Chiral Amino Acid and Peptide Separations – the Next Generation

Denise Wallworth, Sigma-Aldrich, now part of Merck.

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 stereochemical 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 their biological functions. Whilst amino acid analysis and tandem MS sequencing is available, the determination of amino acid stereochemistry in a peptide is critical 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-isomerases, 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. Macrocyclic 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.

The mechanism of crown ether CSPs, available coated onto 5µm silica and (recently added) 3µm immobilised (commercially, CROWNPAK CR-I), relies on the multiple hydrogen bonding interactions between the primary amine of the amino acid and the ether moieties of the crown ether and so requires that the amine is cationic through the use of an acid (pH2-3) in the mobile phase. In practice, amino acids with a secondary amine functionality (such as Proline) generally do not separate because of insufficient interactions [10]. This 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].

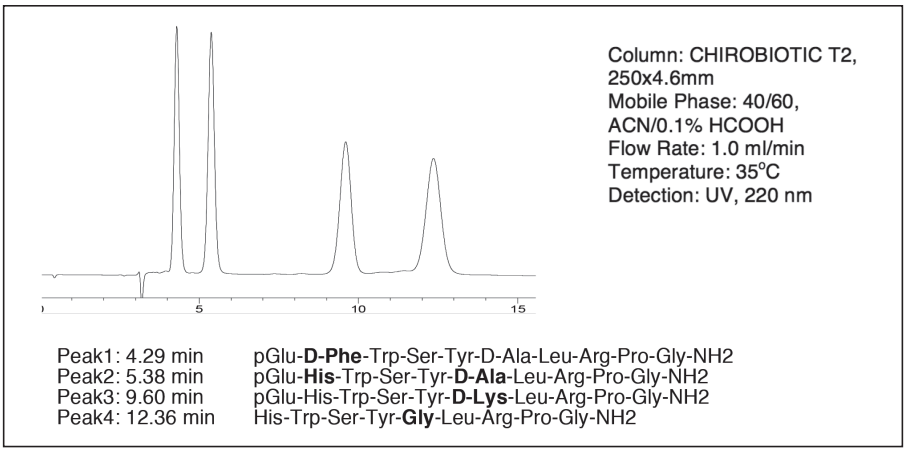

Figure 1. Separation of peptide isoforms of Luteinising hormone-releasing factor, LHRH [Data courtesy of D W Armstrong]

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, hydrophobic, ionic and steric interactions. NMR and HPLC Investigations by Gasparrini 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.

During a study to determine the importance of the glycone moiety in the mechanism of separation of tecoplanin bonded phases [20], it was expected that enantioselectivity would disappear one it was removed. In fact, selectivity remained but changed such that the aglycone version enabled alternative separations and mobile phases for different amino acids. It was made available commercially as CHIROBIOTIC TAG in 2000. In contrast to teicoplanin, this CSP contains a single primary amine that remains charged under usual HPLC conditions.

An interest in the therapeutic effects of changing the conformation of one or more amino acids in a peptide led to the use of teicoplanin for the separation of peptide isoforms, using a simple mobile phase of acetonitrile/formic acid (Figure 1). This method enabled selectivity between peptides with just one amino acid difference, even if not in the terminal position [7].

Zwitterionic Ion Exchange Type CSPs

By combining quinine with (S,S)-trans-2-aminocyclohexane 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 analyte and the zwitterionic CSP can become charged; polar organic solvents in combination with added acid and base ensure that ionisation

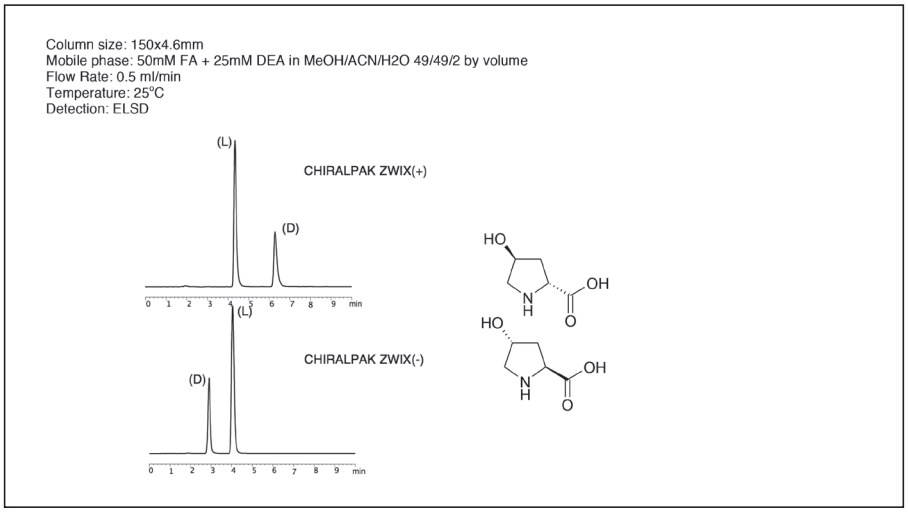

Figure 2. Reversal of elution order of L-enriched trans 4-hydroxy proline on CHIRALPAK ZWIX(+)/ZWIX(-) columns.

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, p−p 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 b-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 particles 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 Sciascera 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.

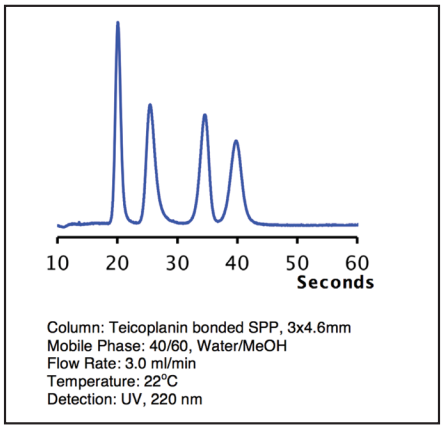

Figure 3. Separation of the dipeptide LeuAla on Teicoplanin bonded SPP particles. [Data courtesy of D W Armstrong]

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/H_2O.

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/H_2O 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

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

CHIRAL STATIONARY PHASE TYPE	CHIRAL LIGAND	COMMERCIAL NAME
Macrocyclic glycopeptide	Teicoplanin	CHIROBIOTIC T
Macrocyclic glycopeptide	Teicoplanin Aglycone	CHIROBIOTIC TAG
Chinchona alkaloid	Quinine, quinidine bonded with ACHSA	CHIRALPAK ZWIX(+) and ZWIX(-)
	Crown Ether	CROWNPAK CR-I
Ligand Exchange	Phenylalanine	Astec CLC-D, CLC-L
	Amino Acid	Nucleosil Chiral
Macrocyclic glycopeptide	Ristocetin A	CHIROBIOTIC R
Brush type	1-(3,5-dinitrobenzamido)-1,2,3,4-tetrahydrophenanthrene	Whelk-O1
	Polycyclic amine	P-CAP
	(S)-valine and 3,5-dinitroaniline urea	Chirex
Inclusion complex	Cyclodextrin	CYCLOBOND 1 2000 DMP
	Cyclodextrin	CYCLOBOND 1 2000 RSP

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.

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