PhD thesis

Liquid and supercritical fluid chromatographic enantioseparation of N^{α}-Fmoc proteinogenic amino acids on *Cinchona* alkaloid-based chiral stationary phases

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2020

University of Szeged

Doctoral School of Pharmaceutical Sciences

Educational program: Pharmaceutical Chemistry and Drug research

Program director: Prof. Dr. Ferenc Fülöp

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Introduction

It is broadly accepted that chirality is universal and chirality at the molecular level plays an essential role in biological systems. In this context, proteins, peptides, canonical amino acids (except glycine), saccharides, enzymes and many metabolites are chiral products being present in living organisms. Under "chiral conditions" (*e.g.*, in a living organism), enantiomeric compounds may behave in different ways. They may differ in their type and range of biological effects as well as their utilization, distribution, metabolism, *etc*. It is well established that pharmacological activity is mostly restricted to one of the enantiomers (eutomer). In several cases, unwanted side effects or even toxic effects may occur with the inactive enantiomer (distomer). Even if the side effects are not that drastic, the inactive enantiomer has to be metabolized, this represents an unnecessary burden for the organism. An example of this is thalidomide, which was introduced as sedative drug and painkiller in the late of 1950s. Another example is amphetamine, where the *S*-(+)-isomer is a few times more potent in central nervous system stimulation than *R*-(–)-amphetamine. The latter, in turn, is slightly more potent in the peripheral system, for example in cardiovascular action. The administration of pure, pharmacologically active enantiomers is therefore of great importance. Therefore, the European (EMA), USA (FDA) and Japanese (PMDA) regulatory authorities nowadays impose strict guidelines for the commercialization of chiral drug substances. Enantioselective identification and quantification methods should be developed for each active pharmaceutical ingredient with chiral properties. In addition pharmacokinetic and toxicological assays should be executed with both pure enantiomers and with the racemate. Based on these fundamental findings, the life science industry has to pay attention to chirality-related phenomena when developing, *e.g.*, biologically active chiral pharmacons. The separation of enantiomers is among the more challenging chromatographic modalities due to the fact that conventional strategies employed to separate achiral analytes are ineffective when applied to enantiomers. Chromatographic methods are the most popular, including gas chromatography (GC), thin-layer chromatography (TLC), capillary electrophoresis (CE), capillary electrochromatography (CEC), supercritical fluid chromatography (SFC), and high-performance liquid chromatography (HPLC) are the most popular techniques. The last quarter of the century has seen a vast growth of diverse chiral technologies, including stereocontrolled synthesis and enantioselective separation and analysis concept. Recently, SFC becomes an alternative technique to HPLC for routine applications in enantioresolution of pharmaceutical compounds, because it may offer several advantages over HPLC in certain circumstances, including improved resolution, faster separations and higher

throughput. These benefits arise from the characteristics of supercritical fluids (SCFs), which are considered green mobile phases. Characteristic features are limited environmental impact, low disposal costs, reduced consumption of toxic solvents and additives, lack of toxicity (in most cases). The reduction in the use of organic solvents results in cost, health and safety benefits, and faster, cleaner sample recovery during experimental procedures. Moreover, SFC is suitable for non-polar pharmaceuticals compounds.

The aim of this work

The primary aim of this work was to develop chiral separation methods for 19 N^{α}-Fmoc-protected protein amino acids on *Cinchona* alkaloid-based zwitterionic and anion-exchanger type chiral stationary phases (CSPs). Two different types of separation techniques were used for separation. One of them is the well-known high-performance liquid chromatography (HPLC) technique, which is the most straightforward and efficient mode used widely. The other separation technique, which uses supercritical fluid as the main component of the mobile phase, is supercritical fluid chromatography (SFC). The effect of the nature and concentration of bulk solvent components, the role of water content in the mobile phase, the nature and concentration of base and acid additives, and the temperature on chromatographic parameters were investigated applying *Cinchona* alkaloid-based chiral stationary phases (CSPs). Thermodynamic parameters were calculated utilizing temperature dependence studies.

Experimental

Chromatography apparatus's

Measurements were carried out on two HPLC systems and one SFC system.

System I: Liquid chromatographic experiments were performed on a Waters Breeze system containing a 1525 binary pump, a 2487 dual-channel absorbance detector, a 717 plus autosampler and Empower 2 data manager software (Waters Chromatography, Milford, MA, USA).

System II: A 1100 Series HPLC system consisted of a solvent degasser, a pump, an autosampler, a column thermostat and a multiwavelength UV-Vis detector from Agilent Technologies (Waldbronn, Germany) as well as 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 from Agilent Technologies.

Both chromatographic systems were equipped with Rheodyne Model 7125 injectors (Cotati, CA, USA) with 20 µl loops. The columns were thermostated in a Spark Mistral column thermostat (Spark Holland, Emmen, The Netherlands) or Lauda Alpha RA8 thermostat (Lauda Dr. R. Wobser Gmbh, Lauda-Königshofen, Germany). The precision of temperature adjustment was ±0.1 °C.

System III: The Waters Acquity Ultra Performance Convergence Chromatography™ (UPC^2, Waters Chromatography) system was equipped with a binary solvent delivery pump, an autosampler with a partial loop volume injector system, a backpressure regulator, a column oven and a PDA detector. The system control and data acquisition Empower 2 software (Waters Chromatography) was used. Experiments were executed with mobile phases composed of liquid CO_2/MeOH in different ratios with various additives. All chromatographic experiments were carried out in isocratic mode at a flow rate of 0.6 mL/min in LC and 2.0 mL/min in SFC mode, with UV detection. The dead time (t_0) was determined by injecting a solution of acetone in MeOH.

Applied columns

The four *Cinchona* alkaloid-based CSPs ZWIX(+)™, ZWIX(–)™, QN-AX and QD-AX were provided by Chiral Technologies Europe (CTE, Illkirch, France). All CSPs comprised 3 µm particles packed into 150 x 3.0 mm I.D. columns.

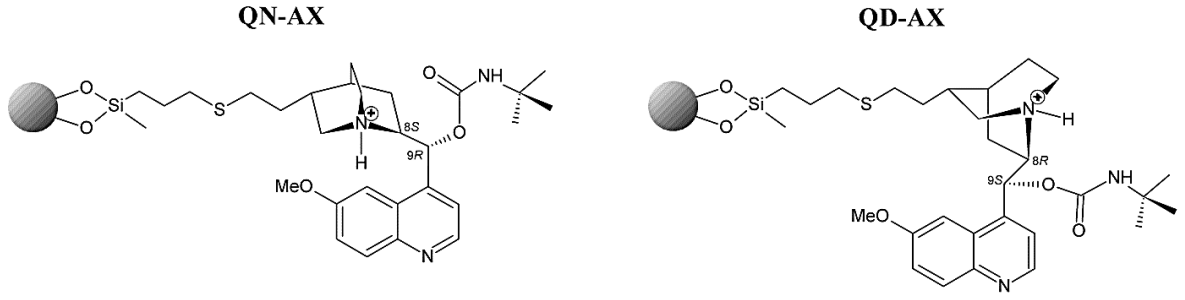

Figure 1. *The structure of anion-exchanger Chiralpak QN-AX and QD-AX CSP*

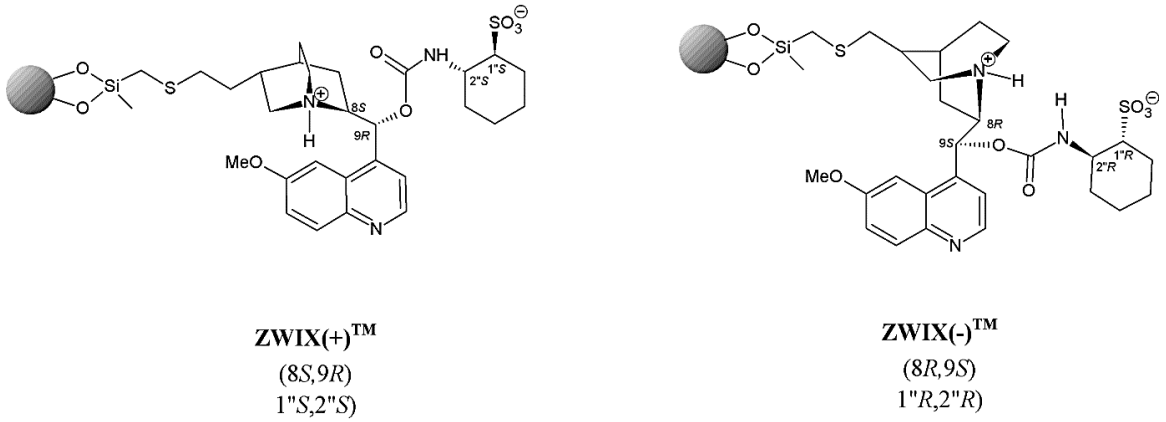

Figure 2. *The structure of zwitterionic Chiralpak ZWIX(+)™ and ZWIX(–)™ CSP*

Results

In my research work, methods were developed utilizing zwitterionic and anion-exchanger type chiral CSPs based on *Cinchona* alkaloids for the separation of N^{α}-Fmoc proteinogenic amino acid enantiomers in LC and SFC mode. In the course of method optimization, several mobile phase compositions and conditions as well as different temperatures affecting the chromatographic parameters were investigated.

1) Effect of mobile phase composition

Experiments were performed at various mobile phase compositions, involving mixtures of MeOH and MeCN in LC mode, and liquid CO_2 and MeOH in the SFC mode with constant ionic strength (the acid-to-base ratio was kept at a constant value of 2:1). The presence of polar solvent had a strong effect on retention, selectivity, and resolution. These values changed (generally decreased) significantly at higher MeOH content in MeCN or CO_2 as bulk solvent. With increasing MeOH content in the mobile phase the polarity of the mobile phase increased promoting the interaction between mobile phase and SA; therefore, retentions decreased in all cases. It is important to note that under all chromatographic conditions applied, ionic interactions between the SO and SA have a decisive role regarding retention. Enantioselectivity is influenced by additional hydrogen bonding as well as aromatic π–π and van der Waals interactions.

2) Role of water content of the mobile phase

The effects of the water content in the mobile phase on the retention, selectivity and resolution were investigated. The presence of water in lower concentrations is beneficial for peak shape, resolution, analysis time, sample, and solubility performance. The addition of small amounts of water to the polar ionic mobile phase shifts the elution system from a nonaquesous PI mode to a HO mode. A few percentage points of H_2O affect solvation of both SO and SA and might reduce the strength of ionic interactions. In LC mode, in most cases, 1.0–2.0% H_2O content in the eluent was advantageous, yielding better peak shapes and higher resolution. In SFC mode, increasing the water content resulted in slightly decreased rentention times. This can be partially explained by the increase of formation of counter-ion via the reaction of CO_2 and H_2O, yielding carbonic acid which dissociates to hydrogen carbonate and proton. Formed hydrogen carbonate and proton act as additional counter-ions in anion chromatographic system. Similar to this behaviour, both α and R_S decreased slightly with increasing water content.

3) Role of nature of base and acid as mobile phase additives

To investigate the effect of the nature of acid and base additives, separations are generally carried out with constant bulk solvent composition and an excess of acid to the base component in the mobile phase ensuring that the base is present in their protonated „ammonium-ion" form. To study the effects of acids and base additives, FA and AcOH were selected as acid additives, and EA, DEA, TEA, PA, and BA as base additives. The amines differed in the degree and nature of their alkyl substitution on the nitrogen atom, while acids FA and AcOH have different strength. The nature of the various acid and base additives in the mobile phase may affect the chromatographic parameters and play an important role in the optimization of the enantioseparation on *Cinchona* alkaloid-based CSPs.

4) Effect of the counter-ion concentration

Retention can be controlled by the type of the counter-ion, but the concentration of the counter-ion also can also affect chromatographic behavior. This means that retention can be greatly influenced by the amounts of co-ions and counter-ions present in the mobile phase. The application of a higher counter-ion concentration should result in lower retention, as the stoichiometric displacement model described. According to this model, linear relationship was found between the logarithm of the retention factor of the first-eluted enantiomer (log k_1) and the logarithm of the counter-ion concentration (log $c_{counter\text{-}ion}$). On anion-exchanger QN-AX and QD-AX CSPs, a „single ionic" ion-exchange mechanism was also suggested.

5) Temperature dependence and thermodynamic parameters

For the investigation of the temperature effect, the van't Hoff plots approach was applied. The temperature range varied between 5–40 ºC in LC mode and between 20–50 ºC in SFC mode. The differences of the standard enthalpy and entropy changes were calculated from the ln α *vs.* 1/*T* curves, where the slope gives $\Delta(\Delta H^{o})$ and the intercept gives $\Delta(\Delta S^{o})$ values. It should be noted, that in all cases the selectivity decreased with increasing temperature, both $\Delta(\Delta H^{o})$ and $\Delta(\Delta S^{o})$ exhibited negative values, *i.e.* the enantioseparation was enthalpically driven. The relative contribution of the enthalphic and entropic terms to the free energy of adsorption can be visualized through the enthalpy/entropy ratio *Q* {$Q = \Delta(\Delta H^{o}) / [298 \times \Delta(\Delta S^{o})]$} calculated at 298 K. A comparison of the *Q* values for the individual analytes revealed that the enantioselective discrimination was enthalpically driven ($Q > 1.0$) in all cases and with few exceptions the highest *Q* values were most often obtained on QN-AX CSP.

6) Determination of elution sequence on different types of Cinchona alkaloids

Cinchona alkaloids (*QN* and *QD*) and *trans*-2-aminocyclohexanesulfonic acid-based chiral SOs and CSPs behave as pseudoenantiomeric CSPs. In fact, they are diastereomers. Elution sequences were determined for all the studied analytes, and a general rule could be observed: D enantiomers eluted first before the L ones on *QN*-based (ZWIX(+)™ and QN-AX) CSPs in both LC and SFC mode. On the other hand, on *QD*-based (ZWIX(-)™ and QD-AX) CSPs the L enantiomers eluted first before D ones in both chromatographic modes.

7) Method development for the enantiomer purity determination of N^{α}-Fmoc proteinogenic amino acids

Determination of the enantiomeric excess (*ee* values) of the starting free amino acids and of the *N*-protected amino acids is highly important in peptide synthesis. The enantiomeric excess used in chemistry is the amount to characterize the composition of mixtures of enantiomers indicating the enantiomeric purity of a substance. A rapid and sensitive chromatographic method protocol was developed for the identification and quantitation of enantiomeric impurities of commercially available N^{α}-Fmoc-protected amino acids on *Cinchona* alkaloid *QN*- and *QD*-based zwitterionic stationary phases. To quantify the amount of enantiomeric impurities, analyses were carried out for the determination of the minor enantiomers in the case of five selected analytes.

List of publications and lectures

Papers related to the thesis

I. **Lajkó G.**, Ilisz I., Tóth G., Fülöp F., Lindner W., Péter A.
Application of *Cinchona* alkaloid-based zwitterionic chiral stationary phases in supercritical fluid chromatography for the enantioseparation of N^{α}-protected proteinogenic amino acids, In: Journal of Chromatography A, 1415 (2015) 134–145.
if:3.981

II. **Lajkó G.**, Grecsó N., Tóth G., Fülöp F., Lindner W., Péter A., Ilisz I.
A comparative study of enantioseparations of N^{α}-Fmoc proteinogenic amino acids on Quinine-based zwitterionic and anion exchanger-type chiral stationary phases under hydro-organic liquid and subcritical fluid chromatographic conditions, In: Molecules, 21 (11) (2016) 1579.
if:2.861

III. **Lajkó G.**, Grecsó N., Tóth G., Fülöp F., Lindner W., Péter A., Ilisz I.
Liquid and subcritical fluid chromatographic enantioseparation of N^{α}-Fmoc proteinogenic amino acids on quinidine-based zwitterionic and anion-exchanger type chiral stationary phases. A comparative study, In: Chirality, 29 (2017) 225–238.
if:1.956
Sum of impact factors: 8.798

Other papers

IV. Ilisz I., Gecse Z., **Lajkó G**., Nonn M., Fülöp F., Lindner W., Péter A.
Investigation of the structure-selectivity relationships and van't Hoff analysis of chromatographic stereoisomer separations of unusual isoxazoline-fused 2-aminocyclopentanecarboxylic acids on *Cinchona* alkaloid-based chiral stationary phases,. In: Journal of Chromatography A, 1384 (2015) 67–75.
if: 3.981

V. Ilisz I., Gecse Z., **Lajkó G.**, Forró E., Fülöp Z., Lindner W., Péter A.
High-Performance liquid chromatographic enantioseparation of cyclic β-amino acids applying zwitterionic chiral stationary phases based on *Cinchona* alkaloids, In: Chirality, 27 (2015) 563–570.
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VI. **Lajkó G.**, Orosz T., Kiss L., Forró E., Fülöp F., Péter A., Ilisz I.
High-Performance liquid chromatographic enantioseparation of fluorinated cyclic β^3–amino acid analogs on polysaccharide-based chiral stationary phases. Comparison with nonfluorinated counterparts, In: Biomedical Chromatography, 30 (2016) 1441–1448.
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VII. **Lajkó G.**, Orosz T., Grecsó N., Fekete B., Palkó M., Fülöp F., Lindner W., Péter A., Ilisz I.
High-Performance liquid chromatographic enantioseparation of cyclic β-aminohydroxamic acids on zwitterionic chiral stationary phases based on *Cinchona* alkaloids, In: Analytica Chimica Acta, 921 (2016) 84–94.
if:4.950

VIII. **Lajkó G.**, Grecsó N., Megyesi R., Forró E., Fülöp F., Wolrab D., Lindner W., Péter A., Ilisz, I.
Enantioseparation of *ß*-carboline derivatives on polysaccharide- and strong cation exchanger-based chiral stationary phases. A comparative study, In: Journal of Chromatography A, 1467 (2016) 188–198.

if: 3.981

IX. Orosz T., Grecsó N., **Lajkó G.**, Szakonyi Z., Fülöp F., Armstrong DW., Péter A., Ilisz I.
Liquid chromatographic enantioseparation of carbocyclic *β*-amino acids possessing limonene skeleton on macrocyclic glycopeptide-based chiral stationary phases. In: Journal of Pharmaceutical and Biomedical Analysis, 145 (2017) 119–126.

if:3.255

X. **Lajkó G.**, Orosz T., Ugrai I., Szakonyi Zs., Fülöp F., Lindner W., Péter A., Ilisz I.
Liquid chromatographic enantioseparation of limonene-based carbocyclic *β*–amino acids on zwittterionic *Cinchona* alkaloid-based chiral stationary phases. In: Journal of Separation Science, 40 (2017) 3196–3204.

XI. Bajtai A., **Lajkó G.**, Szatmári I., Fülöp F., Lindner W., Péter A., Ilisz I.
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. In: Journal of Chromatography A, 1563 (2018) 180–190.

if:2.557

Sum of impact factors: 22.293

Total impact factor: 31.091

Lectures related to this thesis

I. **Lajkó G.**,
N-védett természetes aminosavak enantiomerjeinek szuperkritikus folyadékkromatográfiás elválasztása kinaalkaloid alapú ikerionos állófázisokon,
Szegedi Ifjú Szerves Kémikusok Támogatásáért Alapítvány 14. Tudományos Előadóülése, 29 Apr. (2015). (oral presentation)

II. **Lajkó G.**, Ilisz I., Tóth G., Fülöp F., Lindner W., Péter A.
Enantioseparation of Nα-protected proteinogenic amino acids by supercritical fluid chromatography on *Cinchona* alkaloid-based zwitterionic chiral stationary phases, 10th Balaton Symposium on High-Performance Separation Methods, Siófok, 2-4 September (2015). (poster)

Other lectures

III. Ilisz I., Grecsó N., **Lajkó G.**, Fülöp F., Lindner W., Péter A., Kationos 1,2,3,4-tetrahidroizokinolin analógok királis nagyhatékonyságú folyadékkromatográfiás vizsgálata ikerionos állófázisokon, Elválasztástudományi Vándorgyűlés, Egerszalók, 12-14. November (2014) (poster)

IV. Grecsó N., **Lajkó G.**, Ilisz I., Forró E., Fülöp F., Armstrong DW., Péter A., 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, 28 September (2015). (poster)

V. **Lajkó G.,** Orosz T., Grecsó N., Palkó M., Fülöp F., Lindner W., Péter A. Ilisz I., Enantioseparation of cyclic ß-aminohydroxamic acids by high-performance liquid chromatography on zwitterionic chiral stationary phases based on Cinchona alkaloids, Olomouc, 6-9 June (2016). (poster)

VI. Orosz T., **Lajkó G.,** Grecsó N., Kiss L., Forró E., Fülöp F., Péter A., Ilisz I. 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, 10 October (2016). (poster)

VII. **Lajkó G.,** Orosz T., Grecsó N., Palkó M., Fülöp F., Lindner W., Ilisz I., Péter A. Ciklikus β-aminohidroxámsavak enantiomerjeinek nagyhatékonyságú folyadékkromatográfiás elválasztása kinaalkaloid alapú ikerionos állófázisokon, Elválasztástudományi Vándorgyűlés, Kecskemét, 9–11 November (2016) (poster)