CHIRAL SEPARATION AND DISCRIMINATION OF ENANTIOMERS

Ph.D. THESIS

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1. INTRODUCTION

Drug chirality is now a major theme in the design, discovery, development, launching and marketing of new drugs. Stereochemistry is an essential dimension in pharmacology. The advances in stereoselective bioanalysis led to a new awareness of importance of stereoselective pharmacodynamics and pharmacokinetics, enabling the differentiation of the relative contribution of enantiomers to the overall drug process. The differences in biological properties of enantiomers arise from the differences in protein transport and binding, the kinetics of their metabolism and disappearance and their stability in the environment. Sometimes the pharmacologically inactive enantiomer can exhibit unwanted side effects, antagonistic activities or even toxic effects. Therefore, there is considerable pressure to develop analytical methods for enantiomer separation for enantiomeric purity control, pharmacological studies, pharmacodynamic investigations etc. To control the enantiomeric purity of a given sample, accurate, reliable and robust analytical methods are necessary. High-performance liquid chromatography (HPLC) fully corresponds these terms, therefore HPLC is the most widespread chiral separation technique in analytical and preparative separation and drug discovery. Mass spectrometry (MS) can be a useful and helpful tool for the chiral analysis of racemates.

2. AIMS

The primary aim of this work was to develop chiral HPLC methods for the separation of the enantiomers of racemic *β*-lactams and *β*-amino acids on newly developed chiral columns. Another objective was to study the influence of different parameters such as the nature and composition of the mobile phases, mobile phase modifiers, the buffer concentration, pH, ionic strength, the nature and structure of investigated compounds and chiral selector on the chiral separation process. The effects of the structural features of the investigated analytes and chiral selector on the discrimination between the enantiomers were characterized through the chromatographic parameters (retention factor, separation factor and resolution) and

calculated thermodynamic data.

The compounds investigated with applied chiral stationer phases (CSPs) were the followings:

- enantiomers of aromatic *β*-lactams on (CSPs) containing macrocyclic glycopeptide antibiotic teicoplanin (**Chirobiotic T)** and teicoplanin aglycone (**Chirobiotic TAG)**,
- enantiomers of tricyclic *β*-lactam and bicyclic *β*-amino acids on five macrocyclic glicopeptide-based colums containing teicoplanin (Chirobiotic **T**), teicoplanin aglycone (Chirobiotic **TAG**), vancomycin (Chirobiotic **V**), vancomycin aglycone (Chirobiotic **VAG**), ristocetin A (Chirobiotic **R**) as chiral selector and 3,5-dimethylphenyl carbamate-derivatized *β*-cyclodextrin (Cyclobond **DMP**) based column,
- enantiomers of aromatic, aliphatic and alicyclic β^3-amino acid on a CSP containing *(+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid* as a chiral selector,
- enantiomers of aliphatic and alicyclic β^2-amino acid on an endcapped CSP containing *(+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid* as a chiral selector.

Finally, our aim was to investigate gas phase chiral discrimination of enantiomers of aromatic β^3-amino acids using a kinetic method by mass spectrometry. Besides achieving chiral discrimination, the emphasis was to study the effect of the side chain of β^3-amino acids and reference α-amino acids on chiral discrimination.

3. EXPERIMENTAL

3.1. HPLC apparatus

The HPLC measurements were carried out on a Waters HPLC system consisting of a Waters 1525 binary gradient pump, a 2487 dual absorbance detector and a Breeze Chromatography Manager data system (Waters Chromatography, Milford,

MA, USA). The chromatographic system was equipped with Rheodyne Model 7125 injector (Cotati, CA, USA) with a 20-µL loop. The columns were thermostated in a water bath, with a cooling-heating thermostat (MK 70, Mechanik Prüfgerate, Medlingen, Germany).

3.1.1. Investigated analytes

- Aromatic *β*-lactams,
- tricyclic *β*-lactams,
- bicyclic *β*-amino acids,
- aromatic β^3-amino acids,
- aliphatic, alicyclic β^3-amino acids,
- aromatic, heterocyclic, aliphatic and alicyclic β^2-amino acids.

3.2. MS apparatus

The mass spectrometry measurements were performed using a quadrupole ion trap (QIT) mass spectrometer (Bruker Esquire 3000 plus, Bruker Daltonik GmbH, Bremen, Germany).

3.2.1. Investigated analytes

- aromatic β^3-amino acids

4. RESULTS

We developed methods for the separation of *β*-lactams, *β*-, β^2- és β^3-amino acid enantiomers by using chiral liquid chromatography, and we investigated the process of gas phase chiral discrimination of β^3-amino acid enantiomers by mass spectrometry.

1. Enantiomers of seven aromatic *β*-lactams were separated on CSPs containing macrocyclic glycopeptide antibiotic teicoplanin (Chirobiotic T) and teicoplanin aglycone (Chirobiotic TAG). Upon comparing the two columns, both the selectivity

factor and the resolution values were larger in the case of the Chirobiotic TAG. We have concluded that upon increasing the organic component, the retention of enantiomers decreased significantly in both stationary phases. The separation factor (α) changed on a slight maximum curve; the resolution (R_s) was the best in the case of MeOH. We also determined the elution sequence of the enantiomers, what consistently proved to be *S*<*R*.

2. The direct separation of tricyclic *β*-lactams and bicyclic *β*-amino acids enantiomers was achieved on five macrocyclic glycopeptide- and *β*-cyclodextrin-based column. The results achieved with the different methods (POM, PIM and reversed-phase mode), were compared in systematic chromatographic examinations. Among the macrocyclic glycopeptide selectors, the highest separation factor and resolution were observed in the case of Chirobiotic **TAG**. The $\Delta_{TAG\text{-}T}\Delta(\Delta G^o)$ value calculated from the α clearly showed that in the case of Chirobiotic **T** the carbohydrate moieties found on the selector influenced the chiral discrimination negatively. The Cyclobond **DMP** column indicated good separation efficiency both in reversed- and normal-phase mode. The elution sequence was determined in all cases and a general rule was established for the sequence of elution of the stereoisomers. We witnessed a change of elutional order with different stationary phases in the case of the three *β*-amino acids.

3. HPLC methods were developed for the separation of enantiomers of *β^2- and β^3*-amino acids on a chiral stationary phase containing *(+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid* as a chiral selector. The chromatographic retention and the resolution behavior of fifteen 3-aryl substituted *β^3*-amino acids were found to be dependent in different way on the mobile phase composition. An increase in the content of MeOH in the aqueous mobile phase increases the retention while the α and R_S decrease. The retention factors continuously decreased with increasing the concentration of acidic modifier in the aqueous mobile phase. This might be explained by the lower dissociation of the carboxylic group of amino acid and the crown ether and the effect of the counter ion of acid. The nature and position of the substituents had substantial effects on the retention, but the α changed to a much

lower extent than the retention. Higher k_1', α and R_S were observed for the analytes containing the F-, Cl-, Br- and CF_3- substituents in *para* positions on the aromatic ring comparing with *ortho* and *meta* substituted analogs. *Ortho* position of the substituents was unfavorable regarding the enantioseparation. The elution sequence was determined, what proved to be *R*<*S*.

4. Chiral separation of enantiomers of aliphatic and alicyclic β^3-amino acids was studied on a CSP also containing *(+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid* as a chiral selector. The chromatographic data of different mobile phase compositions revealed that an increase in the content of MeOH in the aqueous mobile phase increased the retention, the selectivity and the resolution. The nature of alcoholic modifier had substantial effects on the retention, but the selectivity changed to a much lower extent than the k' value. At constant mobile phase composition the longer aliphatic chain or branching structure of the substituents on the β-carbon atom resulted in lower retention, but the selectivity and the resolution did not decrease. The elution sequence was *S*<*R*.

5. HPLC methods were developed for the separation of enantiomers of aliphatic and alicyclic β^2-amino acids on an endcapped CSP containing *(+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid* as a chiral selector. The chromatographic data of the analytes revealed that elevation of the content of MeOH in the aqueous mobile phase increased the retention. The nature of acidic modifier influenced substantially only the non-chiral chromatographic process. The nature of the substituent on the α-carbon atom of the analyte exerted a substantial effect on the retention, but the selectivity changed to a much lower extent than the *k'* value. Different chromatographic behavior was observed for aliphatic β^2-amino acids comparing with aromatic ones using different mobile phase compositions. The analytes with aromatic side-chains exhibited better resolution on this type of selector than that for the analytes with aliphatic side-chains. The change of temperature exerted different effects on the β^2-homoamino acids with either aliphatic or aromatic side-chains (Table 2). The lowering of the temperature increased the retention factor in all cases, while the α values for the amino acids with aliphatic side-chains slightly decreased,

and those for the amino acids with aromatic side-chains increased with decreasing temperature. The R_S values in most cases increased with decreasing temperature.

6. Chiral discrimination of seven enantiomeric pairs of β^3-amino acids ($\mathbf{A}_{R\ or\ S}$) was studied by using the kinetic method and trimeric metal-bound complexes $[M^{II}(\mathbf{ref})_2(\mathbf{A}_{R\ or\ S})-H]^+$, with natural and unnatural α-amino acids as chiral reference (**ref**) compounds and divalent metal ions Cu^{II} and Ni^{II} as the center ions (M^{II}). The highest enantioselectivities were achieved for the analytes with benzyl side chain, β^3-amino acids. Benzyl side chain makes the analytes (enantiomers of interest) more flexible allowing more effective $\pi - \pi$ interactions between the analyte and reference molecules. The highest enantioselectivities were obtained using ***L*-Pro**, ***L*-Phg** and ***L*-4-OHPhg** as reference compounds, so the combination of relatively rigid reference compound and more flexible analyte generates the optimal environment for chiral discrimination. For all the analytes studied, interesting behavior was noticed, the Cu^{II} reduced to Cu^{I} when **L-Phg** and **L-4-OHPhg** were used as reference compounds and copper as the central metal ion.

5. LIST OF PUBLICATIONS

5.1. The thesis is based on the following publications

1. R. Berkecz, I. Ilisz, E. Forró, F. Fülöp, D.W. Armstrong, A. Péter, LC enantioseparation of aryl-substituted *β*-lactams using variable-temperature conditions Chromatographia 63 (**2006**) S29.

Impact factor: 1.171

2. R. Berkecz, R. Török, I. Ilisz, E. Forró, F. Fülöp, D.W. Armstrong, A. Péter LC enantioseparation of *β*-lactam and *β*-amino acid stereoisomers and a comparison of macrocyclic glycopeptide- and *β*-cyclodextrin-based columns, Chromatographia 63 (**2006**) S37.

Impact factor: 1.171

3. R. Berkecz, A. Sztojkov-Ivanov, I. Ilisz, E. Forró, F. Fülöp, M.H. Hyun, A. Péter, High-performance liquid chromatographic enantioseparation of *β*-amino acid stereoisomers on a (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based chiral stationary phase, J. Chromatogr. A 1125 (**2006**) 138.

Impact factor: 3.554

4. R. Berkecz, I. Ilisz, F. Fülöp, M.H. Hyun, A. Péter, High-performance liquid chromatographic enantioseparation of β^3--amino acid stereoisomers on a (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based chiral stationary phase, J. Chromatogr. A 1189 (**2008**) 285.

Impact factor: 3.756

5. R. Berkecz, I. Ilisz, Z. Pataj, F. Fülöp, H.J. Choi, M.H. Hyun, A. Péter LC enantioseparation of *β*-amino acids on a crown ether-based stationary phase, Chromatographia 68 (**2008**) S13.

Impact factor: 1.312

6. R. Berkecz, I. Ilisz, A. Misicka, D. Tymecka, F. Fülöp, H.J. Choi, M.H. Hyun, A. Péter HPLC enantioseparation of β^2-amino acids using crown ether-based stationary phase, J. Sep. Sci. 32 (**2009**) 981.

Impact factor: 2.746

7. **R. Berkecz**, A.R.M. Hyyrylainen, F. Fülöp, A. Péter, T. Janáky, P. Vainiotalo, J.M.H. Pakarinen, Chiral discrimination of β^3--amino acids using the kinetic method, J. Mass Spectrom. 45 (**2010**) 1312.

Impact factor$_{(2009)}$: 2.94

Cumulative impact factors: 16.650

5.2. Other publications

1. R. Török, **R. Berkecz**, A. Péter, High-performance liquid chromatographic enantioseparation of alpha-substituted glycine analogs on a quinine-based anion-exchanger chiral stationary phase under variable temperature conditions, J. Chromatogr. A 1120 (**2006**) 61.

Impact factor: 3.554

2. I. Ilisz, **R. Berkecz**, A. Péter, HPLC separation of amino acid enantiomers and small peptides on macrocyclic antibiotic-based chiral stationary phases: a review, J. Sep. Sci. 29 (**2006**) 1305.

Impact factor: 2.535

3. R. Török, **R. Berkecz,** A. Péter, Enantioseparation of phenylalanine analogs on a quinine-based anion-exchanger chiral stationary phase: structure and temperature effects, J. Sep. Sci. 29 (**2006**) 2523.

Impact factor: 2.535

4. I. Ilisz, **R. Berkecz**, A. Péter, Application of chiral derivatizing agents in the high-performance liquid chromatographic separation of amino acid enantiomers: a review, J. Pharm. Biomed. Anal. 47 (**2008**) 1.

Impact factor: 2.629

5. 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-amino acids, J. Sep. Sci. 31 (**2008**) 3688.

Impact factor: 2.746

6. R. Berkecz, I. Ilisz, G. Benedek, F. Fülöp, D.W. Armstrong, A. Péter, High-performance liquid chromatographic enantioseparation of 2-aminomono- and dihydroxycyclopentanecarboxylic and 2-aminodihydroxycyclohexanecarboxylic acids on macrocyclic glycopeptide-based phases, J. Chromatogr. A 1216 (**2009**) 927.

Impact factor: 3.756

7. I. Ilisz, **R. Berkecz**, E. Forró, F. Fülöp, D.W. Armstrong, A. Péter, The role of pi-acidic and pi-basic chiral stationary phases in the high-performance liquid chromatographic enantioseparation of unusual beta-amino acids, Chirality 21 (**2009**) 339.

Impact factor: 2.212

8. I. Ilisz, **R. Berkecz**, A. Péter, Retention mechanism of high-performance liquid chromatographic enantioseparation on macrocyclic glycopeptide-based chiral stationary phases, J. Chromatogr. A 1216 (**2009**) 1845.

Impact factor: 3.756

9. I. Ilisz,. G. Fodor, **R. Berkecz**, R. Iványi, L. Szente, A. Péter, Enantioseparation of beta-substituted tryptophan analogues with modified cyclodextrins by capillary zone electrophoresis, J. Chromatogr. A 1216 (**2009**) 3360.

Impact factor: 3.756

10. Z. Pataj, **R. Berkecz**, I. Ilisz, A. Misicka, D. Tymecka, F. Fülöp. W.D. Armstrong, A. Péter, High-performance liquid chromatographic chiral separation of β^2-amino acids, Chirality 21 (**2009**) 787.

Impact factor: 2.212

11. 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 beta-lactams, Chirality 22 (**2010**) 120.

Impact factor: 2.212

12. I. Ilisz, Z. Pataj, **R. Berkecz**, A. Misicka, D. Tymecka, F. Fülöp. J.H. Choi, M.H. Hyun, A. Péter, High-performance liquid chromatographic enantioseparation of beta(2)-amino acids using a long-tethered (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based chiral stationary phase, J. Chromatogr. A, 1217 (**2010**) 1075.

Impact factor: 3.756

13 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, J. Chromatogr. A, 1217 (**2010**) 2980.

Impact factor: 3.756
Cumulative impact factors: 39.415

5.3. Congress abstracts and posters

High-performance liquid chromatographic enantioseparation of aryl-substituted *β*-lactams
R. Berkecz, I. Ilisz, E. Forró, F. Fülöp, D.W. Armstrong, A. Péter
*6th Balaton Symposium on High-Performance Separation Methods, 7-9 September **2005**, Siófok, Hungary.*

High-performance liquid chromatographic enantioseparation of *β*-lactam and *β*–amino acid stereoisomers
R. Török, **R. Berkecz**, I. Ilisz, E. Forró, F. Fülöp, D.W. Armstrong, A. Péter
*6th Balaton Symposium on High-Performance Separation Methods, 7-9 September **2005**, Siófok, Hungary.*

High-performance liquid chromatographic enantioseparation of aryl-substituted *β*-lactams on macrocyclic glycopeptide antibiotic-based columns
R. Berkecz, I. Ilisz, E. Forró, F. Fülöp, D.W. Armstrong, A. Péter
*The 12th Symposium on Analytical and Environmental Problems, 26 September **2005**, Szeged, Hungary.*

High-performance liquid chromatographic enantioseparation of *β*-lactam and *β*–amino acid stereoisomers
R. Török, **R. Berkecz**, I. Ilisz, E. Forró, F. Fülöp, D.W. Armstrong, A. Péter
*The 12th Symposium on Analytical and Environmental Problems, 26 September **2005**, Szeged, Hungary.*

High performance liquid chromatographic enantioseparation of aryl substituted *β*-lactams
R. Berkecz, I. Ilisz, E. Forró, F. Fülöp, D.W. Armstrong, A. Péter
In Peptides 2006, Proceedings of the 29th European Peptide Symposium,
eds.: K. Rolka, P. Rekowski, J. Silberring,
*KENES International, **2007**, Geneva, Switzerland. 298*

High-performance liquid chromatographic enantioseparation of *β*^{3}-amino acid stereoisomers on a (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based chiral stationary phase
A. Péter, **R. Berkecz**, I. Ilisz, F. Fülöp, G. Hauspie, M.H. Hyun
*31st International Symposium on High Performance Liquid Phase Separations and Related Techniques HPLC **2007**, Ghent, Belgium.*

5.4. Lectures

High-performance liquid chromatographic enantioseparation of *β*-amino acid stereoisomers on a crown-ether based chiral stationary phase
Workshop on International Cooperation in Science & Technology between Flanders-Hungary, *20 october* ***2006,*** *Brussels, Belgium.*

Investigation of *β*-amino acid by mass spectrometry
A XXXIX. Kromatográfiás Továbbképző Tanfolyam, 28 January ***2008****, Szeged, Hungary.*

Investigation of β^3-amino acid by mass spectrometry
Ifjú Szerves Kémikusok Támogatásáért Alapítvány Előadóülése, ***2008****, Szeged, Hungary.*