

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ENANTIOSEPARATION OF UNNATURAL β -AMINO ACIDS ON CHIRAL STATIONARY PHASES

Summary of PhD Thesis

László Sipos

Supervisors:

Prof. Ferenc Fülöp, Ph.D., D.Sc.

Prof. Antal Péter, Ph.D., D.Sc.



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**University of Szeged
Doctoral School of Pharmaceutical Sciences**

Programme of Pharmaceutical Chemistry and Drug Research

Programme Director: Prof. Ferenc Fülöp, Ph.D., D.Sc.

Institute of Pharmaceutical Chemistry

**Supervisors: Prof. Ferenc Fülöp, Ph.D., D.Sc.
Prof. Antal Péter, Ph.D., D.Sc.**

László Sipos

**High-performance liquid chromatographic enantioseparation of
unnatural β -amino acids on chiral stationary phases**

Final Exam Committee:

Head: Prof. György Dombi, Ph.D., D.Sc.

Members: Tamás Martinek, Ph.D.

Pál Szabó, Ph.D.

Reviewer Committee:

Head: Prof. György Dombi, Ph.D., D.Sc.

Reviewers: Prof. Mária Báthori, Ph.D., D.Sc.

Prof. Péter Molnár, Ph.D., D.Sc.

Members: Livia Fülöp, Ph.D.

Anita Sztojtkov-Ivanov, Ph.D.

INTRODUCTION

High-performance liquid chromatography (HPLC) (and also other chromatographic techniques) is an extremely versatile technique to separate chemical materials – or enantiomers in chiral chemistry – and also to clean *i.e.* a medical agent from impurities.

The importance of chirality has been appreciated and addressed by the pharmaceutical industry for decades. As technologies for measuring and making enantiopure materials have improved, the production of enantiopure pharmaceuticals has become commonplace, with many of the top selling drugs in the world now being sold in enantiopure form. Consequently, the subject of chirality and the pharmaceutical industry is a topic of considerable recent interest and importance.

Most of the molecules of importance to living systems are chiral, *e.g.* amino acids, sugars, proteins and nucleic acids. An interesting feature of these chiral biomolecules is that in nature they usually exist in only one of the two possible enantiomeric forms.

The physical and chemical properties of the enantiomers are equal therefore their discrimination was too difficult in the past. For discrimination of the enantiomers a chiral environment is required. The chiral separation is achieved with two methods in chromatography, the indirect and the direct methods. In this study the direct method was used by application of chiral stationary phases (CSPs).

β -Amino-acids allow for greater diversity opposite α -amino-acids, so the research of them have received increased attention. Because of their unique biological, neurological and pharmaceutical activity, unnatural amino acids are utilized as building blocks, molecular scaffolds, conformational constraints, or pharmacologically active products. β -Amino-acids are key components of numerous bioactive molecules such as peptides, alkaloids or β -lactam antibiotics.

AIMS

The aim of this work is to investigate chiral separation of two types of β -amino acids on two different types of CSPs such as macrocyclic glycopeptide- and crown ether-based CSPs.

1 Enantioseparation of monoterpene-based β -amino acids is planned to be investigated on the macrocyclic glycopeptides-based CSPs such as on teicoplanin, ristocetin A and vancomycin-based columns. The influence of the pH, mobile phase

composition, the structure of the molecules, the sugar moieties of the selectors and the temperature on chiral recognition are planned to be investigated.

2 Enantioseparation of isoxazoline-fused 2-aminocyclopentanecarboxylic acid analogs will be studied on macrocyclic antibiotics-based CSPs, to develop new chromatographic methods for their chiral separation. For this purpose the method will be optimized changing the chromatographic conditions (pH, mobile phase composition, temperature, etc.).

3 For the enantioseparation of isoxazoline-fused 2-aminocyclopentanecarboxylic acid analogs new types of crown ether-based selectors are planned to be used. With variation of chromatographic conditions the goal was to determine the main interactions which contribute to the chiral recognition. The effect of the nature of “spacer” used for immobilization of selector will be studied, too.

4 The separation efficiency of the two types of CSPs will be compared on the bases of the chromatographic data obtained for isoxazoline-fused 2-aminocyclopentanecarboxylic acid analogs.

In the case of all measurements the chromatographic parameters (retention factors, selectivity factor and resolution) and in the case of temperature dependence the thermodynamic parameters will be collected.

EXPERIMENTAL

Apparatus

Our measurements were carried out with three HPLC systems.

System I: An M-600 low-pressure gradient pump, equipped with an M-996 photodiodearray detector and a Millennium³² 2.1 Chromatography Manager data system (all Waters Chromatography, Milford, MA, USA).

System II: A 1525 binary pump, a 2487 dual-channel absorbance detector, a 717 plus autosampler and Empower 2 data manager software (all Waters Chromatography, Milford, MA, USA).

System III: An L-6000 pump (Hitachi Ltd., Tokio, Japan), an SPD-6AV UV-VIS detector (Shimadzu Corporation, Japan) and Borwin data software (Merck, Darmstadt, Germany).

All of the HPLC systems were equipped with a Rheodyne 7125 injector with a 20- μ l loop (Rheodyne, Cotati, CA, USA).

A Thermo Orion 420 pH-meter was employed for pH measurements.

The columns were thermostated in a water bath and a cooling-heating thermostat. The accuracy of temperature adjustment was ± 0.1 °C.

The chromatographic system was conditioned by passing the eluent through the column until a stable baseline signal and reproducible retention factors were obtained for the subsequent injections. This procedure was always followed when a new mobile phase or temperature was chosen.

Applied columns

Crown ether-based CSP: (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based CSPs, 150 \times 4.6 mm I.D., 5 μ m particle size.

Macrocyclic glycopeptide-based CSPs: teicoplanin-containing Chirobiotic T and T2, teicoplanin aglycone-containing Chirobiotic TAG, vancomycin-containing Chirobiotic V, vancomycin aglycone-containing Chirobiotic VAG and ristocetin A-containing Chirobiotic R, 250 \times 4.6 mm I.D., 5 μ m particle size.

Investigated compounds

The investigations were extended to monoterpene-based 2-aminocyclopentane-carboxylic acids (**Fig. 1**) and isoxasoline-fused 2-aminocyclopentanecarboxylic acids (**Fig. 2**). These analytes were prepared in Institute of Pharmaceutical Chemistry, in Szeged.

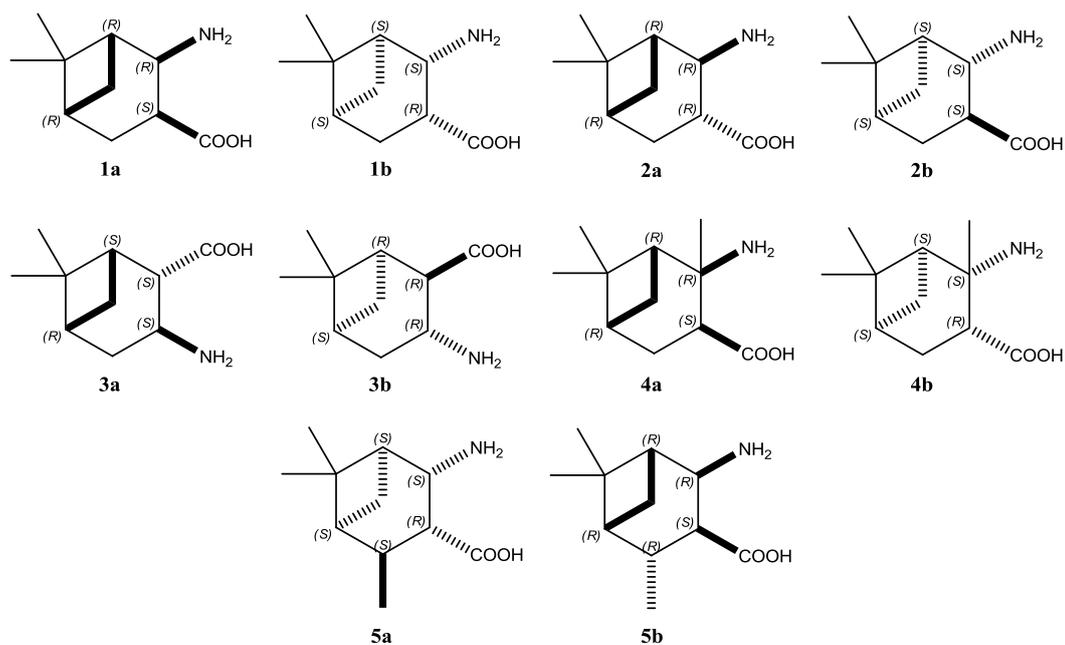


Figure 1. Structures of monoterpene-based β -amino acids

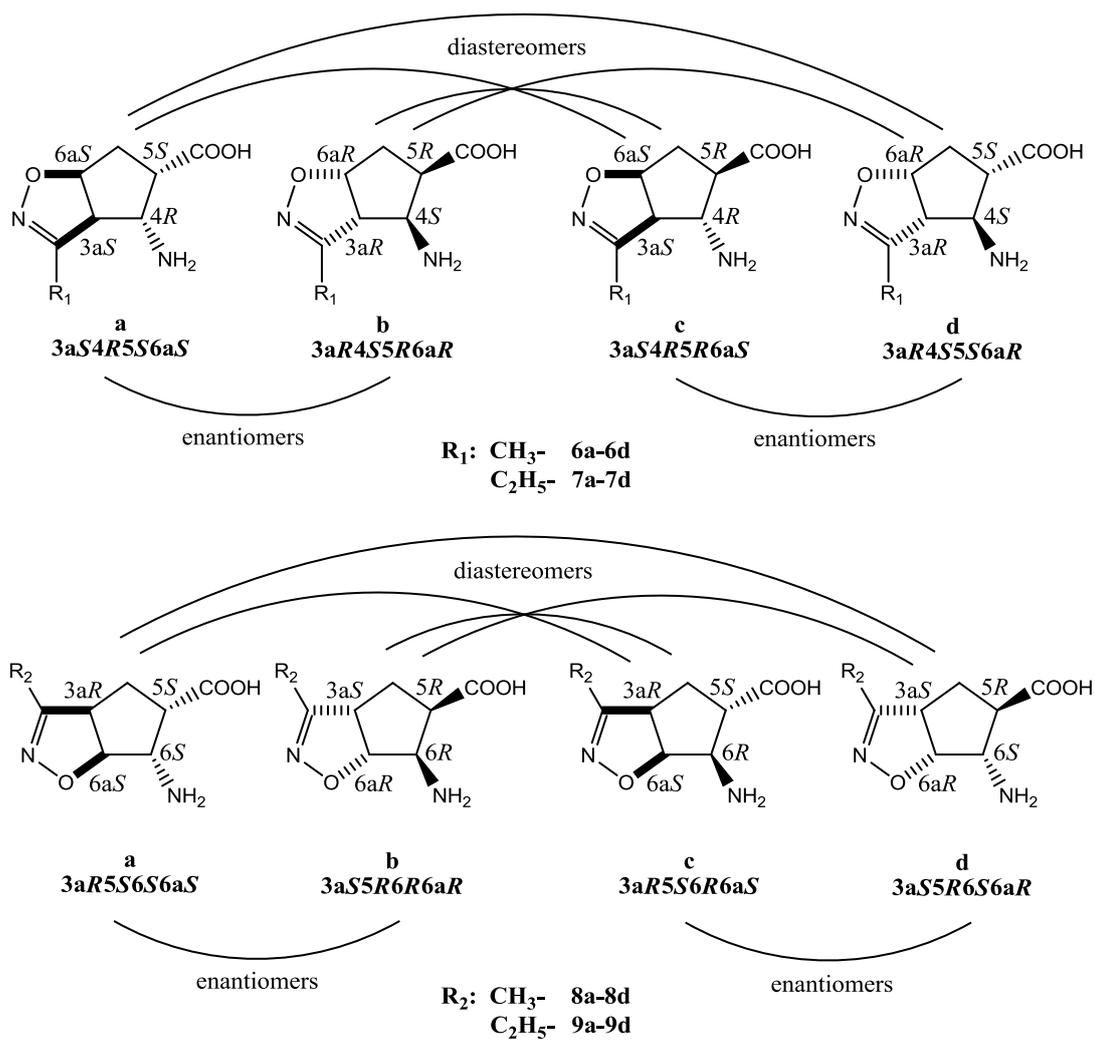


Figure 2. Structures of isoxazoline-fused 2-aminocyclopentanecarboxylic acid analogs

RESULTS

Enantioseparation of monoterpene-based 2-aminocyclopentane carboxylic acids on macrocyclic antibiotic-based CSPs

Reversed-phase, polar organic and polar ionic experimental conditions were applied in this project. Applied CSPs were teicoplanin-based Chirobiotic T, T2, TAG, ristocetin A-based Chirobiotic R and vancomycin-based Chirobiotic V and VAG columns.

The effect of pH of the mobile phase was investigated. On Chirobiotic T column increasing the pH of mobile phase decreased the retention factors, while selectivity slightly and resolution considerably increased.

On the basis of the effect of pH measurement, the mobile phases contained 0.1% TEAA (pH=4.1) buffer and alcohol modifier. On Chirobiotic T, T2, TAG columns with increasing alcohol content the retentions were changed according to a U-shaped curve for all analytes (except analyte **3** on T and T2 columns). The explanation of this result was that in water-rich eluent the hydrophobic interactions increase the k' values, while in alcohol-rich mobile phase the hydrophilic interaction chromatography (HILIC) interaction increases the retention. In case of analyte **3** the different behavior could be attributed to the opposite position of amino and carboxy groups. In general, the selectivity factors and resolutions neither described a U-shaped curve nor increased with increasing alcohol content. The other three CSPs (Chirobiotic R, V and VAG) did not provide good results, the best value for resolution was $R_S=1.00$.

The elution order was not changed under the measurements, except in the case of analyte **2** where the elution sequence differed when the mobile phase was changed from reversed-phase to polar-ionic mode.

The effect of temperature was also investigated on T and TAG columns between 10 and 40°C and the thermodynamic parameters were determined in all cases. In most cases the enantioseparations were enthalpically-driven, while in the case of analyte **3** a rare entropically controlled separation was observed. Baseline resolution was achieved in all cases.

Enantioseparation of isoxazoline-fused 2-aminocyclopentanecarboxylic acids

Because of the importance of isoxazolines, their investigation is necessary in pharmaceutical industry. Therefore these systems were investigated on two types of CSPs.

Investigation on macrocyclic antibiotic CSPs

With application of MeOH as mobile phase additive in most cases the retention factors described a U-shaped curve. However, in case of **6a,6b** on T, T2 and TAG and **7a,7b** on T and T2 columns k' increased with increasing MeOH content. For all of the analytes, the Chirobiotic TAG column proved to be the most useful, the best resolutions were reached on it. In case of **8a,8b** and **9a,9b** analogs outstanding results were shown also on the V and VAG columns. As concerns the elution sequence it differed only on TAG CSP for *trans* analogs.

The effect of temperature was investigated between 5-45°C (in 10°C increments) on Chirobiotic T and TAG CSPs. Thermodynamic parameters were determined in all cases and in one case the process of separation was entropically-driven for **8a,8b** on Chirobiotic TAG. On Chirobiotic T for analogs **6c,6d** and **7c,7d** the T_{iso} temperature (where the enantioselectivity disappears) was 37°C. The change of elution order for analytes **6c,6d** below and above 37°C was demonstrated.

As for these types of analytes four pairs of diastereomers exist, the separation of four diastereomers (four enantiomers) was achieved. In case of **7a-7d** and **8a-8d** the eight enantiomers were separated in one chromatographic run but the separation of **6a-6d** and **9a-9d** needs two chromatographic runs.

Investigation on crown ether-containing CSPs

The primary interaction between protonated amino group of amino acids and crown ether is the inclusion complex formation. Further interactions are needed to fulfill the requirement of three-point interaction for chiral resolution.

The effects of the alcohol (MeOH, EtOH, IPA) content of the mobile phase showed that in most cases, k' increased with increasing alcohol content. This suggests that the retention behavior may be controlled by a mechanism of HILIC at high alcohol contents. Although in some cases, in MeOH containing mobile phase on **Crown 1** and for all analytes on **Crown 3** a U-shaped retention curve was observed. At higher water content,

the retention factor increased with increasing water content; this was probably due to enhanced hydrophobic interactions between the analyte and the CSP in the water-rich mobile phases.

Comparing retention behavior of the three crown ether-containing CSPs with application of mobile phases containing large or small amount of water k_1' was larger on **Crown 3** when H₂O content was larger and on **Crown 1** when alcohol content was larger. In the first case, the most apolar **Crown 3** in water-rich mobile phase favors the hydrophobic interactions between **Crown 3** and analyte resulting in larger k_1' . In the second case, the most polar **Crown 1** in alcohol-rich eluent system favors the HILIC interactions resulting in the large k_1' values.

Since the inclusion complex formation requires protonation of primary amino group of amino acids, addition of acids to mobile phase is needed. A comparison of the chromatographic data obtained by using HCOOH, AcOH, TFA, HClO₄, H₂SO₄ or H₃PO₄ as acidic modifier demonstrates that in most cases the larger k' values were obtained on the application of AcOH or HCOOH, while other acids resulted in the lower k' . Also larger selectivity and resolution were obtained using AcOH and HCOOH, and in some cases H₂SO₄ (when EtOH or IPA was used). As concerns the effect of concentration of acid modifier the increase of AcOH and H₂SO₄ content decreased the k' values. α was increased with increasing acid concentration, however the R_S values decreased with increasing concentration for *trans* analogs, and increased for *cis* analogs.

The structures of analytes also influenced the chiral recognition. On all CSPs the values of selectivity and resolution were better for analytes **8a,8b** and **9a,9b** than for analytes **6a,6b** and **7a,7b**, the position of R-substituent influenced the chromatographic parameters. The *trans* analogs exhibited different chromatographic behavior.

The elution order was determined in all cases, but no general rule could be found to describe the elution behavior of these compounds.

PUBLICATIONS

1. **László Sipos**, István Ilisz, Zoltán Pataj, Zsolt Szakonyi, Ferenc Fülöp, Daniel W. Armstrong, Antal Péter
High-performance liquid chromatographic enantioseparation of monoterpene-based 2-amino carboxylic acids on macrocyclic glycopeptide-based phases
Journal of Chromatography A, 1217 (2010) 6956-6963. i.f.: 4.192
2. **László Sipos**, István Ilisz, Melinda Nonn, Ferenc Fülöp, Zoltán Pataj, Daniel W. Armstrong, Antal Péter
High-performance liquid chromatographic enantioseparation of unusual isoxazoline-fused 2-aminocyclopentanecarboxylic acids on macrocyclic glycopeptide-based chiral stationary phases
Journal of Chromatography A, 1232 (2012) 142-151. i.f.: 4.192
3. **László Sipos**, István Ilisz, Anita Aranyi, Zsanett Gecse, Melinda Nonn, Ferenc Fülöp, Myung Ho Hyun, Antal Péter
High-performance liquid chromatographic enantioseparation of unusual isoxazoline-fused 2-aminocyclopentanecarboxylic acids on (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based chiral stationary phases
Chirality, 24 (2012) 817-824. i.f.: 2.982

Total impact factors: 11.370

POSTERS

1. **László Sipos**, István Ilisz, Zoltán Pataj, Zsolt Szakonyi, Ferenc Fülöp, Daniel W. Armstrong, Antal Péter
High-performance liquid chromatographic enantioseparation of monoterpene-based 2-aminocyclopentanecarboxylic acids on macrocyclic glycopeptide-based stationary phases
Elvlasztástudományi Vándorgyűlés, 10-12 November 2010, Tapolca, Hungary. Abstr.: P-37.
2. **László Sipos**, Melinda Nonn, Lóránd Kiss, Ferenc Fülöp, Daniel W. Armstrong, Antal Péter
High-performance liquid chromatographic enantioseparation of isoxazoline-fused cispentacin derivatives on chiral stationary phases
36th International Symposium on High Performance Liquid Phase Separations and Related Techniques, 19-23 June 2011, Budapest, Hungary. Abstr.: P-79.

LECTURES

1. *High-performance liquid chromatographic enantioseparation of monoterpene-based 2-aminocyclopentanecarboxylic acids on macrocyclic glycopeptide-based stationary phases*

XXXIII. Kémiai Előadói Napok, 25-27 October 2010, Szeged, Hungary.