Theses of the doctoral (Ph.D.) dissertation

ANALYSIS OF THE INTERACTIONS OF *CINCHONA* AND POLYSACCHARIDE-BASED CHIRAL STATIONARY PHASES

Attila Bajtai

Supervisor:
Prof. Dr. István Ilisz

University of Szeged
Faculty of Pharmacy
Institute of Pharmaceutical Analysis
Doctoral School of Chemistry

2021

1. INTRODUCTION

Understanding and describing how living systems work is a natural human need which dates back millennia. Part of this is the deeper understanding of diseases that limit the quality and content of human life. Reducing the symptoms of pathological conditions and combating them is one of the most important motivations for drug research. In addition, drug research is a value-creating process that is also a path to scientific cognition. A high level of integration of the constantly evolving knowledge of various fields of research, such as biology, chemistry and medicine, is essential for its success. Thus, drug development is based on the harmonised joint efforts of several researchers. Analytical chemistry is a part of this process, its results are crucial, from exploratory research through pre- and clinical development all the way to registration. Since the molecules that make up living organisms form a chiral system, such as amino acids, proteins, sugars, enzymes, polynucleotides, thus special attention should be paid to similar chiral components which also interact with them.

Thorough investigation and regulation of the amount of chiral components in the final product - in accordance with the regulatory requirements, which are becoming stricter every year - is crucial during drug development. What is more, in the case of chiral molecules, it is of paramount importance that both the raw materials and the intermediates in the production are of sufficient purity and this should be supported by continuous monitoring. This expectation which dates back more than 50 years has encouraged generations of researchers to develop the most efficient methods possible, leading to the widespread adoption of chiral chromatographic techniques.

Besides pharmaceutical industry, several other areas affecting the quality of human life, such as food industry, cosmetics industry or plant protection, also use the results of chiral chromatography, thus it is important for future perspectives to be built on solid foundations.

2. AIMS

During my work my aim was to develop liquid chromatographic methods in order to separate stereoisomers of biologically and pharmaceutically important compounds, and to study the separation performance of chiral columns with diverse operating principles by separating molecules of various structures.

I intended to investigate:

- the separation of biologically active dipeptides on *cinchona* alkaloid-based stationary phases using high-performance liquid chromatography (HPLC),
- basic and amphoteric indole analogues separation on *cinchona* alkaloid-based zwitterionic and on derivatized polysaccharide-based stationary phases with the use of HPLC and supercritical fluid chromatography (SFC) methods,
- the separation of 1,2,3,4-tetrahydroquinoline-based and 1-naphthol compounds and their structural analogues on derivatized polysaccharide-based stationary phases with the use of HPLC and SFC methods.

I have aimed to interpret the impact of the eluent composition and the quality and quantity of the polar modifier (alcohol) on the separation through the determination of the investigated compounds' chromatographic parameters. I intended to draw conclusions from the interactions between the studied compound and the chiral selector by systematically changing the structure of the investigated compounds. For the ion-exchanger stationary phases I wished to explore the effect of counter-ion concentration on the separation by changing the mobile phase's acid and base content. In addition, my goal was to gain a deeper understanding of the thermodynamic background of the separation mechanism by examining the impact of temperature on the chromatographic parameters.

3. EXPERIMENTAL SUBSTANCES AND METHODS

The investigated analytes have biological importance and they were prepared in the laboratories of our cooperating partners. I used HPLC grade solvents during my work.

Chromatographic separations were performed with two different stationary phase families.

Chiralpak QN-AX, *Chiralpak QD-AX*, *Chiralpak ZWIX(+)* and *Chiralpak ZWIX(-)* columns, distributed by Chiral Technologies Europe (CTE, Illkirch, France), were used as a *cinchona* alkaloid-based chiral ion-exchanger stationary phase. The physical properties of the columns are identical: 150 mm × 3,0 mm, the particle size is 3 μm.

Among the derivatized polysaccharide-based chiral stationary phases distributed by CTE, I have studied the *Chiralpak IA, Chiralpak IB, Chiralpak IC, Chiralpak ID, Chiralpak IE, Chiralpak IF* and *Chiralpak IG* columns. These columns have identical physical properties too: 250 mm × 4,6 mm, the particle size is 5 μm.

I achieved my research results using three chromatographic systems:

The I. chromatographic system was an 1100 Series HPLC system from Agilent Technologies (Waldbronn, Germany), which 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). Data acquisition and analysis were carried out with Chemstation chromatographic data software.

The II. chromatographic system consisted of a Waters 1525 binary pump, a Waters column thermostat, a Waters 2487 dual-channel absorbance detector, a Waters 717 plus autosampler and the Empower 2 chromatographic data software (Waters, Milford, MA, USA).

The III. system was a Waters Acquity UPC^2 chromatographic system (Waters Corporation, Milford, MA, USA) with which the SFC measurements were carried out. This system is constituted by a solvent degasser, a pump,

an autosampler, a column thermostat, a photodiode array detector, a backpressure regulator and the Empower 2 chromatographic data software.

I used either the aforementioned chromatographic thermostats or an Alpha RA 8 (Lauda, Germany) liquid thermostat for the measurements of the temperature dependence.

4. NEW SCIENTIFIC RESULTS

T1. I experimentally demonstrated that using HPLC and SFC techniques with polysaccharide-based stationary phases, the reduction of the mobile phase's alcohol content caused an increase in retention for the studied compounds in all cases, but the enantioselectivity varied in several cases.

Using the derivatized polysaccharide-based stationary phases under normal-phase and subcritical conditions, I investigated the effect of the amount of alcohol contained within the eluent on the separation. I observed that the mobile phase, which became more apolar in parallel with the reduction of the alcohol content of the eluent, increased the retention of the components in both HPLC and SFC techniques. In contrast, the selectivity showed little change or increase in case of the HPLC technique, while under SFC conditions, selectivity can typically be described by a small change or maximum curve. This chromatographic behaviour can be explained by the formation of different spatial structures of the selector under the influence of the mobile phases.

T2. In the case of both HPLC and SFC techniques, I proved that the retention of ampholytic compounds is less dependent on the amount of modifiers added when using zwitterionic stationary phases compared to basic compounds.

Upon separation of the test compounds with *cinchona* alkaloid-based ion-exchange stationary phases, the amount of ions in the chromatographic system determined the retention of the enantiomers. By varying the concentrations of acid and base modifiers, I established that regardless of the structure of the investigated components, when using HPLC and SFC techniques, the stoichiometric displacement model can be used to describe the chromatographic behaviour of the compounds. Based on the model, examining basic and ampholytic compounds with mono- and zwitterionic stationary phases, I found that the double ion pairing interaction between the ampholytic compounds and the zwitterionic selector is responsible for the more stable binding.

T3. I have demonstrated that *cinchona* alkaloid-based columns can be used effectively to separate not only enantiomers but also diastereomeric compounds.

I successfully separated several ampholytic dipeptide enantiomers with various compositions and structures by using a polar-ionic mobile phase and a *cinchona* alkaloid-based column with the HPLC method. Based on the results obtained in this way, I concluded that the aromatic side chains near the terminus of the dipeptides, with an H-bridge, by forming π-π interactions or by using steric effects, play a decisive role in the separation. In addition, the flexibility of dipeptides with a small aliphatic side chain facilitates attachment to the selector due to their structure. In view of these interactions, I have succeeded in separating diastereomers of dipeptide compounds, which involves a considerable expansion of the use of zwitterionic stationary phases.

T4. Based on the analysis of the HPLC and SFC techniques, I found that the structure of the polysaccharide backbone and the quality and spatial position of the related modifiers have a decisive influence on the process of chiral recognition.

The polar ampholytic indole compounds investigated on polysaccharide-based stationary phases had no retention under normal-phase HPLC conditions, but could be successfully separated under subcritical conditions. Thus, during the use of the two techniques, the interactions responsible for chiral recognition differed depending on the chromatographic conditions. One of the reasons for the different chromatographic behaviour of the SFC method may be the *in situ* formation of alkyl carbonic acid in the eluent or the presumed alcohol accumulation and polarity change on the surface of the selector. Overall, the use of SFC methods resulted more often in successful separations than the HPLC methods, however, in many cases baseline separation could be also achieved using HPLC techniques.

T5. By applying SFC technique with polysaccharide-based stationary phases I found that temperature change can have the opposite effect on selectivity depending on the quality of the modifiers connected to the selector.

As a result of increased temperature, the retention of 1,2,3,4-tetrahydroisoquinoline derivatives decreased in all cases using polysaccharide-based stationary phases under subcritical conditions. By using the *Chiralpak IA* column containing an amylose-based *tris*(3,5-dimethylphenylcarbamate) modifier, I observed that the selectivity changed in contrast with increasing temperature. By contrast, using the *Chiralpak IE* column containing a *tris*(3,5-dichlorophenylcarbamate) modifier, based on amylose, the selectivity changed in parallel with temperature. Calculating the apparent thermodynamic values, I found that the separation of the tested compounds was enthalpy-driven for the *Chiralpak IA* column and entropy-controlled for the *Chiralpak IE* column. Thus, I have shown that by replacing the methyl groups on the polysaccharide-based selector with chlorine, the energetic processes of chiral separation can be greatly altered.

5. LIST OF PUBLICATIONS

My ID in the Hungarian Collection of Scientific Publications (MTMT) is 10058359.

Journal publications defining the basis of the dissertation

1. **A. Bajtai**, Gy. Lajkó, I. Szatmári, F. Fülöp, W. Lindner, I. Ilisz, A. Péter
   Dedicated comparisons of diverse polysaccharide- and zwitterionic Cinchona alkaloid-based chiral stationary phases probed with basic and ampholytic indole analogs in liquid and subcritical fluid chromatography mode
   Journal of Chromatography A, 1563, (**2018**) 180-190
   Impact factor$_{(2018)}$: 3,858 (Journal ranking: Q1)

2. **A. Bajtai**, Gy. Lajkó, G. Németi, I. Szatmári, F. Fülöp, A. Péter, I. Ilisz
   High-performance liquid chromatographic and subcritical fluid chromatographic separation of α-arylated ß-carboline, N-alkylated tetrahydroisoquinolines and their bioisosteres on polysaccharide-based chiral stationary phases
   Journal of Separation Science, 42, (**2019**) 2779-2787
   Impact factor$_{(2019)}$: 2,878 (Journal ranking: Q2)

3. **A. Bajtai**, I. Ilisz, D.H.O. Howan, G. K. Tóth, G.K.E. Scriba, W. Lindner, A. Péter
   Enantioselective resolution of biologically active dipeptide analogs by high-performance liquid chromatography applying Cinchona alkaloid-based ion-exchanger chiral stationary phases
   Journal of Chromatography A, 1611, (**2020**) 1-12
   Impact factor$_{(2019)}$: 4,049 (Journal ranking: Q1)

Sum of impact factor: **10,785**

Additional journal publications also related to the topic of dissertation

1. **A. Bajtai**, B. Fekete, M. Palkó, F. Fülöp, W. Lindner, M. Kohout, I. Ilisz, A. Péter
   Comparative study on the liquid chromatographic enantioseparation of cyclic β-amino acids and the related cyclic β-aminohydroxamic acids on Cinchona alkaloid-based zwitterionic chiral stationary phases
   Journal of Separation Science, 41, (**2017**) 1216-1223
   Impact factor$_{(2017)}$: 2,415 (Journal ranking: Q2)

2. I. Ilisz, **A. Bajtai**, W. Lindner, A. Péter
   Liquid chromatographic enantiomer separations applying chiral ion-exchangers based on Cinchona alkaloids
   Journal of Pharmaceutical and Biomedical Analysis, 159, (**2018**) 127-152
   Impact factor$_{(2018)}$: 2,983 (Journal ranking: Q1)

3. T. Orosz, **A. Bajtai,** T.M. Le, D. Tanács, Zs. 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, 43, (**2019**) 1-11
   Impact factor$_{(2019)}$: 1,728 (Journal ranking: Q2)

4. I. Ilisz, **A. Bajtai**, A. Péter, W. Lindner
   Chiral Separations: Cinchona Alkaloid-Based Zwitterionic Chiral Stationary Phases Applied for Liquid Chromatographic Enantiomer Separations: An Overview
   Chiral Separations-Methods and Protocols
   Editor: G.K.E. Scriba, **2019**, Humana Press, New York, NY, USA, book chapter

5. I. Ilisz, **A. Bajtai**, I. Szatmári, F. Fülöp, W. Lindner, A. Péter
   Enantioseparation of ß-carboline, tetrahydroisoquinoline and benzazepine analogues of pharmaceutical importance: Utilization of chiral stationary phases based on polysaccharides and sulfonic acid modified Cinchona alkaloids in high-performance liquid and subcritical fluid chromatography
   Journal of Chromatography A, 1615, (**2020**) 1-10
   Impact factor$_{(2019)}$: 4,049 (Journal ranking: Q1)

6. **A. Bajtai**, I. Ilisz, A. Péter, W. Lindner
   Liquid chromatographic resolution of natural and racemic Cinchona alkaloid analogues using strong cation- and zwitterion ion-exchange type stationary phases. Qualitative evaluation of stationary phase characteristics and mobile phase effects on stereoselectivity and retention
   Journal of Chromatography A, 1609, (**2020**) 1-13
   Impact factor$_{(2019)}$: 4,049 (Journal ranking: Q1)

7. **A. Bajtai**, I. Ilisz, R. Berkecz, F. Fülöp, W. Lindner, A. Péter
   Polysaccharide-based chiral stationary phases as efficient tools for diastereo- and enantioseparation of natural and synthetic Cinchona alkaloid analogs
   Journal of Pharmaceutical and Biomedical Analysis, 193, (**2021**) 113724
   Impakt faktor$_{(2019)}$: 3,209 (Journal ranking: Q1)

Sum of all scientific article impact factor: **29,218**

Oral presentations related to the topic of dissertation

1. I. Ilisz, A. Péter, T. Orosz, **A. Bajtai**, Gy. Lajkó
   Enantiomeric Separations by Ion Exchanger-Based Chiral Stationary Phases
   11th Balaton Symposiumon on High-Performance Separation Methods
   2017. September 6-8. Siófok, Hungary.

2. T. Orosz, E. Forró, F. Fülöp, W. Lindner, Gy. Lajkó, **A. Bajtai**, A. Péter, I. Ilisz
   Enantioseparation of cyclic β-amino acids on ion-exchanger-based chiral stationary phases
   23rd International Symposium on Analytical and Environmental Problems
   2017. October 9-10. Szeged, Hungary.

3. **A. Bajtai**, Gy. Lajkó, I. Szatmári, F. Fülöp, W. Lindner, I. Ilisz, A. Péter
   Comparative study for the characterization of enantiorecognitions obtained by supercritical fluid chromatography and high-performance liquid chromatography
   Applications of Supercritical Fluids
   2018. May 17. Budapest, Hungary.

4. **A. Bajtai**, D. Tanács, T. Orosz, Gy. Lajkó, I. Szatmári, F. Fülöp, W. Lindner, I. Ilisz, A. Péter
   Enantiomer separation of chiral tetrahydroisoquinoline analogs by supercritical fluid chromatography and high-performance liquide chromatography
   24th International Symposium on Analytical and Environmental Problems
   2018. October 9. Szeged, Hungary.

5. I. Ilisz, **A. Bajtai**, A. Péter
   Királis szelektorok alkalmazási lehetőségei enantiomerek elválasztására a nagyhatékonyságú folyadékkromatográfiában
   Elválasztástudományi vándorgyűlés 2018
   2018. November 8-10. Tapolca, Hungary.

6. **A. Bajtai**, D. Tanács, E. Forró, F. Fülöp, W. Lindner, A. Péter, I. Ilisz
   High-performance liquid chromatographic enantioseparation of some amino compounds with pharmaceutical relevance on ion-exchanger-based chiral stationary phases

26th International Symposium on Analytical and Environmental Problems
2020. november 23. Szeged, Magyarország.

Poster presentations related to the topic of dissertation

1. I. Ilisz, **A. Bajtai**, Zs. Szakonyi, F. Fülöp, D.W. Armstrong, A. Péter
   HPLC enantioseparation of carbocyclic β-amino acids possessing limonene skeleton on macrocyclic glycopeptide-based chiral stationary phases
   45th International Symposium on High-Performance Liquid Phase Separations and Related Techniques
   2017. June 18-22. Prague, Czech Republic.

2. **A. Bajtai**, B. Fekete, M. Palkó, F. Fülöp, W. Lindner, A. Péter, I. Ilisz
   Application of Cinchona alkaloid-based chiral zwitterionic stationary phases for the enantioseparation of cyclic β-aminohydroxamic acids
   11th Balaton Symposium on High-Performance Separation Methods
   2017. September 6-8. Siófok, Hungary.

3. T. Orosz, G. Németi, **A. Bajtai**, Zs. Szakonyi, F. Fülöp, I. Ilisz, A. Péter
   Chiral high-performance liquide and supercritical fluid chromatographic enantioseparations of limonene-based vicyclic aminoalcohols and aminodiols on polysaccharide chiral stationary phases
   24th International Symposium on Analytical and Environmental Problems
   2018. October 8-9. Szeged, Hungary.

4. **A. Bajtai**, Gy. Lajkó, D. Tanács, I. Szatmári, F. Fülöp, W. Lindner, I. Ilisz, A. Péter Királis elválasztási mechanizmusok vizsgálata poliszacharid és ikerionos állófázisokon **Elválasztástudományi vándorgyűlés 2018**
   2018. november 8-10. Tapolca, Hungary.

5. T. Orosz, **A. Bajtai**, G. Németi, Zs. Szakonyi, F. Fülöp, I. Ilisz, A. Péter
   Új, limonén alapú biciklusos aminoalkoholok és aminodiolok elválasztása királis folyadékkromatográfiával és szuperkritikus fluid kromatográfiával **Elválasztástudományi vándorgyűlés 2018**
   2018. November 8-10. Tapolca, Hungary.

6. I. Ilisz, **A. Bajtai**, I. Szatmári, F. Fülöp, W. Lindner, A. Péter
   Chiral high-performance liquid and subctirical fluid chromatography study of some pharmaceutically important β-carboline tetrahydroisoquinoline and benzazepine analogs **48th International Symposium on High-Performance Liquid Phase Separations and Related Techniques**
   2019. június 16-20. Milan, Italy.

7. A. Péter, **A. Bajtai**, G. Tóth, W. Lindner, G.K.E. Scriba, I. Ilisz
   Comparative study of enantioseparations of natural and unnatural dipeptides on Cinchona alkaloid based chiral stationary phases
   48th International Symposium on High-Performance Liquid Phase Separations and Related Techniques
   2019. June 16-20. Milan, Italy.

8. **A. Bajtai**, A. Péter, W. Lindner, I. Ilisz
   Application of Polysaccharide-based Chiral Stationary Phases for the Enantioseparation of Natural and Synthetic Cinchona Alkaloid Analogues
   12th Balaton Symposium on High-Performance Separation Methods
   2019. September 11-13. Siófok, Hungary.