Chiral Amino Acid and Peptide Separations – the Next Generation

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Chiral chromatography became well established in the 1980’s as a routine technique in pharmaceutical, food and environmental applications and now largely replaces earlier techniques that used derivatisation of the enantiomers with a chiral reagent and their separation on standard, achiral HPLC columns. For amino acids, the ability to separate as free amino acid enantiomers eliminates uncertainty in the determination of enantiomeric configuration compared to the use of a chiral derivatisation reagent. Several chiral stationary phases (CSPs) are available for this separation (Table 1), but whilst HPLC stationary phases have undergone a revolution with the introduction of sub-2μm and superficially porous particles (SPP), CSPs have generally remained on 3 and 5μm silica supports. This article reviews current methods, technology and new research that should bring UHPLC advantages to chiral HPLC.

Chiral Amino Acids and Peptides

Although all proteinogenic amino acids are present in nature as L-enantiomers (and so their biological interactions are stereochemically predictable), enzymatic posttranslational modifications can result in the incorporation of D-amino acids in some proteins, notably in small molluscs [1]. They are also abundant components of peptidoglycan cell walls of bacteria [2]. Further, D-Serine has been known for some time to act as a neurotransmitter, activating the N-methyl-D-aspartate (NMDA) receptor: it originates by synthesis in the brain from its L-enantiomer [3,4]. The ability to easily enantiomerically separate amino acids is therefore important to our understanding of nature and human biology.

Enantiomerically pure amino acids are frequently used as chiral building blocks in asymmetric synthesis such that monitoring both purity and reaction progress are important applications of chiral separations [5,6]. Additionally, because of the clinical monitoring of biomarkers [5] and peptides of therapeutic importance [7], interest in the effective separations of chiral amino acids has continued to grow over recent years.

For bioactive peptides, an analysis of their amino acid sequences is an essential key to understanding possible post-translational modifications and unexpected digressions from homochirality in which the natural L-isomer is replaced by the D-isomer. The action of peptidyl-aminoacyl-L/D-isomerase, for instance, can convert an L-enantiomer to its D-counterpart during biological peptide synthesis [8]. D-residues in a peptide linkage can also result from age-dependent racemization [9].

Chiral Separations

The separation of free amino acids has been made possible by several different CSPs that include Crown Ether, Ligand Exchange, while N-protected amino acids can be separated using brush type and cyclodextrin CSPs. Macro cyclic glycopeptide and chinchona alkaloid CSPs generally have the ability to separate both. Table 1 outlines the most commonly used CSPs for the direct separation of free and N-protected amino acids.

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CSP is compatible with LC-MS, as shown in a recent study [10], in which LC-TOFMS with an isocratic ACN/Water/TFA mobile phase enabled the separation of 18 of the proteinogenic amino acids without derivatisation.

Ligand Exchange separations use a more unusual mobile phase of CuSO4, useful if the amino acid does not have a UV chromophore. These CSPs utilise a bonded D- or L-amino acid (phenylalanine, in the case of Supelco CLC columns, for example) and so can be used for the reversal of elution order in trace analysis by simply changing columns [11,12]. Brush type and protein based CSPs have been also been utilised for the separation of some derivatised amino acids [13].

However, phases based on ionisable teicoplanin and the zwitterionic quinine/quinidine selectors have become the broadest ranging and most useful today (see later sections on these). The main reason for this is the broad scope for separations as both separate free natural and synthetic α, β, γ-amino acids, whether primary or secondary, aliphatic or aromatic, cyclic or acyclic, in addition to small peptides (and of other amphoteric compounds). The prevalence of LC-MS is another reason; both are fully compatible. Interestingly, teicoplanin bonded CSPs have also been used in clinical applications to separate isobaric biomarker amino acids (example, glutamine and lysine[14]).
Mobile phases

Most mobile phases in use for the macrocyclic glycopeptide and chinchona alkaloid based CSPs are of either polar organic or aqueous organic composition. Polar organic mobile phases are principally a non-aqueous methanol / acetonitrile mix, with the addition of acid and base where control of the ionisable moieties of the glycopeptide CSP and solutes is required. The polar organic was first developed by Armstrong in 1993 for use with cyclodextrin CSPs (commercially, CYCLOBOND, [15]) and was then extended to the macrocyclic glycopeptide phases when they were launched in 1994. In recent years, polar organic has also been adopted by several other CSPs including brush type CSPs for general chiral separations [16]. For amino acid separations on the chinchona alkaloid based CSPs [17], formic acid, diethlyamine and water are usually added in small quantities to control the comparative strengths of anionic and cationic interactions. Simple alcohol water mixes are also used with the macrocyclic glycopeptide phases, adding buffer when the amino acid has ionisable functional groups additional to the alpha zwitterion moiety.

Macrocyclic glycopeptides CSPs

The variety of functional groups of macrocyclic glycopeptide phases (known commercially as CHIROBIOTIC) provides high selectivity for ionisable and zwitterionic compounds, including amino acids. Since just after their launch in 1994, the use of the macrocyclic glycopeptide, teicoplanin has been successfully used for the separation of a wide range of amino acid types and small peptides [18]. The range of enantioselectivity of teicoplanin is based on its ability to offer multiple mechanisms that includes hydrogen bonding, dipole-dipole, π-π, van der Waals, NMR and HPLC Investigations by Gasparini et al [19] indicated that free carboxylic groups can strongly interact by hydrogen bonding with the CSP peptide amide moieties, providing high selectivity for a wide range of free and N-protected amino acids and peptides. The amine group of the amino acid can be free or blocked with N-bonded functional groups such as 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC, trade mark Waters Corporation), benzoyl, N-tert-butoxycarbonyl (tBOC), carboxybenzyl (CBZ), 5-(dimethylamino)naphthalene-1-sulphonyl (dansyl) and 9-fluorenylmethoxycarbonyl (FMOC), making this method suitable for the monitoring of peptide synthesis. In each case for all amino acids, the elution order is L before D and the mobile phase alcohol / water, adding formic acid for acidic amino acids and ammonium acetate buffer for the basic ones.

Zwitterionic Ion Exchange Type CSPs

By combining quinine with (S,S)-trans-2-amino cyclohexane sulphonic acid (ACHSA), or quinidine with the (R,R) form, and bonding at the C-9 position via a carbamate linkage, two new 3 and 5μm brush type chinchona alkaloid CSPs (commercially, CHIRALPAK ZWIX(+) and ZWIX(-), respectively) were commercially introduced in 2012 [21,22,23]. The phases incorporate a weak anionic and a strong cationic interaction site. By selecting particular mobile phase conditions, both the amphoteric amino acid anlyate and the zwitterionic CSP can become charged, polar organic solvents in combination with added acid and base ensure that ionisation
occurs, positively charged at the protonated nitrogen atom of the bicyclic quinuclidine moiety and negatively charged at the sulphonic acid functional group. These CSPs separate amino acids by an electrostatic interaction between the charged species, supported (structure dependant) by hydrogen bonding, Van der Waals forces, π–π stacking and hydrophobic interactions.

A recent study [17] showed the influence of steric effects. Enantioselectivity increases with the side chain length when it is linear and increases with the bulkiness and rigidity of the side chain when it is branched. Steric effects may also be responsible for the longer retention of α-amino acids on these CSPs. There are indications that the amino group may not always be necessary for enantioselectivity, as evidenced by the separations achieved for N-protected amino acids, such that the CSP behaves in such cases as a chiral anion exchange rather than a zwitterionic CSP.

The main advantage of these phases is the ability to provide reversal of elution order (Figure 2) by switching columns, although the resolution may differ slightly mainly because the two CSPs are not exact enantiomeric versions of the same selector.

### The next generation: Superficially and Fully Porous Particles

Higher performance smaller particle size CSPs have been made available over recent years utilising 3μm and sub-2μm particles; in contrast, CSPs for amino acid separations have until recently largely remained in 5 and 3 μm formats. Since their launch in 2006 for reversed phase separations [24], superficially porous particles (SPP) have continued to gain success and widespread application. A porous layer over a solid core of the SPP improves mass transfer kinetics since analytes cannot diffuse into the particle. It was realised that higher efficiencies and faster separations could be readily achieved from the resulting reduced band broadening [25]. The narrower particle size distribution provided by this technology leads to a more homogeneously packed bed and a consequential higher back pressure (albeit lower than that for sub-2μm columns)[26], such that these phases could be used on both HPLC and the then new UHPLC instruments. Later research [27] also showed that the increased efficiencies of these phases is largely due to improvements in the A and B term of the van Deemter equation (eddy and longitudinal diffusion, respectively) resulting from homogeneously packed columns.

Recently, several studies have been carried out to incorporate this technology into chiral applications. The practical difficulties of bonding more bulky chiral selectors onto sub-2μm particles along with the tendency of the particles to aggregate were overcome in a study by Sciasciera et al, creating a sub-2μm version of the brush-type CSP Whelk-O1 [28].

The axial and radial temperature gradients that result from frictional heating in SPPs [29] can have a marked effect on CSPs made from these phases when mass transfer effects are more prominent.

A new fully porous particle (FPP) was recently developed that has an extremely high efficiency and a reduced plate height of 1.7 in narrow bore columns [31]. This was investigated as a potential particle for CSPs resulting in very fast separations in seconds rather than minutes [32,33]. Traditional 5μm and new 1.9μm TPP teicoplanin bonded phases were compared [33] and showed a 3-4-fold increase in efficiency (N/m) with a reduction in reduced plate height from 3.5 to 2.5. Methionine, for instance, exhibited a resolution of 3.0 in MeOH/H2O in under 40 seconds on a 5 x 4.6mm 1.9μm FPP teicoplanin column. It was noted that the increased permeability of this CSP enabled fast separations at high flow rates without excessive frictional heating. High speed peptides separations were also demonstrated; a separation of the dipeptide DL-Leu-DL-Ala was possible in less than one minute (Figure 3) [30].

Gasparrini et al [32] in contrast utilised a novel bonding chemistry on the same 1.9μm TPP that resulted in a protonated amino group on the teicoplanin structure, such that this CSP maintains a zwitterionic character, as evidenced by the separation of a range of hydrophobic, neutral and permanently charged solutes. This developmental CSP provided a high selectivity of 2.25 to 10.7 for a range of N-protected amino acids in RP with a 10 cm column length that minimises the impact of extra column effects. Reducing this to 2cm maintained relative efficiencies and resulted in extremely fast separations. BOC-D,L-Met, for example, separated in under 1 minute in RP, with a resolution of 2.20 and average efficiency of (93,575 N/m) at 2 mL/min. Ultra-fast separations using a 1cm column were also explored and

![Figure 3. Separation of the dipeptide LeuAla on Teicoplanin bonded SPP particles. (Data courtesy of D W Armstrong)](Image 226x468 to 363x627)

A further study [30] showed that in high water content mobile phases, axial temperature gradients improved mass transfer and countered any loss in efficiency due to radial temperature gradients and eddy diffusion. This resulted in a significantly increased efficiency for teicoplanin bonded SPP; high resolutions of 1.6 to 3.0 were achieved in less than one minute for a range of amino acids in MeOH/H2O.

### Table 1. Commonly used Chiral Stationary Phases for Amino Acid separations

<table>
<thead>
<tr>
<th>CHIRAL STATIONARY PHASE TYPE</th>
<th>CHIRAL LIGAND</th>
<th>COMMERCIAL NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macro cyclic glycopeptide</td>
<td>Teicoplanin</td>
<td>CHIROBIOTIC T</td>
</tr>
<tr>
<td>Macro cyclic glycopeptide</td>
<td>Teicoplanin Aglycone</td>
<td>CHIROBIOTIC TAG</td>
</tr>
<tr>
<td>Chinchona alkaloid</td>
<td>Quinine, quinidine bonded with ACHSA</td>
<td>CHIRALPAK ZWIX(+ and ZWIX(-)</td>
</tr>
<tr>
<td>Ligand Exchange</td>
<td>Phenylalanine</td>
<td>Astec CLC-D, CLC-L</td>
</tr>
<tr>
<td>Amino Acid</td>
<td>Crown Ether</td>
<td>CROWNPAK CR-I</td>
</tr>
<tr>
<td>Macro cyclic glycopeptide</td>
<td>Ristocetin A</td>
<td>CHIROBIOTIC R</td>
</tr>
<tr>
<td>Brush type</td>
<td>1-(3,5-dinitrobenzamido)-1,2,3,4-tetrahydrophenanthrene</td>
<td>Whelk-O1</td>
</tr>
<tr>
<td>Poly cyclic amine</td>
<td>P-CAP</td>
<td>Chirex</td>
</tr>
<tr>
<td>(S)-valine and 3-dinitroaniline urea</td>
<td>Cyclodextrin</td>
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<tr>
<td>Inclusion complex</td>
<td>Cyclodextrin</td>
<td>CYCLOBOND 1 2000 DMP</td>
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<td></td>
<td>Cyclodextrin</td>
<td>CYCLOBOND 1 2000 RSP</td>
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provided this same separation in 11 seconds in HILIC mode (Rs 1.04). Interestingly, the unusual exclusion effect occasionally seen in teicoplanin CSPs caused by repulsion of the negatively charged carboxylic acid group on the phase with a negatively charged analyte, is absent in this phase.

Conclusions

Further developments in the application of SPP and FPP CSPs for free amino acid separations will be of great interest. It is likely that instrument optimisation is likely to be as important as selectivity optimisation. Methods may be affected more by injection cycle time than particle efficiencies since these may be longer than the actual separation time. It will also be very critical to use low dispersion injection needles, low volume detector flow cells and low internal diameter connection tubing to make the most out of these developments.

Fast separations such as these could also be useful in preparative chromatography, with the potential for reduced solvent consumption. Perhaps most importantly, the speed of separation is so fast that on-line monitoring of asymmetric synthesis becomes feasible.

References

26. Application Note 183 Supelco, Improving HPLC Performance: Relationship between particle size, column efficiency and column pressure

New Conductivity and pH Monitor Works with Virtually Any Chromatography System

Gilson has expanded its chromatography product line with the launch of the VERITY® 1810 Conductivity and pH Monitor, an easy-to-use, compact device that can be integrated with virtually any chromatography system.

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