristocetin A) and (b) C18 stationary phases. Isocratic separation conditions for (a): 80/20 ACN/Water with 0.1% ammonium trifluoroacetate in both solvents at a flow rate of 0.4 mL/min. Gradient separation conditions for (b): 75% A/25% B hold for five minutes to 50% B at 25 min at a flow rate of 0.5 mL/min where A is 0.1% ammonium acetate in water and B is ACN. Small arrows indicate beginning and end of gradient. Reprinted from Soukup-Hein et al. [84].