Direct stereo-selective separations of amino acids and small peptides
on cinchona derived zwitterionic chiral columns by HPLC

[1]Tong Zhang (tzhang@chiral.fr), [1]Emilie Holder, [1]Pilar Franco, [2]Elena Eksteen

[1] Chiral Technologies Europe, 160, Bd. Gonthier d'Andernach, 67404 Illkirch, France

[2] Chiral Technologies, Inc. 800, North Five Points Road, West Chester, PA 19380, USA

Introduction

Synthetic peptides occupy an important position in therapeutics and drug discovery. They represent the effective and innovative solutions for new and/or unmet medical requirements.

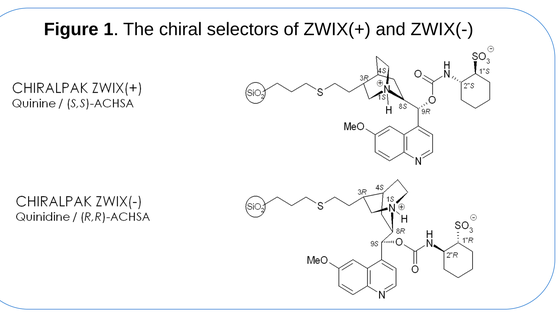
In concert with the peptide synthesis and their applications, appropriate analytical methods are needed for assays of the chiral amino acid and small peptide building blocks, for monitoring and controlling the stereochemistry of the synthetic process, for assessing the structural integrity (amino acid composition, sequence and chirality) of the final peptides, as well as for determining the constituents of the samples back from biological media. HPLC, often combined to MS detection, has been the most frequently used technique for these purposes. However, developing efficient and reliable methods involving the molecular chirality information for control and characterization of synthetic peptides remains challenging.

In response to such challenges, the newly developed zwitterionic chiral stationary phases, namely CHIRALPAK® ZWIX(+) and CHIRALPAK® ZWIX(-), have been explored in HPLC for direct stereo-selective resolution of amino acids and oligopeptides (di- and tripeptides mainly) using LC-MS compatible mobile phase conditions.

The chiral stationary phases (CSPs)

CHIRALPAK ZWIX(+) and CHIRALPAK ZWIX(-) are cinchona alkaloid-derived zwitterionic CSPs. The chiral selectors are designed in a combinatorial approach, incorporating a weak anionic and a strong cationic interaction sites into a single chemical moiety [1-2]. They are synthesized by the fusion of quinine or quinidine with enantiomerically pure trans-2-aminocyclohexanesulfonic acid ((*R*,*R*)- or (*S*,*S*)-ACHSA)) at C-9 position via a carbamate linkage. Quinine combines with (*S*,*S*)-ACHSA to form the ZWIX(+) chiral selector unit being then immobilised onto mercaptopropyl silica (particle size: 3µm for analytical columns; 5µm for semi-preparative columns). The ZWIX(-) CSP is made in a similar way but via the combination of quinidine with (*R*,*R*)-ACHSA. Both columns are commercially available from Daicel Corporation.

Figure 1. The chiral selectors of ZWIX(+) and ZWIX(-)



Based on synergistic double ion-pairing interactions with the ampholytes (analytes) and assisted by hydrogen bonding, π-π stacking and Van der Waals forces, these CSPs enable enantio- and/or stereo-selective separation of a great variety of zwitterionic compounds, typically amino acids and small peptides [3-5]. ZWIX(+) and ZWIX(-) behave pseudo-enantiomerically and provide the feature of reversing enantiomer elution order by simple column switching from ZWIX(+) to ZWIX(-) and vice versa. The same columns exhibit chiral recognition for N-derivatives of amino acids as well [1-2].

MS-compatible mobile phase system

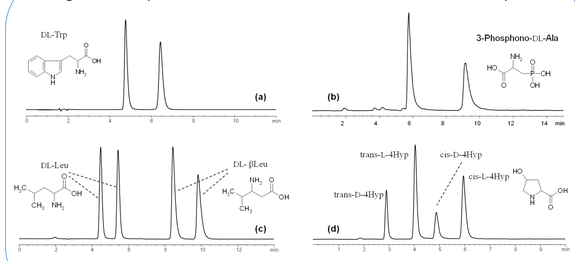
Due to its appropriate proton activities, methanol is the basic mobile phase component for ZWIX(+) and ZWIX(-) columns. In combination with a low percentage of water, it provides generally good solubility to amino acids and peptides, is a suitable medium for ionisation of the chiral selector and the amphoteric analytes and affords efficient solvation to all the ionised groups involved in the double ion-pairing chromatographic equilibria. The eluting strength of the mobile phase can be adjusted for weaker by incorporating polar non-protic solvents (e.g. ACN or THF); for stronger by adding more water.

In addition, the combined presence of acidic and basic additives (being the co- and counter-ions) in a suitable ratio is necessary to regulate the interactions via displacement effects. The combination of 50 mM formic acid (FA) and 25 mM diethylamine (DEA) has been effectively used as the generic mobile phase additives. The replacement of DEA with ammonia is straightforward in the sake of the improved ionization performance for MS.

Direct enantiomer resolution of amino acids

A large series of chiral amino acids of diverse structures have been successfully resolved into enantiomers using ZWIX(+) and ZWIX(-) columns by following a generic experimental scheme [5]. The examples are given in Figure 2.

Figure 2. Separation of enantiomers of amino acids - Examples

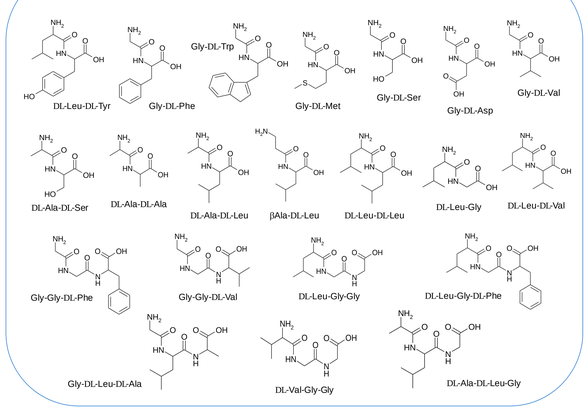


Chromatographic conditions
Flow rate: 0.5 mL/min Temperature: 25°C Detection: UV 254nm for (a); ELSD for (b,c,d) Additives: 50 mM FA + 25 mM DEA
Column: 150x3 mm i.d., (a,c) ZWIX(+); (b,d) ZWIX(-) Bulk mobile phase: (a,b) MeOH/H_2O 98:2 v/v/v; (c,d) MeOH/ACN/H_2O 49:49:2 v/v/v

Stereo-selective separation of small peptides

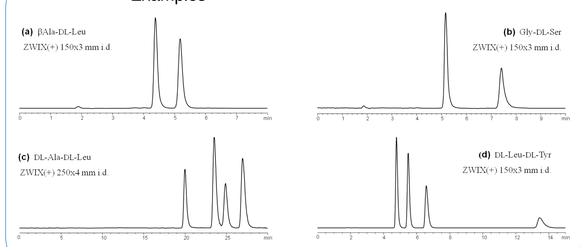
Due to the very limited access to the peptide samples, only the commercially available di- and tripeptide standards containing one or two stereogenic centers are tested (Figure 3) on the same columns.

Figure 3. Structure of the di- and tripeptides in test



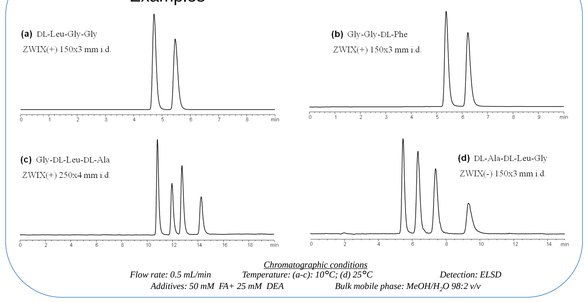
For stereo-selective separation of the peptides, the same experimental scheme is followed as for amino acids. Among the 21 peptides tested, 14 of them can be fully resolved into enantiomers or diastereomers on ZWIX(+) and/or ZWIX(-) columns, including DL-Leu-DL-Tyr, DL-Leu-DL-Val, DL-Ala-DL-Leu-Gly and Gly-DL-Leu-DL-Ala. Partial resolution is achieved with some others. The separations are exemplified in Figures 4-5.

Figure 4. Separation of enantiomers/diastereomers of dipeptides - Examples



Chromatographic conditions
Flow rate: 0.5 mL/min Temperature: (a-b): 25°C; (c-d) 10°C Detection: ELSD Additives: 50 mM FA+ 25 mM DEA
Bulk mobile phase: (a,b) MeOH/H_2O 98:2 v/v; (c) MeOH/THF/H_2O 49:49:2 v/v/v; (d) MeOH/ACN/H_2O 49:49:2 v/v/v

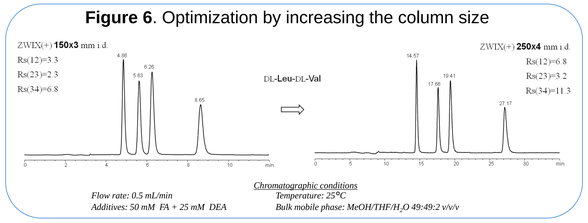
Figure 5. Separation of enantiomers/diastereomers of tripeptides - Examples



Chromatographic conditions
Flow rate: 0.5 mL/min Temperature: (a-c): 10°C; (d) 25°C Detection: ELSD
Additives: 50 mM FA+ 25 mM DEA Bulk mobile phase: MeOH/H_2O 98:2 v/v

The retention factor, stereo-selectivity and degree of resolution are dependent on the compound structure, the mobile phase and the temperature. These parameters can play important roles in method optimization. The use of a longer column may also an alternative for enhancing the resolution (Figure 6). ZWIX(+) and ZWIX(-) are proved to be complementary in selectivity and in elution order.

Figure 6. Optimization by increasing the column size



Chromatographic conditions
Temperature: 25°C
Flow rate: 0.5 mL/min Additives: 50 mM FA + 25 mM DEA Bulk mobile phase: MeOH/THF/H_2O 49:49:2 v/v/v

Control of elution order

Due to their opposite configuration at four stereogenic centres (8*S*,9*R*,1"*S*, 2"*S* for ZWIX(+); 8*R*,9*S*,1"*R*, 2"*R* for ZWIX(-)), the two chiral selectors behave as pseudo-enantiomers. The elution order of enantiomers can be reversed on ZWIX(+) and ZWIX(-) in a quasi-systematic way, although their separation performance may not be exactly equal towards each analyte. This can be a valuable instrumental tool for reliable determination of enantiomeric excess, for assessing the impurity profile of the synthetic peptides and for adapting the analytical method to the real application requirements.

Compound	ZWIX(+)	ZWIX (-)
DL-Ala	L/ D	D/ L
DL-Asp	L/ D	D/ L
4-Chloro-DL-Phe	L/ D	D/ L
DL-Cys	L/ D	D/ L
DL-DOPA	L/ D	D/ L
DL-Gln	L/ D	D/ L
cis-DL-4Hpr	L/ D	D/ L
trans-DL-4Hpr	L/ D	D/ L
DL-His	L/ D	D/ L
DL-Ile	L/ D	D/ L
DL-Kyn	L/ D	D/ L
DL-Leu	L/ D	D/ L
DL-Met	L/ D	D/ L
DL-Nle	L/ D	D/ L
DL-α-Phg	L/ D	D/ L
DL-Phe	L/ D	D/ L
DL-Pro	L/ D	D/ L
DL-Tyr	L/ D	D/ L
DL-Thr	L/ D	D/ L
3-(2-Thienyl)-DL-Ala	L/ D	D/ L
DL-Val	L/ D	D/ L
DL-Methotrexate	D/ L	L/ D
N-α-BOC-DL-His	D/ L	L/ D
DL-Trp	D/ L	L/ D

Due to the lack of the enantiomer/diastereomer standards, no information on elution order could be given for the series of peptides in test. However, the elution order of a number of amino acids is reported in the table above.
The enantiomer elution order is predictable to a certain extent. It is related to the structural features of the analyte and the chiral selector but regardless of the chromatographic conditions.

Remarks and perspectives

Owing to their chiral recognition ability to ampholytes, CHIRALPAK® ZWIX(+) and ZWIX(-) are versatile for chiral analysis of free amino acids and small peptides. They are also able to separate enantiomers of N-blocked amino acids. They can be effective tools for chiral assays of amino acids/peptides building blocks, their N-derivatives and final synthetic peptides in terms of amino acid composition, molecular chirality and the structurally related impurities. The fact that they are able to separate stereoisomers of small peptides (or fragments of large peptides), these chiral columns may be substantially suitable if milder conditions should be applied to the hydrolysis of certain peptides to avoid the degradation, enantiomerization or epimerization of the hydrolysates. One of the most significant advantages using ZWIX(+) and ZWIX(-) lies in the possibility to analyze the hydrolysates without pre-column derivatization. The option of hyphenation with a MS detector and the complementarity between ZWIX(+) and ZWIX(-) related to selectivity, retentivity and elution order may make them an attractive tool box for characterization of synthetic peptides.

Our further efforts will be focused on the separation of stereoisomers of more complex peptides of larger sizes, preferably with some real samples from peptide synthesis.

References

[1] C.V. Hoffmann, R. Pell, M. Lämmerhofer, W. Lindner, Anal. Chem. 80 (2008) 8780.
[2] C.V. Hoffmann, R. Reischl, N.M. Maier, M. Lämmerhofer, W. Lindner, J. Chromatogr. A, 1216 (2009) 1157.
[3] S. Wernisch, W. Lindner, J. Chromatogr. A 1269 (2012) 279.
[4] T. Zhang, E. Holder, P. Franco, W. Lindner, J. Sep. Sci. 37 (2014) 1237.
[5] T. Zhang, E. Holder, P. Franco, W. Lindner, J. Chromatogr. A, in press, DOI: 10.1016/j.chroma.2014.06.012