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Application of Commercially Available Crown Ether Chiral Stationary Phases for Separation of Tetrapeptide Stereoisomers

TOMS UPMANIS[1]; HELENA KAŽOKA[1]

[1] *Latvian institute of Organic Synthesis, 21 Aizkraukles Street, LV-1006 Riga, Latvia*

Correspondence: upmanis@osi.lv

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1. Introduction

Over the past decade, peptide drug discovery has experienced a revival of interest as the pharmaceutical industry has come to appreciate how this class of compounds can be an excellent complement or even a preferable alternative to small molecule drugs [1]. The essential biological functions of small peptide drugs often depend on peptide stereochemistry. With the exception of glycine, all other amino acids exist as D and L enantiomers, therefore, peptides can exist as several stereoisomers, possessing different biological properties [2,3]. During synthesis, storage or metabolic processes stereoisomers may experience racemization, resulting in complex enantiomeric / epimeric mixtures, therefore, enantiomeric purity control of peptide analytes is an important challenge in the biological and medical sciences and is necessary for the pharmaceutical industry.

Among the numerous commercially available chiral stationary phases (CSPs), crown ether-based CSPs have been proven to be very effective for the resolution of chiral analytes containing primary amino groups [4].

This work is an extension to our previous research [5] regarding the chiral resolution of tetrapeptides, using Tyr-Arg-Phe-Lys-NH_2 (see ***Figure 1A***; LDLL isomer is also known as μ-opioid receptor agonist DALDA), as a model structure.

Efforts to extend the use of chiral crown ether-based CSPs in resolution of all sixteen tetrapeptide stereoisomers were made by employing the opposite characteristics of *S-* and *R*-3,3′-diphenyl-1,1′-binaphthyl crown ether selectors (see ***Figure 1B***, commercially sold as CROWNPAK CR-I columns).

2. Materials and methods

All 16 stereoisomers of Tyr-Arg-Phe-Lys-NH_2 tetrapeptide were synthesized at the Latvian Institute of Organic Synthesis by the author. Chromatographic measurements were performed on Waters Alliance (Waters Corporation, Milford, MA, USA) LC systems equipped with 2695 separations module consisting of quaternary pump, degasser, autosampler and column heater, Waters 2489 dual λ absorbance detector was used for detection of analytes. The output signal was monitored and processed using Waters Empower 2 software. CROWNPAK CR-I (+) and CR-I (-) columns (3.0 mm (i.d.) × 150 mm, 5 μm particle size) based on *S- and R*-(3,3′-diphenyl-1,1′-binaphthyl)-20-crown-6 immobilized on silica was purchased from Chiral Technologies Europe (Illkirch, France). Mixtures of acetonitrile and 50mM $HClO_4$/water solution were used as a mobile phase. Chromatographic runs were performed at flow rate of 0.4 mL/ min. Detection was accomplished via measurement of UV absorption at 220 nm and injection volume was set at 5 μl. The analytical samples consist of mixtures eight LXXX stereoisomers and a mixture of their corresponding DXXX enantiomers (0.5 mg/ mL of each stereoisomer in mobile phase). Elution order was determined by injecting configurationally known samples.

3. Results

It is well known, that the chiral selector in CROWNPAK CR-I columns - *S- and R*-(3,3′-diphe-

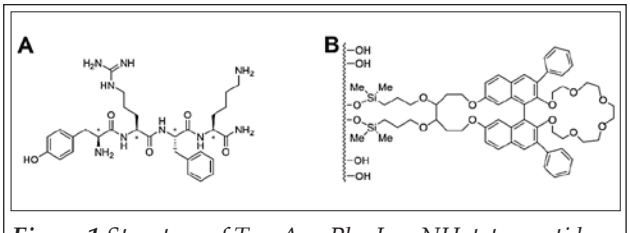

Figure 1 *Structure of Tyr-Arg-Phe-Lys-NH_2 tetrapeptide - **A** and chiral stationary phase in CROWNPAK columns: (S-(3,3′-diphenyl-1,1′-binaphthyl)-20-crown-6) for CR-I(+) and (R-(3,3′-diphenyl-1,1′-binaphthyl)-20-crown-6) for CR-I(-) immobilized on silica] –**B***

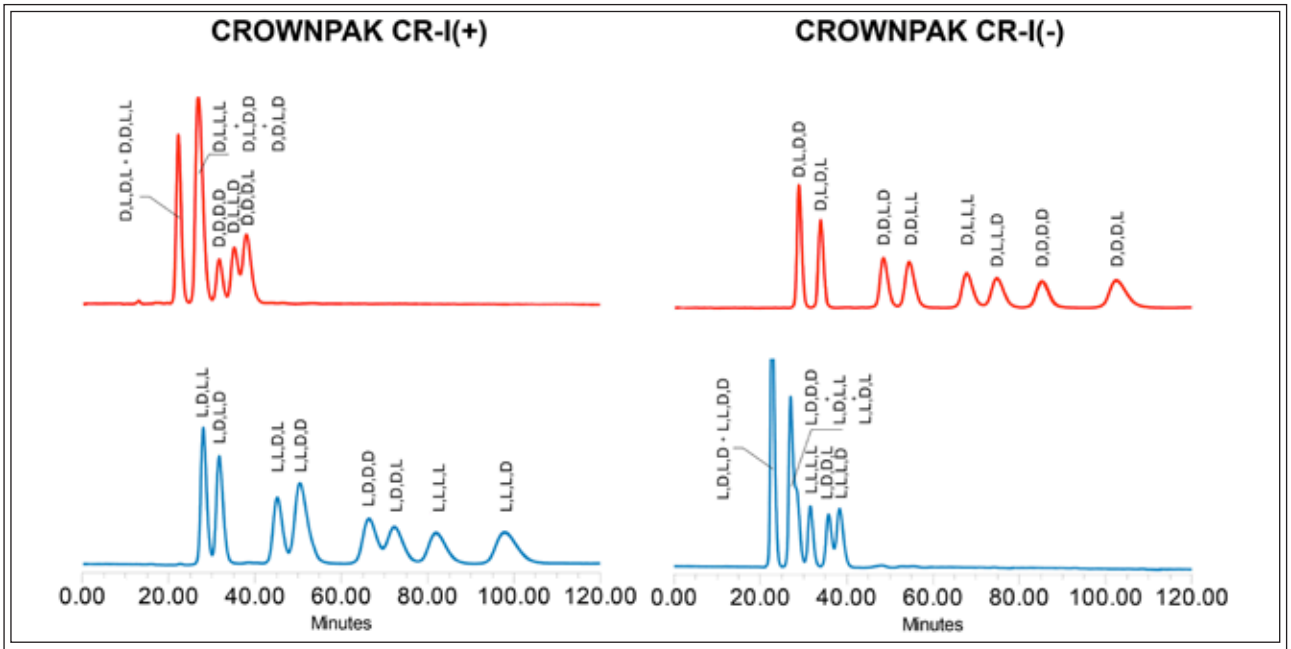

Figure 2 *Separation chromatograms of mixed standard solutions of eight* DXXX *(in red) and* LXXX *(in blue) Tyr-Arg-Phe-Lys-NH_2 stereoisomers on CROWNPAK CR-I(+) and CROWNPAK CR-I(-) columns. Mobile phase: ACN / 50mM $HClO_4$ = 15/85 (v/v).*

nyl-1,1'-binaphthyl)-20-crown-6 are both each other enantiomers, therefore, opposite elution order is expected for analyte enantiomers. A mixed standard solution of eight LXXX stereoisomers and a mixture of their corresponding DXXX enantiomers of Tyr-Arg-Phe-Lys-NH_2 was injected into LC system for evaluation of stereoisomer separation ability on both crown ether CSPs.

It was determined that the best stereoisomer separation can be achieved under reversed phase mode (15% ACN). Tetrapeptide enantiomers possessing LXXX configuration (L-Tyr at the N-terminus, see ***Figure 1A***) retained stronger than their D-antipodes (DXXX) on CROWNPAK CR-I (+) column and vice versa for CR-I (-) column.

As shown in ***Figure 2***, a mixture of eight LXXX tetrapeptide stereoisomers was separated on CROWNPAK CR-I (+) column. It appears, that dxxx tetrapeptide stereoisomers, that could not be resolved on the CR-I (+) column can be resolved on CR-I (-) column. This property could be used in the analysis of a real life samples in order to be able to identify the optical impurities.

4. Conclusions

Reversed phase LC conditions were found to achieve the best separation for a mixture of Tyr-Arg-Phe-Lys-NH2 stereoisomers. The combined analysis on both columns of the Tyr-Arg-Phe-Lys-NH2 sample is a convenient approach that would provides a broader insight within real life sample optical purity. These LC conditions allow the identification and quantification of sixteen Tyr-Arg-Phe-Lys-NH2 from the mixture.

5. Acknowledgements

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