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Short Communication

High-performance liquid chromatography of *cis–trans* isomers of proline-containing dipeptides III. Comparative studies with different stationary phases

A. Wutte[a], G. Gübitz*[,a], S. Friebe[b], G.-J. Krauss*[,b]

[a]*Institute of Pharmaceutical Chemistry, Karl-Franzens-University of Graz, Universitätsplatz 1, A-8010 Graz, Austria*
[b]*Department of Biochemistry and Biotechnology, Martin-Luther-University of Halle, Weinbergweg 16a, D-06120 Halle, Germany*

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Abstract

Different stationary phases were compared for their capacity to separate *cis–trans* isomers of proline-containing dipeptides for equilibrium and isomerization studies. Both reversed-phase chromatography on an RP-18 or a graphitized carbon phase (Hypercarb S) and ligand-exchange chromatography on an L-proline–Cu(II) phase can be used effectively to separate the *cis–trans* isomers.

1. Introduction

Conformational changes of the peptide bond occurring in peptides with a C-terminal proline or N-alkylamino acids have been studied using different spectroscopic and kinetic methods [1–5]. Several approaches for the investigation of dynamic equilibria have been reported, including *cis–trans* isomerism of proline-containing peptides by high-performance liquid chromatography (HPLC). Melander et al. [6] and others described experiments to obtain quantitative data on the conformational changes of the peptidyl–proline imidic bond by low-temperature RP-HPLC. According to Melander et al. [6], differing hydrophobic interactions of the conformers are responsible for the resolution. Recently, we introduced cyclodextrin-bonded silicas for the chromatographic separation of peptide-bond isomers [7,8]. The conformers of proline-containing di- and oligopeptides were resolved with high efficiency using steric discrimination by inclusion complexation. Even peptides with two peptidyl–proline bonds split into their conformer peaks. Similar results concerning dipeptides have been obtained when using an RP-8 phase and β-cyclodextrin as additive to the mobile phase [8].

This paper deals with comparative studies of different HPLC supports representing the reversed-phase mode (RP-18 phase and a graphitized carbon phase) and the ligand-exchange mode using an L-proline–Cu(II) bonded phase, previously developed for the resolution of enantiomers [9,10]. Further, the influence of β-

* Corresponding author.

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cyclodextrin as an additive in combination with the graphitized carbon phase was studied.

2. Experimental

2.1. Materials

Optically pure dipeptides were purchased from Bachem Biochemica (Heidelberg, Germany) and β-cyclodextrin from Merck (Darmstadt, Germany). The separations were carried out on a 250 × 4 mm I.D. Separon SG × RP-18 (7 μm) column from Tessek (Prague, Czech Republic) and a 100 × 4.6 mm I.D. Hypercarb S (7 μm) column from Shandon Scientific (Astmore, UK). The 250 × 4.6 mm I.D. L-proline–Cu(II) ligand-exchange chromatographic (LEC) column (5 μm) was prepared as reported previously [9,10].

2.2. Apparatus

HPLC separations were performed with a Merck–Hitachi HPLC system consisting of an L-6200 intelligent pump, an L-4000 UV detector and a computing integrator. A Lauda RM6 thermostat regulated cooling of the columns.

2.3. Chromatographic conditions

Chromatographic experiments were performed isocratically using phosphate buffer–acetonitrile eluents of different composition and pH. The analyte absorptions were monitored at 223 nm. Peak splitting as a result of isomerization processes was verified by reinjection of the fractionated peaks, whereby the same retention time was obtained in this second separation for the peak collected.

3. Results and discussion

3.1. Separation on RP-18 phase

Efficient separations with significant α-values were obtained for Ala–Pro, Leu–Pro, Ile–Pro and Phe–Pro on the RP-18 phase at low temperature (Table 1). According to previous studies by Melander et al. [6] on an RP-8 phase, the elution order was interpreted to be *trans* before *cis*, owing to the stronger hydrophobic interactions of the larger hydrophobic surface of the *cis* isomer with the reversed phase. The absolute order of elution, however, was not determined either by Melander et al. or in this study.

The ratio of the *cis* and *trans* isomers in solution was found to be dependent on temperature, solvent composition and pH. This conforms with the investigations of Henderson and Horvath [11]. In an aqueous solution of Leu–Pro freshly prepared at 0°C, the first isomer eluted, postulated as *trans*, was significantly dominant, reflecting the presumed original ratio. After exposing the solution to room temperature, the equilibrium changed (Fig. 1). These observations were confirmed on the other phases used.

Table 1
k' and α values for the *cis–trans* isomers of dipeptides on different phases

Dipeptide	Hypercarb S			RP-18			L-Proline–Cu(II)		
	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α
Ala–Pro	3.4	6.6	1.9	3.7	4.7	1.2	6.0	7.6	1.2
Ile–Pro	5.2	11.4	2.1	6.0	12.5	2.0	7.5	10.3	1.3
Leu–Pro	5.0	12.8	2.6	5.7	9.0	1.5	7.3	10.5	1.4
Phe–Pro	16.0	67.0	4.1	9.5	38.3	4.0	15.6	48.3	3.1

Hypercarb S and RP-18: mobile phase, 0.05 *M* phosphate buffer (pH 6.2)–acetonitrile (92:8). L-Proline–Cu(II): mobile phase, 0.05 *M* phosphate buffer (pH 4.5)–acetonitrile (92:8). Temperature, 0°C; flow-rate, 1 ml/min.

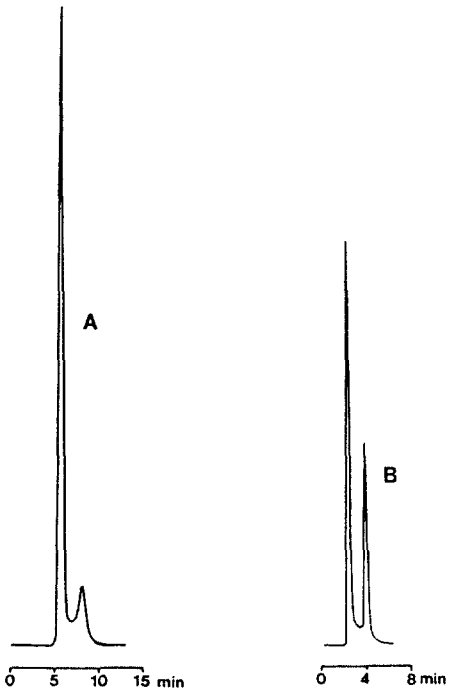

Fig. 1. Isomerization of Leu–Pro. Column, RP-18; mobile phase, 0.05 *M* phosphate buffer (pH 6.2)–acetonitrile (92:8); flow-rate, 1 ml/min. (A) Injection of an aqueous solution of Leu–Pro freshly prepared at 0°C; (B) isomerization after storage of the solution at room temperature.

3.2. Separation on Hypercarb S phase

All dipeptides show higher retention than with the octadecylsilica column (Table 1). The retention mechanism is assumed to be influenced by the layer structure of the graphite [12] and also by hydrophobic interactions.

Experiments using an acidic (pH 4.5) or a basic (pH 8) eluent resulted in extremely high retention values without peak splitting. An eluent of pH 6.2 was found to be most effective in separating the two isomers. To decrease the retention time of the second peak and so to reduce the plateau between the two peaks caused by relaxation of the molecules, a higher ionic strength of the buffer and an organic modifier were used. Fig. 2 demonstrates the influence of temperature and organic modifiers on the separation of the conformers of Leu–Pro.

Phe–Pro showed an extremely high retention time with only two spikes and a marked plateau between them. In contrast to Ala–Pro, Leu–Pro and Ile–Pro, the peak ratio of the conformers of Phe–Pro was reversed. This peak ratio is analogous to that found on reversed phases. The retention time decreased and the resolution improved when β-cyclodextrin (β-CD) was added to the mobile phase (Fig. 3). This phenomenon can be explained by steric hindrance of isomer interconversion caused by the formation of an inclusion complex. The plateau observed between the isomer peaks in separations without β-CD was found to be significantly reduced. These results are in accordance with previous investigations using β-CD as a mobile phase additive in combination with reversed phases or by using chemically bonded β-CD phases [7,8].

To study peak splitting as a result of peptide bond isomerization, fractions collected from both peaks of Ile–Pro were reinjected. After exposure of the solution to ambient temperature for 45 min, relaxation to the original peak ratio was observed.

3.3. Separation by ligand-exchange chromatography

The third type of stationary phase investigated was a chemically bonded L-proline–Cu(II) phase, previously used for the resolution of enantiomers [9,10]. The idea was to obtain a discriminant steric fixation of the isomers by chelate complexation and the isomers were in fact resolved at low temperature. Without copper on the column there was no separation (Fig. 4).

Surprisingly, the peak ratio was the reverse of that on the RP-18 and Hypercarb S phases for all the dipeptides investigated, indicating a possibly reversed elution order due to a different separation mechanism (compare Fig. 1 with Fig. 4). A freshly prepared aqueous solution of Leu–Pro showed a dominant second peak, presumably the *trans* isomer. The same behaviour was observed for all dipeptides except Phe–Pro. Injection of a fraction of the second peak of Ile–Pro collected from RP-18 on to the L-proline column resulted in a dominant first peak (Fig. 5). By analogy, if a fraction of the first peak was collected and

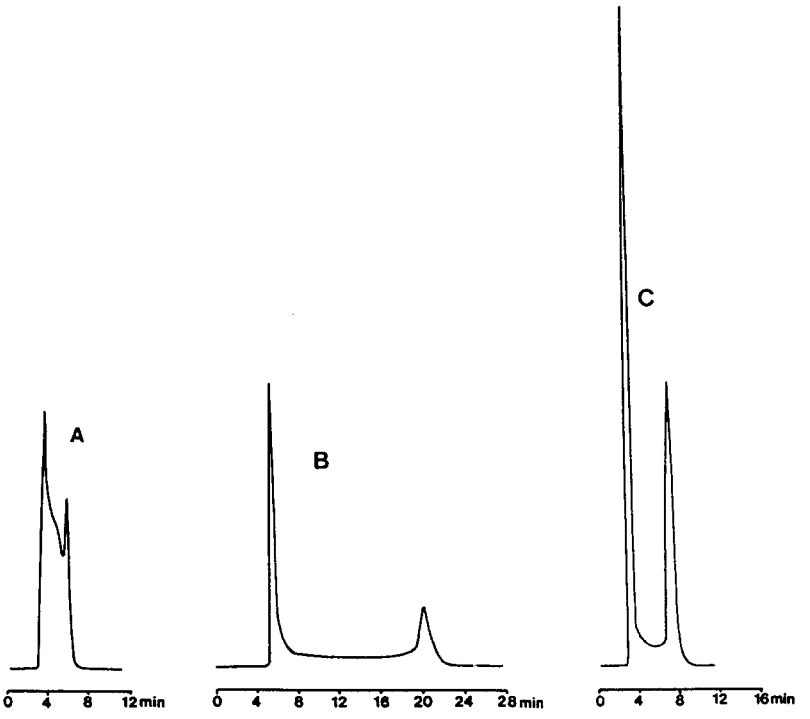

Fig. 2. Separation of the *cis–trans* isomers of Leu–Pro on Hypercarb S: influence of temperature and organic modifier on the resolution. (A) Temperature, 23°C; mobile phase, 0.05 *M* phosphate buffer (pH 6.2); flow-rate, 1 ml/min. (B) Temperature 5°C; mobile phase as in (A). (C) Temperature, 5°C; mobile phase, 0.05 *M* phosphate buffer (pH 6.2)–acetonitrile (92:8).

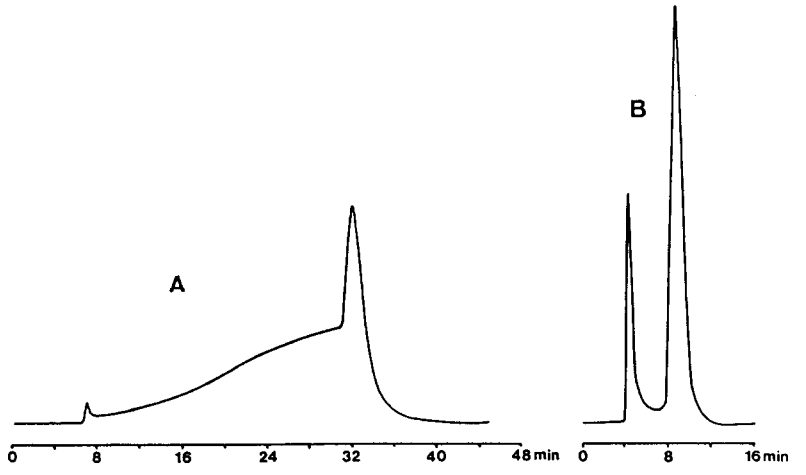

Fig. 3. Separation of the *cis–trans* isomers of Phe–Pro on Hypercarb S: influence of cyclodextrin addition. Temperature, 5°C; flow-rate, 1 ml/min. Mobile phase: (A) 0.05 *M* phosphate buffer (pH 6.2)–acetonitrile (90:10); (B) 0.05 *M* phosphate buffer (pH 6.2)–acetonitrile (90:10) + 0.01 *M* β-cyclodextrin.

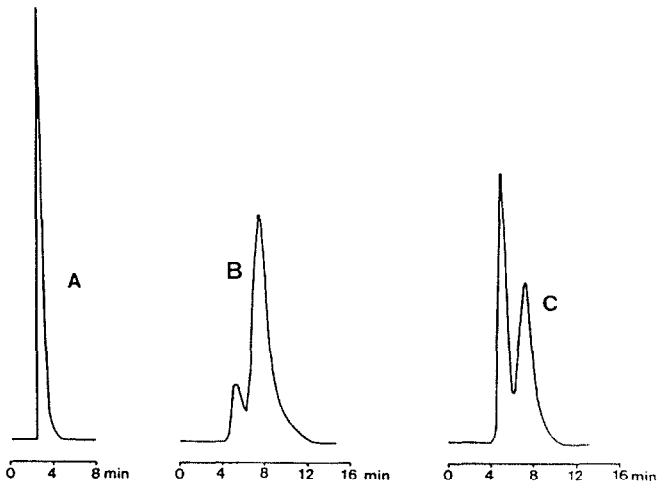

Fig. 4. Separation of the *cis–trans* isomers of Leu–Pro by ligand-exchange chromatography. Flow-rate, 1 ml/min; temperature, 0°C. (A) Column, L-proline without Cu(II); mobile phase, 0.05 *M* phosphate buffer (pH 4.5)–acetonitrile (92:8). (B) Injection of an aqueous solution of Leu–Pro freshly prepared at 0°C. Column, L-proline–Cu(II); mobile phase, 0.05 *M* phosphate buffer (pH 4.5) + 10^{-4} *M* copper(II) sulfate–acetonitrile (92:8). (C) Isomerization after storage of the solution at room temperature for 45 min. Conditions as in (B).

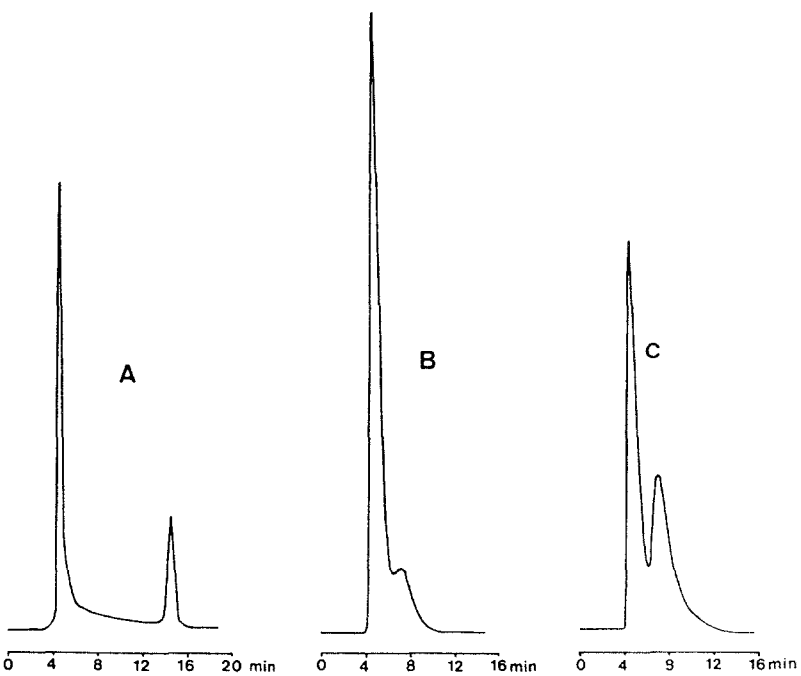

Fig. 5. Reinjection of a fraction of the second peak of Ile–Pro collected from the RP-18 column on to the L-proline–Cu(II) column. (A) Separation of an aqueous solution of Ile–Pro on the RP-18 column. Mobile phase, 0.05 *M* phosphate buffer (pH 4.5)–acetonitrile (98:2); temperature, 0°C; flow-rate, 1 ml/min. (B) Reinjection of a fraction of the second peak on to the L-proline–Cu(II) column. Conditions as in Fig. 4B. (C) Isomerization after storage of the fraction at room temperature for 45 min.

injected on to the L-proline column, the second peak was dominant. These results were confirmed in reverse by collecting fractions from the L-proline column and transferring them on to the RP-18 column.

4. Conclusions

Conformational changes of the peptide bond in proline-containing dipeptides were studied using HPLC with stationary phases utilizing different separation principles. It was shown that in addition to the conformer separations on reversed-phase and β-cyclodextrin-bonded silica described recently, a graphitized carbon phase and a chemically bonded L-proline–Cu(II) phase are additional tools for the investigation of *cis–trans* isomerism in proline-containing peptides. Based on the different discrimination mechanism, the elution order of the peptide conformers can change. The real elution order of the *cis* and *trans* forms can only be established by NMR analysis immediately after low-temperature isomer fractionation.

The composition of the mobile phase, pH, temperature and flow-rate are variables affecting the dynamic equilibrium and, accordingly, the resolution of peptide conformers in the corresponding chromatographic system.

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