Theses of the doctoral (Ph.D.) dissertation

ENANTIOSELECTIVE CHROMATOGRAPHIC ANALYSIS OF POTENTIAL PHARMACONS

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1. INTRODUCTION

These days we live in a world of rapidly developing instrumental analytical techniques, mainly driven by pharmaceutical industry. The number of published papers in the last decades proves the increasing demand for enantiomeric pure forms of compounds in the process of drug research and production. This is preeminently understandable considering the fact that each enantiomer has different biological effects when a chiral compound enters a chiral selective living organism. Thus, while one isomer may produce the desired therapeutic effect (eutomer), the other (distomer) may be inactive or in worse cases it may induce unwanted, even toxic changes in the human body. The guidelines of today's operative pharmacopoeias strictly regulate the chiral purity of active pharmaceutical ingredients, which require the introduction of novel and faster analytical methods with lower detection limits and operation costs. Controlling chiral purity is essential no matter how the enantiomeric pure compounds are produced.

In my dissertation the studied compounds have a significant role in the research field of pharmaceutical chemistry. Preceding the investigation of their pharmacological and biological effects, a possible strategy for the verification of their chiral purity is the application of direct chromatographic techniques using chiral stationary phases.

2. AIMS

The primary aim of my work was to perform an enantioselective liquid chromatographic analysis of potential pharmacons having a great importance in pharmaceutical chemistry, to optimize the separation of stereoisomers and to characterize the separation performance of newly developed chiral stationary phases from chromatographic aspects.

The compounds investigated were:

- enantiomers of carbocyclic *β*-amino acids possessing limonene skeleton on macrocyclic glycopeptide-based chiral stationary phases,
- enantiomers of *N*-substituted cyclic *β*-amino acids using *Cinchona* alkaloid- and sulfonic acid-based chiral zwitterionic stationary phases,
- enantiomers of limonene-based bicyclic 1,3-aminoalcohols and 1,3,5- and 1,3,6-aminodiols on polysaccharide-based chiral stationary phases.

Via the direct chiral liquid chromatographic analysis of the model compounds I intended to study what effects the nature and composition of the mobile phase and the nature of the polar modifier (alcohol) have on the chiral separation.

Another goal of my work was to investigate the effects of the structural features of the studied compounds and the applied selectors on the chiral discrimination by monitoring the changes of the chromatographic parameters.

An additional aim was to study the influence of the column temperature on the chiral separation and to determine the thermodynamic parameters which can be exploited to understand the possible separation mechanisms.

3. APPLIED SUBSTANCES AND EXPERIMENTAL METHODS

The investigated analytes were mainly synthetic compounds prepared by our cooperating partners. HPLC grade solvents and high purity water were used for the measurements.

The chromatographic characterization of the separation performance of three different types of chiral stationary phases was carried out by the application of:

- macrocyclic glycopeptide-based chiral stationary phases: *Chirobiotic™ R*, *T* and *TAG*,
- zwitterionic chiral stationary phases*: Chiralpak® ZWIX(+)* and *ZWIX(-)*,
- derivatized polysaccharide-based chiral stationary phases: *Lux Cellulose-1*, *Lux Cellulose-2*, *Lux Cellulose-3*, *Lux Cellulose-4*, *Lux i-Cellulose-5*, *Lux Amylose-1* and *Lux Amylose-2*.

Measurements were performed on four chromatographic systems.

The HPLC systems were:

- System I: Waters Breeze system consisting of a 1525 binary pump, a Waters 2487 dual-channel absorbance detector, a 717 Plus autosampler, a Waters column thermostat (Waters, Milford, MA, USA).
- System II: Waters chromatographic system consisting of a M-600 pump and a 2996 PDA detector (Waters, Milford, MA, USA) connected to a Jasco 2031 Plus refractive index (RI) detector (Jasco

Tokyo, Japan) and Rheodyne Model 7125 injector with 20 µl loop (Cotati, CA, USA).

- System III: The 1100 Series HPLC system from Agilent Technologies (Waldbronn, Germany) consisted of a solvent degasser, a pump, an autosampler, a column thermostat, a multiwavelength UV-Vis detector and a corona-charged aerosol detector from ESA Biosciences, Inc., (Chelmsford, MA, USA).

For SFC measurements a Waters Acquity Ultra Performance Convergence Chromatography™ system was applied (UPC^2, Waters Chromatography, Milford, MA, USA) containing a binary solvent delivery pump, an autosampler, a backpressure regulator, a column oven and a PDA detector (Waters, Milford, MA, USA).

Data acquisition and analyses were carried out with Empower 2 chromatographic data software (Waters, Milford, MA, USA) in the cases of HPLC systems I-II and the Waters UPC^2 SFC system. For HPLC system III ChemStation software was applied (Agilent Technologies).

4. NEW SCIENTIFIC RESULTS

During my work I performed the enantioselective chromatographic separation of compounds bearing pharmaceutical importance, namely **limonene-based carbocyclic *β*-amino acids, *N*-substituted cyclic *β*-amino acids and limonene-based bicyclic 1,3-aminoalcohols and 1,3,5- and 1,3,6-aminodiols** using chiral stationary phases. As a result of the optimization of the separation of stereoisomers and the investigation of the chemical structure related chiral recognition, I achieved the following new scientific results:

T1. I determined the optimal mobile phase composition for the high-performance separation of the investigated analytes.

For the separation of limonene-based carbocyclic *β*-amino acids on macrocyclic glycopeptide stationary phases it was established that the retention increased with the increasing concentration of water which can be explained by enhanced hydrophobic interactions inside the "basket" of the selector. In MeOH-rich mobile phase the values of the retention factor increased due to the formation of the ionic and dipolar interactions between the studied analytes and applied selectors.

In the case of the separation of *N*-substituted cyclic *β*-amino acids on zwitterionic stationary phases an increase in MeCN content in the mobile phase was found to result in increased retention factors in most cases. The observed chromatographic behavior can be explained by the decreased solvation shell of the ionized compounds which enforces the electrostatic attraction due to the reduced distances of the involved charged sites.

Investigating the enantioseparation of limonene-based bicyclic aminoalcohols and aminodiols accomplished by normal-phase HPLC (NP-LC) and

supercritical fluid chromatography (SFC) on polysaccharide-based chiral stationary phases, an increased content of apolar *n*-hexane or CO_2 in the mobile phase resulted in an increased retention that can be explained by the formation of a typical normal phase chromatographic behavior. According to my results, increasing the carbon chain length of applied alcohol generally resulted in an increase in retention with only a minor effect on enantiodiscrimination. This observed chromatographic behavior proved that the solvation of the limonene analogs reduced in these solvents and the studied compounds stayed in the stationary phase rather than in the mobile phase.

T2. Applying the stoichiometric displacement model I established the role of ionic interactions in the separation mechanism of the studied compounds, furthermore, I demonstrated the applicability of the model on the characterization of the retention behavior based on the ion-pairing process in the cases of macrocyclic glycopeptide-based and zwitterionic chiral stationary phases.

I was first to prove that ion-interactions mechanism is operative in chiral discrimination using macrocyclic glycopeptide-based chiral stationary phases. The obtained slopes of $lg\ k_1$ vs. $lg\ c$ plots (with an absolute value of 0.05-0.26 on *Chirobiotic™ TAG* column) showed that teicoplanin aglycon exhibits a "zwitterionic character" in the course of enantioseparation of limonene-based carbocyclic *β*-amino acids.

My results based on the enantioseparation of *N*-substituted amino acids highlighted the outstanding characteristic of zwitterionic columns that by changing the respective counter-ion concentration, the retention can be conveniently regulated without significantly altering selectivity values. The

determined slopes of $lg\ k_1$ vs. $lg\ c$ plots ranged between 0.12-0.41 on *ZWIX(+)* and 0.18-0.33 on *ZWIX(-)* columns, respectively, which indicated the existence of two pairs of electrostatic interaction mechanisms between the zwitterionic selector and the investigated ionizable compounds.

T3. I revealed characteristic structure-retention relationships. My results showed that both the structure of studied compounds and applied selectors had a significant effect on chiral recognition.

The calculated $\Delta(\Delta G°)_{TAG}–\Delta(\Delta G°)_T$ values revealed that in the presence of sugar moieties on the teicoplanin selector, the enantiorecognition of limonene-based carbocyclic *β*-amino acids is sterically hindered. Comparing the separation performances of the investigated macrocyclic glycopeptide chiral stationary phases, the teicoplanin aglycon selector appeared more suitable for the enantioseparation of the investigated compounds.

It was proved that with an increase in the degree of substitution of the amino group of *N*-substituted cyclic *β*-amino acids the retention decreased. The highest enantioselectivity values were observed for the amidinated amino acids, which can be explained by an additional hydrogen bonding increment on top of the electrostatic interactions. Comparing the two zwitterionic selectors, the *ZWIX(-)* column was more selective for the separation of the studied analytes, however, for the *N*-amidino compounds the *ZWIX(+)* afforded more effective separations.

On the basis of my results, it can be stated that the extra OH-group on the aminodiol compounds contributed to stronger H-bond interactions with the selector which resulted in higher retention and selectivity. Comparing the chromatographic behavior of methylbenzyl- and dibenzyl-substitution on the

N-atom a higher retention and selectivity was observed for dibenzyl derivatives due to the enhanced π-π interactions with the selector.

T4. I studied the effect of column temperature on the chiral separation. It was established that the temperature had a complex influence on the chiral separations, changing in different direction the tendencies of chromatographic parameters (retention and selectivity).

Studying the effects of column temperature on the chiral separations my results demonstrated that the values of the retention factor, selectivity and resolution generally decreased with increasing temperature. In the case of limonene-based carbocyclic *β*-amino acid compounds I observed a chromatographic behavior different from the so-called thermodynamic effect most often experienced; decreasing retention with increasing column temperature was accompanied with higher enantioselectivity and in many cases the resolution also improved. Under SFC conditions, the resolution of the separation of bicyclic aminoalcohol and aminodiol analoges improved in many cases with increasing temperature which can be explained by an increase of the kinetic efficiency of the applied column. Chromatographic data were utilized to construct *van't Hoff* plots and thermodynamic parameters were calculated. According to my results the enantioselective discrimination of *N*-substituted cyclic *β*-amino acids and limonene-based bicyclic aminoalcohols and aminodiols was in most cases enthalpically-driven, while the chiral recognition of limonene-based carbocyclic *β*-amino acids was mostly enthalpically controlled.

T5. The elution sequences preeminently important for the individual identification of enantiomer pairs were determined in most cases.

I established that neither the configuration of the carbon atom attached to the carboxyl group nor the configuration of the carbon atom attached to the amino or 2-propyl group determined the elution sequence of limonene-based carbocyclic *β*-amino acids. Using the pseudo-enantiomeric quinine- and quinidine-based chiral stationary phases I demonstrated that the enantiomers of *N*-substituted cyclic *β*-amino compounds can be made to elute in reversed order upon changing the applied zwitterionic columns without the need for time-consuming development of new chromatographic methods. In SFC mainly the structure of the polysaccharide backbone (cellulose or amylose) affected the elution sequence of limonene-based bicyclic aminoalcohols and aminodiols, while in NP-LC both the polysaccharide backbone and the structure of the selector did so.

5. LIST OF PUBLICATIONS

My ID in the Hungarian Collection of Scientific Publications (MTMT) is 10055660.

Journal publications defining the basis of the dissertation:

1. **T. Orosz,** N. Grecsó, G. Lajkó, Z. Szakonyi, F. Fülöp, D. W. Armstrong, I. Ilisz, A. Péter
Liquid chromatographic enantioseparation of carbocyclic *β*-aminoacids possessing limonene skeleton on macrocyclic glycopeptide-based chiral stationary phases
Journal of Pharmaceutical and Biomedical Analysis, 145, 119-126, 2017.
IF: 2.831

2. **T. Orosz,** E. Forró, F. Fülöp, W. Lindner, I. Ilisz, A. Péter
Effects of *N*-methylation and amidination of cyclic *β*-amino acids onenantioselectivity and retention characteristics using *Cinchona* alkaloid- and sulfonic acid-based chiral zwitterionic stationaryphases
Journal of Chromatography A, 1535, 72-79, 2018.
IF: 3.858

3. **T. Orosz,** A. Bajtai, T. Minh Le, D. Tanács, Z. Szakonyi, F. Fülöp, A. Péter, I. Ilisz
Chiral high-performance liquid and supercritical fluid chromatographic enantioseparations of limonene-based bicyclic aminoalcohols and aminodiols on polysaccharide-based chiral stationary phases
Biomedical Chromatography, 33, (5), e4517, 2019.
IF: 1.748

Σ IF: 8.437

Additional journal publications also related to the topic of dissertation:

1. G. Lajkó, **T. Orosz**, L. Kiss, E. Forró, F. Fülöp, A. Péter, I. Ilisz
High-performance liquid chromatographic enantioseparation of fluorinated cyclic β^3-amino acid derivatives on polysaccharide-based chiral stationary phases. Comparison with nonfluorinated counterparts
Biomedical Chromatography, 30, 1441-1448, 2016.

IF: 1.613

2. G. Lajkó, **T. Orosz**, N. Grecsó, M. Palkó, F. Fülöp, A. Péter, I. Ilisz
High-performance liquid chromatographic enantioseparation of cyclic *β*-aminohydroxamic acids on zwitterionic chiral stationary phases based on *Cinchona* alkaloids
Analytica Chimica Acta, 921, 84-94, 2016.

IF: 4.950

3. G. Lajkó, **T. Orosz**, I. Ugrai, Z. Szakonyi, F. Fülöp, W. Lindner, A. Péter, I. Ilisz
Liquid chromatographic enantioseparation of limonene-based carbocyclic *β*-amino acids on zwitterionic *Cinchona* alkaloid-based chiral stationary phases
Journal of Separation Science, 40, 3196-3204, 2017.

IF: 2.415

4. D. Tanács, **T. Orosz**, Z. Szakonyi, T. Minh Le, F. Fülöp, A. Péter
High-performance liquid chromatographic enantioseparation of isopulegol-based *β*-amino lactone and *β*-amino amide analogs on polysaccharide-based chiral stationary phases focusing on the change of the enantiomer elution order
Journal of Chromatography A, https://doi.org/10.1016/j.chroma.2020.461054

IF: 3.858

Σ IF: 12.836

ΣΣ IF: 21.273

Book chapter:

1. I. Ilisz, **T. Orosz**, A. Péter
High-performance liquid chromatography enantioseparations using macrocyclic glycopeptide-based chiral stationary phases: An overview
Chiral Separations, Methods and Protocols, 3rd Edition, 201-237, 2019.
Szerkesztő: Gerhard K. E. Scriba, Könyvfejezet

Poster presentations related to the topic of dissertation:

1. **T. Orosz,** G. Németi, A. Bajtai, Z. Szakonyi, F. Fülöp, I. Ilisz, A. Péter
Chiral high-performance liquid and supercritical fluid chromatographic enantioseparations of limonene-based bicyclic aminoalcohols and aminodiols
24th International Symposium on Analytical and Environmental Problems, Szeged, Hungary, 2018.

2. **T. Orosz**, G. Németi, A. Bajtai, Zs. Szakonyi, F. Fülöp, I. Ilisz, A. Péter
Új, limonén alapú biciklusos aminoalkoholok és aminodiolok elválasztása királis folyadékkromatográfiával és szuperkritikus fluid kromatográfiával
Elválasztástudományi Vándorgyűlés, Tapolca, Hungary, 2018.

3. **T. Orosz**, A. Péter, F. Fülöp, W. Lindner, I. Ilisz
Effects of *N*-substitution on the elution order of cyclic β^3-amino acid enantiomers on *Cinchona* alkaloid-based zwitterionic and anion exchanger type chiral stationary phases
45th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Prague, Czech Republic, 2017.

4. **T. Orosz**, A. Péter, F. Fülöp, W. Lindner, I. Ilisz
Study of the effects of structural characteristics on the elution order of cyclic β3-amino acid enantiomers on anion exchanger-type chiral stationary phases by high-performance liquid chromatography
11th Balaton Symposium on High-Performance Separation Methods, Siófok, Hungary, 2017.

5. G. Lajkó, **T. Orosz**, Z. Szakonyi, F. Fülöp, W. Lindner, A. Péter, I. Ilisz
Liquid chromatographic enantioseparation of limonene-based carbocyclic *β*3-amino acids on Cinchona alkaloid-based chiral stationary phases
11th Balaton Symposium on High-Performance Separation Methods, Siófok, Hungary, 2017.

6. **T. Orosz,** G. Lajkó, N. Grecsó, A. Péter, I. Ilisz
High-performance liquid chromatographic study on the enantioseparation of fluorine containing cyclic amino acid derivatives
Proccedings of the 22nd International Symposium on Analytical and Environmental Problems, Szeged, Hungary, 2016.

7. G. Lajkó, **T. Orosz**, N. Grecsó, M. Palkó, F. Fülöp, W. Lindner, A. Péter, I. Ilisz
Enantioseparation of cyclic *β*-aminohydroxamic acids by high-performance liquid chromatography on zwitterionic chiral stationary phases based on *Cinchona* alkaloids
Advances in Chromatography and Electrophoresis & Chiranal, Olomouc, Czech Republic, 2016.

8. G. Lajkó, **T. Orosz**, N. Grecsó, M. Palkó, F. Fülöp, W. Lindner, A. Péter, I. Ilisz
Ciklikus *β*-aminohidroxámsavak enantiomerjeinek nagyhatékonyságú folyadékkromatográfiás elválasztása kinaalkaloid alapú ikerionos állófázisokon
Elválasztástudományi Vándorgyűlés, Kecskemét, , Hungary, 2016.

9. N. Grecsó, G. Lajkó, **T. Orosz,** L. Schönstein, F. Fülöp, A. Péter, I. Ilisz
Enantioseparation of amino alcohol analogs possessing 1,2,3,4-tetrahydroisoquinoline Skeleton and its derivatives using polysaccharide-based chiral stationary phases
Proceedings of the 21st International Symposium on Analytical and Environmental Problems, Szeged, Hungary, 2015.

Oral presentations related to the topic of dissertation:

1. **T. Orosz,** A. Bajtai, D. Tanács, Z. Szakonyi, F. Fülöp, A. Péter, I. Ilisz
Chiral separation of limonene-based bicyclic aminoalcohols and aminodiols on polysaccharide-based chiral stationary phases
1st Hungarian-Polish Interdisciplinary Scientific Symposium, Szeged, Hungary, 2019.

2. A. Bajtai, D. Tanács, **T. Orosz**, G. Lajkó, I. Szatmári, F. Fülöp, W. Lindner, I. Ilisz, A. Péter
Enantiomer separation of chiral tetrahydroisoquinoline analogs by supercritical fluid chromatography and high-performance liquid chromatography
24th International Symposium on Analytical and Environmental Problems, Szeged, Hungary, 2018.

3. **T. Orosz**, E. Forró, F. Fülöp, L. Wolfgang, G. Lajkó, A. Bajtai, A. Péter, I. Ilisz
Enantioseparation of cyclic *β*-amino acids on ion-exchanger-based chiral stationary phases
Proceedings of the 23rd International Symposium on Analytical and Environmental Problems, Szeged, Hungary, 2017.

4. I. Ilisz, A. Péter, **T. Orosz**, A. Bajtai, G. Lajkó
Enantiomeric separations by ionexchanger-based chiral stationary phases
11th Balaton Symposium on High-Performance Separation Methods, Siófok, Hungary, 2017.

5. I. Ilisz, **T. Orosz**, A. Péter, N. Grecsó, G. Lajkó, F. Fülöp
Exploring the applicability of polysaccharide-based chiral stationary phases for the enantioseparations of compounds of pharmacological interest in high-performance liquid chromatography
Advances in Chromatography and Electrophoresis & Chiranal, Olomouc, Czech Republic, 2016.

6. I. Ilisz, **T. Orosz,** N. Grecsó, G. Lajkó, F. Fülöp, W. Lindner, A. Péter
Új típusú ikerionos királis állófázisok alkalmazása a nagyhatékonyságú folyadékkromatográfiában
Elválasztástudományi Vándorgyűlés, Kecskemét, Hungary, 2016.

7. A. Péter, I. Ilisz, G. Lajkó, **T. Orosz**, F. Fülöp
Királis kromatográfia poliszacharid-alapú állófázisokon
Elválasztástudományi Vándorgyűlés, Kecskemét, Hungary, 2016.