Chiral Separation and Discrimination in High-Performance Liquid Chromatographic Systems

Ph.D. THESIS

Zoltán Pataj

Supervisors:

Antal Péter Ph.D., D.Sc.

István Ilisz Ph.D.

UNIVERSITY OF SZEGED
Faculty of Science and Informatics
Department of Inorganic and Analytical Chemistry
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1. Introduction

The physical, biological and chemical properties determined by the symmetry and asymmetry play an especially important role in nature. Many of the organic materials from which different organisms are built up are chiral, so it is easy to understand that the presence of the enantiomers of a chiral compound in the organism can result in different interactions and therefore different effects and the pathways of the metabolism of the stereoisomers can also differ. Accordingly, a demand has arisen in pharmaceutical and other therapeutic fundamental research for the preparation of stereoisomers in enantiomerically pure form, and for the analytical qualification and quantification of pure chiral products.

Peptide-based receptor research involves the interactions of receptors with peptides with known conformations. Peptides with known conformations are prepared by the application of sterically hindered amino acids, which have special structures and special functional groups that can form conformational blocks in the structures of peptides. The preparation of peptides with known structures requires the application of enantiomerically pure amino acids.

Chirality is now a major theme in the design, discovery, development, launching and marketing of new drugs. The advances in stereoselective bioanalysis have led to a new awareness of the importance of stereoselective pharmacodynamics and pharmacokinetics, enabling differentiation of the relative contributions of enantiomers to the overall drug process.

There are several possibilities for the preparation of pure enantiomers, e.g. using enantioselective synthetic routes or the separation of enantiomers after the preparation of racemic mixtures. Such separations can be carried out by the traditional resolution processes or through the use of preparative chiral chromatographic techniques. The chromatographic processes play an important part not only in the production of the enantiomers, but also in the quality control of the chiral products. Chiral high-performance liquid chromatography (HPLC) is curently one of the most dynamically developing and most widely applied branches of the chiral analytics.

2. Aims

The primary aim of this work was to develop chiral HPLC methods for the separation of the enantiomers of racemic β-2-, β-3- and γ-amino acids, β-lactams and aminonaphthols on newly developed chiral columns.

The compounds investigated were:

a) enantiomers of aliphatic and aromatic β-2- and β-3-amino acids on **macrocyclic glycopeptide antibiotic**-based chiral stationary phases (CSPs);

b) enantiomers of γ-amino acids on **macrocyclic glycopeptide**-based columns;

c) enantiomers of aliphatic and aromatic β-2-amino acids on an end-capped CSP containing **(+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid** as chiral selector;

d) enantiomers of β-lactams on CSPs containing **derivatized polysaccharides**; and

e) enantiomers of various aminonaphthol analogs on **polysaccharide**-based CSPs.

Another objective was to study the influence on the chiral separation process of parameters such as the nature and composition of the mobile-phase, mobile-phase modifiers (the natures and concentrations of the alcoholic and acidic modifiers) and the structures of the investigated compounds and chiral selectors. The effects of the structural features of the investigated analytes and chiral selectors on the discrimination between the enantiomers were characterized through the chromatographic parameters (retention factor, separation factor and resolution) and calculated thermodynamic data. In some cases, the effects of temperature were studied and thermodynamic parameters were determined.

3. Experimental

3.1. Apparatus

For direct chromatographic methods, several different types of CSPs were applied, including macrocyclic glycopeptide-based crown ether-based and polysaccharide-based CSPs. All of the chiral selectors were covalently bonded to the silica support.

The system applied apparatus were as follows:

I. Chromatographic system: an M-600 low-pressure quaternary gradient pump, an M-996 photodiode array detector and Millenium 32 Chromatography Manager software (Waters, Milford, USA).

II. Chromatographic system: a 1525 high-pressure binary gradient pump, a 487 dual-wavelength detector, a 717 autosampler, an in-line degasser and Breeze Chromatography Manager software (Waters, Milford, USA).

3.2. Investigated analytes

- β-2-Amino acids
- β-3-Amino acids
- γ-Amino acids
- β-Lactams
- Betti bases
- Aminonaphthols

4. Results

We developed methods for separation of the enantiomers of β-2-, β-3- and γ-amino acids, β-lactams and aminonaphthols by using chiral liquid chromatography.

4.1. Enantioseparation of unnatural amino acids on macrocyclic glycopeptide-based columns

The enantiomers of *β*-2-, *β*-3- and *γ*-amino acid analogs were separated on macrocyclic glycopeptide-based CSPs, i.e. Chirobiotic T, T_2, TAG and R. Separation could be accomplished in reversed-phase mode by using 0.1% Triethylammonium acetate (TEAA) buffer (pH 4.1)/MeOH mobile-phases with different compositions and at different temperatures.

a) For analytes with an alkyl side-chain, the retention factor progressively increased as the MeOH content of the mobile-phase was increased; this was probably due to the HILIC (hydrophilic interaction) effect. Similar behavior was found for *γ*-amino acid analogs. For analytes with an aromatic side-chain, a U-shaped curve was observed. At higher water content, the retention factor increased again with increasing water content, which was probably due to enhanced hydrophobic interactions in the water-rich mobile-phase.

All α values increased slightly with increasing MeOH content for all investigated unnatural amino acids (higher increases were observed for *β*-2-amino acids with aromatic side-chains). The R_S values progressively increased with increasing MeOH content on all CSPs.

b) The values of the chromatographic parameters depended on the structures of the compounds and on the CSPs applied. The possible interactions depend strictly on how the enantiomers fit into the aglycone cavity, which is determined by the structures of the analytes and CSPs and also by the mobile-phase composition.

c) Investigation of the selective and non-selective interactions and the role of the sugar moieties were important for an understanding of the chiral separation mechanism.

d) Linear van't Hoff plots were observed in the studied temperature range 7–45 ºC, and the apparent changes in enthalpy (ΔH^o), entropy (ΔS^o) and free energy (ΔG^o) were calculated. The values of the thermodynamic parameters depended on the

structures of the compounds and on the CSPs applied.

e) The elution sequence was determined in some cases, and was found to be *(R)* < *(S)* for *β*-amino acids. For *γ*-amino acids it emerged that the first-eluting enantiomer for analyte ***γ*-1** was 1*(S)*,3*(R)* and that for analyte ***γ*-3** was 2*(R)*,4*(S*) on all CSPs.

4.2. Enantioseparation of *β*-2-amino acids on crown ether-based columns

This research work demonstrated that the long-tethered crown ether CSP is quite successful for direct enantioseparation of the investigated *β*-2-amino acids. The chromatographic behavior (*k'*, α and R_S) proved to be dependent on the natures and concentrations of the acidic and alcoholic modifiers in the H_2O/alcohol/acidic modifier mobile-phase system.

a) The application of HCOOH, AcOH, Trifluoroacetic acid (TFA), $HClO_4$ or H_2SO_4 at the same concentration (10 mM) resulted in different levels of pH. A decrease of the pH in the mobile-phase system decreased the retention factors by 70–80%. The largest *k'* values were in most cases obtained on the application of HCOOH or AcOH, while $HClO_4$ and H_2SO_4 resulted in the lowest *k'* values. The change of the pH exerted only slight effect on the α and R_S values.

b) The nature of the alcohol influenced the retention and resolution, but the change in *k'* did not appear to correlate with the carbon numbers of the alcohols. It was found that MeOH and EtOH in most cases gave larger *k'* values than those for PrOH or IPA. The nature of the alcohol caused slight changes in α and significant effects on the resolution, but no general rule could be established. In most cases, the smallest resolution was observed on the application of PrOH, whereas the application of MeOH or IPA resulted in the largest R_S values.

c) The effects of the MeOH content of the mobile-phase on the retention, selectivity and resolution were investigated. For all analytes, a U-shaped curve was observed. At higher water content, the retention factor increased with increasing water content due to the enhanced hydrophobic interactions in the water-rich mobile-phase. As the content of MeOH in the aqueous mobile-phase was increased, *k'*

increased again.

d) The chromatographic retention and resolution were found to be dependent on the structures of the analytes and the nature of the substituents in the β position.

e) The thermodynamic parameters revealed the separation was enthalpically favored, in contrast with that on the macrocyclic glycopeptide-based CSPs. The elution sequence was found to be *(S) < (R)*.

4.3. Enantioseparation of β-lactams on polysaccharide-based columns

HPLC methods were developed for the separation of the enantiomers of 19 β-lactams. The direct separations were performed on CSPs containing either amylose-tris-3,5-dimethylphenyl carbamate (AmyCoat^{TM} column) or cellulose-tris-3,5-dimethylphenyl carbamate (CelluCoat^{TM} column) as chiral selector in the normal-phase mode.

a) By variation of the nature and the content of the alcoholic modifier, the separation of the stereoisomers was optimized; as a result, baseline resolution was achieved for the β-lactams in at least one chromatographic system. The change in *k'* did not appear to correlate with the carbon number of the alcohols. It emerged that MeOH gave a smaller *k'* on the AmyCoat^{TM} CSP and a higher *k'* on the CelluCoat^{TM} CSP. The enantioselectivity did not change dramatically when different alcohols were applied in the same molar concentration.

b) The alcohol content of the mobile-phase influenced the chromatographic behavior considerably. The retention factor decreased strongly with increasing alcohol content, whereas the changes in α and R_S differed. For the β-lactam analogs, slight changes in α were registered with increasing IPA content, whereas R_S changed in parallel with *k'*, i.e. R_S in most cases decreased with decreasing *k'*.

c) For most of the analytes investigated, a structure-retention relationship was observed on both CSPs. With increasing number of carbon atoms attached to the β-lactam ring, *k'* usually increased. Besides polar interactions, π–π interactions between

the phenyl groups of the CSP and an aromatic group of the solute may play some role in chiral recognition. At constant mobile-phase composition, the *para*-substituted analog was separated with similar enantioselectivity on both CSPs, whereas for the *ortho*-substituted analog CelluCoat™ CSP and for the *meta*-substituted analog the AmyCoat™ CSP proved to be much more efficient.

d) The elution sequence was determined in all cases, but no general rule could be established. The AmyCoat™ and CelluCoat™ columns appeared to be highly complementary.

4.4. Enantioseparation of aminonaphthol analogs with one or two asymmetric center(s) on polysaccharide-based columns

This work demonstrated that the developed methods are quite successful for the direct enantioseparation of new aminonaphthol analogs possessing one or two chiral centers. The direct separations were performed on CSPs containing either amylose-tris-3,5-dimethylphenyl carbamate (AmyCoat™ column) or cellulose-tris-3,5-dimethylphenyl carbamate (CelluCoat™ and Chiralcel OD-H columns) with various *n*-heptane/alcohol/DEA mobile-phase systems.

a) By variation of the nature and the content of the alcoholic modifier, the separation of the enantiomers was optimized. The alcohol content of the mobile-phase strongly influenced the chromatographic behaviour. In the *n*-heptane/IPA/DEA mobile-phase system, the retention factors decreased with increasing alcohol content, while the changes in separation factor and resolution varied. The nature of the alcoholic modifier exerted considerable effects on the retention, selectivity and resolution. At constant alcohol concentration, the retention factors of the first-eluting enantiomers increased with increasing alcohol chain length, especially for those with branched and bulky side-chains, such as IPA, BuOH and *t*-BuOH. Increasing carbon number was disadvantageous for polar interactions between the mobile-phase and the analyte; the overall resultant was that the retention factor increased with increasing alcohol chain length.

b) At the eluent composition *n*-heptane/IPA/DEA = 40/60/0.1 (v/v/v), similar or lower retention factors of the first-eluting enantiomers were observed on CelluCoat than on Chiralcel OD-H for all of the Betti base analogs. For the second-eluting enantiomers, different behavior was found on the two cellulose-based columns. The separation factors were higher for the 2-naphthol analogs on Chiralcel OD-H than for the 1-naphthol analogs on CelluCoat. As far as resolution was concerned, R_S was in all cases higher on the CelluCoat CSP than on Chiralcel OD-H.

For the aminonaphthol analogs the two polysaccharide-based CSPs appeared to be highly complementary.

c) It was reported by Meyer that the steric effect of a substituent on the reaction rate is characterized by the size-descriptor V^a. The results revealed that the chromatographic parameters *k'* and α correlated with V^a. The retention factors depended strongly on the volumes of the substituents: bulkier substituents inhibited the interaction with the selector, and the retention decreased.

For aminonaphthol analogs with two chiral center, the presence of an *N*-atom in the *ortho* position, close to the chiral center, in most cases hindered the interaction with the CSP, resulting in smaller *k'*, α and R_S values, while the presence of an *N*-atom in the *meta* or especially the *para* position was favorable for chiral recognition. The size of the aromatic ring system (phenyl or naphthyl) also influenced the chromatographic behavior.

d) The elution sequence was determined in some cases for Betti base analogues and in all cases for aminonaphthol analogs with two chiral centers. No general rule could be established.

5. Publications

5.1. The thesis is based on the following publications

2008:
Z. Pataj, I. Ilisz, R. Berkecz, A. Misicka, D. Tymecka, F.Fülöp, D. W. Armstrong, A. Péter *Comparison of performance of Chirobiotic T, T2 and TAG columns in the separation of β^2- and β^3-homoamino acids* Journal of Separation Science, 31, 3688-3697
Impact factor: **2.746**

2009:
Z. Pataj, R. Berkecz, I. Ilisz, A. Misicka, D. Tymecka, F. Fülöp, D. W. Armstrong, A. Péter *High-performance liquid chromatographic chiral separation of β^2-homoamino acids* Chirality, 21, 787-798
Impact factor: **2.680**

I. Ilisz, **Z. Pataj**, R. Berkecz, I. Szatmári, F. Fülöp, A. Péter *Comparsion of separation performances of cellulose-based chiral stationary phases in high-performance liquid chromatographic enantioseparation of aminonaphthol analogues* Chromatographia, 70, 723-729
Impact factor: **1.098**

2010:
Z. Pataj, I. Ilisz, R. Berkecz, E. Forró, F. Fülöp, A. Péter *Comparison of separation performances of amylose- and cellulose-based stationary phases in the high-performance liquid chromatographic enantioseparation of stereoisomers of β-lactams* Chirality, 22, 120-128
Impact factor: **2.892**

I. Ilisz, **Z. Pataj**, R. Berkecz, A. Misicka, D. Tymecka, F. Fülöp, H. J. Choi, M. H. Hyun, A. Péter *High performance liquid chromatographic enantioseparation of β^2-amino acids using a long tethered (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based chiral stationary phase* Journal of Chromatography A, 1217, 1075-1082
Impact factor: **4.194**

Z. Pataj, I. Ilisz, R. Berkecz, E. Forró, F. Fülöp, A. Péter *Comparsion of separation performances of amylose- and cellulose-based stationary phases in the high-performance liquid chromatographic enantioseparation of stereoisomers of β-lactams* Procedia Chemistry, 2, 116-119
Impact factor: **0.000**

Z. Pataj, I. Ilisz, A. Aranyi, E. Forró, F. Fülöp, D. W. Armstrong, A. Péter *LC separation of γ-amino acid enantiomers* Chromatographia, 71, 13-19
Impact factor: **1.075**

I. Ilisz, **Z. Pataj**, R. Berkecz, I. Szatmári, F. Fülöp, A. Péter *High performance liquid chromatographic enantioseparation of aminonaphthol analogs on polysaccharide-based chiral stationary phases* Journal of Chromatography A, 1217, 2980-2985
Impact factor: **4.194**

Total impact factor: **18.879**

5.2. Other publications

2008:
R. Berkecz, I. Ilisz, **Z. Pataj**, F. Fülöp, H.J. Choi, M. Ho Hyun, A. Péter *LC enantioseparation of β-amino acids on a crown ether-based stationary phase* Chromatographia, 68, 13-18
Impact factor: **1.312**

R. Berkecz, I. Ilisz, F. Fülöp, **Z. Pataj**, M. Ho Hyun, A. Péter *High-performance liquid chromatographic enantioseparation of β-3-homo-amino acid stereoisomers on a (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based chiral stationary phase* Journal of Chromatography A, 1189, 285-291
Impact factor: **3.756**

2010:
I. Ilisz, **Z. Pataj**, A. Péter *Macrocyclic glycopeptide-based chiral stationary phases in high performance liquid chromatographic analysis of amino acid enantiomers and related analogs* Macrocyclic Chemistry (book chapter); Editors, Daniel W. Fitzpatrick, Henry J. Ulrich; Nova Science Publishing, 2010

I. Ilisz, R. Iványi, **Z. Pataj**, J. Kupai, P. Huszthy, I. Szatmári, F. Fülöp, A. Péter *Capillary electrophoretic enantioseparation of Betti bases with cyclodextrins and crown ether as chiral selectors* Chromatographia, 71, 115-119
Impact factor: **1.075**

I. Ilisz, **Z. Pataj**, A. Aranyi, A. Péter *Chiral HPLC separation of amino acid enantiomers and epimers of small, biologically important peptides* Mini Reviews in Medicinal Chemistry, 10, 287-298
Impact factor: **2.622**

L. Sipos, I. Ilisz, **Z. Pataj**, Zs. Szakonyi, F. Fülöp, D. W. Armstrong, A. Péter *High-performance liquid chromatographic enantioseparation of monoterpene-based 2-amino carboxylic acids on macrocyclic glycopeptide-based phases* Journal of Chromatography A, 1217, 6956-6963
Impact factor: **4.194**

2011:
A. Aranyi, I. Ilisz, **Z. Pataj**, I. Szatmári, F. Fülöp, A. Péter
High-performance liquid chromatographic enantioseparation of 1-(phenylethylamino)- or 1-(naphthylethylamino)methyl-2-naphthol analogs and a temperature-induced inversion of the elution sequence on polysaccharide-based chiral stationary phases Journal of Chromatography A, 1218, 4869-4876
Impact factor$_{2010}$: **4.194**

A. Aranyi, **Z. Pataj**, I. Ilisz, I. Szatmári, F. Fülöp, D. W. Armstrong, A. Péter
High-performance liquid chromatographic enantioseparation of Betti base analogs on a newly developed isopropyl carbamate-cyclofructan6-based (IP-CF6) chiral stationary phase Chirality, 23, 549-556
Impact factor$_{2010}$: **2.892**

I. Ilisz, **Z. Pataj**, A. Aranyi, A. Péter *Macrocyclic antibiotic selectors in direct HPLC enantioseparations* Separation and Purification Reviews, accepted
Impact factor$_{2010}$: **2.429**

Total impact factor: **41.353**

5.3. Posters

2007:
I. Ilisz, R. Iványi, G. Tóth, **Z. Pataj**, A. Péter *Capillary electrophoretic enantioseparation of substituted amino acids with cyclodextrins* 7th Balaton Symposium on High-Performance Separation Methods, Siófok, 5-7. September 2007.

A. Péter, R. Berkecz, I. Ilisz, **Z. Pataj**, F. Fülöp, M. Ho Hyun *HPLC enantioseparation of β-amino acids on chiral crown ether and β-cyclodextrin-based stationary phases* 7th Balaton Symposium on High-Performance Separation Methods, Siófok, 5-7. September 2007.

2008:
A. Péter, **Z. Pataj**, R. Berkecz, I. Ilisz, A. Misicka, D. Tymecka, F. Fülöp, D.W. Armstrong *High-performance liquid chromatographic chiral separation of β-2-homo amino acids* 20th International Symposium on Chirality, Geneva, 5-7. July 2008.

2009:
A. Péter, **Z. Pataj**, I. Ilisz, E. Forró, F. Fülöp *HPLC enantioseaparation of β-lactams on Amycoat and Cellucoat chiral stationary phases* 5th Symposium on Separations and Related Techniques of the Nordic Society of Separation Science, Tallin, 26-29. August 2009.

Z. Pataj, I. Ilisz, E. Forró, F. Fülöp, D. W. Armstrong, A. Peter *High-performance liquid chromatographic enantioseparation of γ-amino acid stereoisomers on macrocyclic glycopeptide-based columns* 8th Balaton Symposium on High-Performance Separation Methods and 15th International Symposium on Separation Sciences, Siófok, 2-4. September 2008.

A. Peter, **Z. Pataj**, I. Ilisz, E. Forró, F. Fülöp *HPLC enantioseparation of β-lactams* 8th Balaton Symposium on High-Performance Separation Methods and 15th International Symposium on Separation Sciences, Siófok, 2-4. September 2008.

I. Ilisz, J. Kupai, P. Huszthy, R. Iványi, **Z. Pataj**, I. Szatmáry, F. Fülöp, A. Péter *Capillary electrophoretic enantioseparation of Betti bases with cyclodextrins and crown ether as chiral selectors* 8th Balaton Symposium on High-Performance Separation Methods and 15th International Symposium on Separation Sciences, Siófok, 2-4. September 2008.

2010:
A. Péter, A. Aranyi, I. Ilisz, **Z. Pataj**, I. Szatmári, F. Fülöp *High-performance liquid chromatographic enantioseparation of aminonaphthol analogs on polysaccharide-based chiral stationary phases* ISC 28th International Symposium on Chromatography, Valencia, 12-16. September 2010.

2011:
Z. Pataj, I. Ilisz, I. Szatmári, F.Fülöp, D. W. Armstrong, A.Péter *The application of a newly developed isopropyl carbamate-cyclofructane6-based (IP-CF6) chiral stationary phase for HPLC enantioseparation of Betti base analogs* 36th International Symposium on High-Performance Liquid Phase Separations and Related Techniques, Budapest, 19-23. June 2011.

A. Aranyi, I. Ilisz, **Z. Pataj**, I. Szatmári, F.Fülöp, A. Péter *HPLC enantioseparation and a temperature-induced inversion of the elution sequence of 1-(phenylethylamino)- or 1-(naphthylethylamino)methyl-2-naphthol analogs* 36th International Symposium on High-Performance Liquid Phase Separations and Related Techniques, Budapest, 19-23. June 2011.

I. Ilisz, G. Fodor, **Z. Pataj**, I. Szatmári, F. Fülöp, L. Szente, A. Péter *Capillary electrophoretic enantioseparation of aminonaphthol analogs* 36th International Symposium on High-Performance Liquid Phase Separations and Related Techniques, Budapest, 19-23. June 2011.