ELSEVIER

Journal of Chromatography A, 874 (2000) 21–26

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Chromatographic resolution of tryptophan enantiomers with L-Leu–L-Leu–L-Leu peptide
Effects of mobile phase composition and chromatographic support

Deborah B. Kaufman[a], Timothy Hayes[b], Joseph Buettner[b], David J. Hammond[c], Ruben G. Carbonell[a,*]

[a]*Department of Chemical Engineering, North Carolina State University, Box 7905, Raleigh, NC 27695-7905, USA*
[b]*Bayer Corporation, NCSU Centennial Campus, 1017 Main Campus Drive, Suite 3800, Raleigh, NC 27606, USA*
[c]*V.I. Technologies, Inc., Audubon Center, 3960 Broadway, New York, NY 10032, USA*

Received 25 August 1999; received in revised form 7 December 1999; accepted 14 December 1999

Abstract

Tryptophan enantiomers have been separated by zwitterion pair chromatography using L-leucine–L-leucine–L-leucine peptide as the zwitterion pairing agent. The peptide ligand is adsorbed onto an octadecylsilane support with excess ligand present in bulk solution. This article examines the roles of the hydrophobic matrix and the mobile phase components on tryptophan enantiomer binding and resolution. Capacity factors and enantioselectivites are given for both hydrophobic and hydrophilic matrices using mobile phases containing Leu–Leu–Leu peptide and/or salt. A decrease in selectivity upon the addition of mobile phase salt suggests that quadrupolar ion-pairing contributes to chiral recognition. Results indicate that binding is significantly reduced and separation is not achieved when Leu–Leu–Leu is coupled onto cross-linked or polymerized hydrophilic resins as well as onto macroporous polystyrene resin. However, resin-immobilized Leu–Leu–Asp–Leu–Leu–Leu, Leu–Leu–Glu–Leu–Leu–Leu, and Leu–Leu–Leu–Glu–Leu–Leu peptides, with ion-pairing sites designed to mimic the Leu–Leu–Leu-saturated C_{18} support, also do not resolve tryptophan enantiomers. This suggests the Leu–Leu–Leu structure is critical for enantiomer resolution. Because D- and L-tryptophan are separated in the absence of bulk Leu–Leu–Leu, chiral discrimination is believed to occur at the surface of the octadecylsilane support.

Keywords: Enantiomer separation; Mobile phase composition; Stationary phases, LC; Amino acids; Tryptophan

1. Introduction

Chromatography is ‘‘winning a place in chiral separations’’ by offering high selectivities, 100% yields of both enantiomers, low development costs and short production times [1]. To achieve separation, a minimum of three interactions, at least one that is stereodependent, must occur between the enantiomer and the affinity ligand [2]. To develop affinity ligands for the large-scale separation of enantiomers, the mechanisms of enantiomer binding and resolution must be examined.

In this paper, we investigate the effect of the chromatographic support and mobile phase composition on the binding and chiral discrimination of tryptophan enantiomers by the affinity ligand L-Leu–

*Corresponding author. Fax: +1-919-5153-465.

0021-9673/00/$ – see front matter
PII: S0021-9673(99)01299-6

L-Leu–L-Leu. Knox and Jurand first reported this separation using an octadecylsilane (C_{18}) column with L-Leu–L-Leu–L-Leu (LLL) peptide as an additive to pH 6.3 phosphate buffer [3]. Because LLL and tryptophan exist as zwitterions at this pH, Knox and Jurand postulated that quadrupolar ion pairing provides two of the three interactions necessary for chiral discrimination. This hypothesis is also supported by the work of Ravichandran and Rogers, who observed a decrease in enantioselectivity (α) as the eluent pH deviated from the center of the pH ''window'' where both tryptophan and LLL occur as zwitterions [4].

Also critical to this separation are the surface density of Leu–Leu–Leu on the reversed-phase packing and the peptide ligand structure. Tryptophan enantiomers are not resolved until a substitution of ca. 0.65 μmol Leu–Leu–Leu/m^2 resin is achieved at the C_{18} surface [3–5]. Mobile phases containing L-Val–L-Val–L-Val, L-Val–L-Val, or L-Leu–L-Leu fail to separate D- from L-tryptophan [4].

The nature of the chromatographic support, in addition to ligand structure, may affect chiral resolution [6,7]. Ravichandran and Rogers observed that residual silanol groups do not affect tryptophan retention and enantioselectivity [4]; however, the role of the reversed-phase packing itself was not considered. It is possible that the hydrophobic support promotes separation by concentrating tryptophan at the surface. Chiral discrimination with Leu–Leu–Leu could occur either at this surface or in bulk solution.

To answer these questions, we have immobilized the L-Leu–L-Leu–L-Leu affinity ligand onto both hydrophilic and hydrophobic chromatography supports. Because Leu–Leu–Leu immobilization removes a potential ion-pairing site, we have synthesized peptides to mimic the Leu–Leu–Leu-saturated C_{18} (Leu–Leu–Leu–C_{18}) system of Ravichandran and Rogers, namely Leu–Leu–Asp–Leu–Leu–Leu (LLDLLL), Leu–Leu–Glu–Leu–Leu–Leu (LLELLL), and Leu–Leu–Leu–Glu–Leu–Leu (LLLELL). Each peptide is covalently attached through its C-terminal leucine; therefore, the N-terminal amine and the carboxyl side groups of Asp or Glu provide ion-pairing sites. The peptides differ slightly in structure at the negatively-charged ion-pairing site and/or in the main chain distance between ion-pairing sites. By adding salt to the mobile phase in the Leu–Leu–Leu–C_{18} system, we further tested the quadrupolar ion-pairing theory.

In the following sections, we report the effects of mobile phase salt and Leu–Leu–Leu on the chromatographic separation of tryptophan enantiomers in a Leu–Leu–Leu–C_{18} column. Capacity factors and enantioselectivities are compared for the various mobile phases. The effect of the chromatographic support on tryptophan resolution by Leu–Leu–Leu and related peptides is also presented.

2. Experimental

2.1. Materials

Sodium chloride, L-leucine–L-leucine–L-leucine, D-tryptophan and L-tryptophan were obtained from Sigma (St. Louis, MO, USA); HPLC-grade methanol and acetic acid were purchased from Fisher Scientific (Pittsburgh, PA, USA). Deionized (dI) water was purified by a Barnstead nanopure water purification system (Dubuque, IA, USA). A 100 mm×4.6 mm I.D. Hypersil 120 Å ODS CAP column (10 μm spherical particles) was purchased from Alltech (Deerfield, IL, USA).

2.2. Resin derivatization and column packing

Leu–Leu–Leu, LLDLLL, LLELLL, and LLLELL peptides were synthesized directly onto polyhydroxylated methacrylate (Toyopearl AF-Amino-650M resin; TosoHaas, Montgomeryville, PA, USA) and cross-linked polystyrene (ArgoPore-NH_2; Argonaut Technologies, San Carlos, CA, USA) resins via standard Fmoc (9-fluorenylmethoxycarbonyl) chemistry following the procedure of Buettner et al. [8]. A Gilson AMS422 Multiple Peptide Synthesizer (Middleton, WI, USA) was employed for the synthesis. The peptide compositions were confirmed and the degree of substitution of the resins calculated by quantitative amino acid analysis (Commonwealth Biotechnologies, Richmond, VA, USA). The LLDLLL, LLELLL, and LLLELL methacrylate res-

ins were synthesized to a density of approximately 1.1 μmol peptide per m^2 of resin, and the molar ratio of leucine to aspartic acid or glutamic acid residues ranged from 5.3 to 5.6. The surface coverage of Leu–Leu–Leu–methacrylate resin was determined to be 1.4 μmol peptide per m^2 of resin.

Peptide densities on macroporous polystyrene resin averaged 0.7 μmol peptide per m^2 of resin. The molar ratio of leucine to aspartic or glutamic acid residues on the LLDLLL, LLELLL and LLLELL polystyrene supports are 4.3, 4.6 and 5.0, respectively.

Peptide-resins were also sequenced on a Hewlett-Packard N-Terminal Protein Sequencer G1000A at Protein Technologies Labs., Texas A&M University, TX, USA [9]. All supports, excluding LLDLLL resin, yielded complete signals. On the LLDLLL TosoHaas and LLDLLL polystyrene supports, sequencing halted at the Asp residue. The reason is unknown.

Dried peptide-resins were packed into 100 mm× 4.6 mm I.D. OmegaChrom polyether ether ketone (PEEK) columns (Thomson, Springfield, VA, USA) under vacuum, then washed with over five column volumes of methanol–water (50:50).

2.3. Chromatography

Chromatography experiments were performed at room temperature in triplicate. All aqueous mobile phases were adjusted to pH 6.3, filtered through a 0.2-μm nylon membrane, and degassed before use. Unless otherwise noted, 10-μl samples of 2.4 m*M* D- or L-tryptophan in buffer A (1 m*M* phosphate buffer, $Na_2HPO_4+H_3PO_4$) were injected and monitored at 280 nm.

For the experiments on the C_{18} and the hydrophilic affinity supports, a Perkin-Elmer Series 200 LC pump (Norwalk, CT, USA), a Spectroflow 783 variable-wavelength detector (Applied Biosystems, Ramsey, NJ, USA), and a Dynamax data acquisition module (Rainin, Ridgefield, NJ, USA) were employed. Tryptophan samples were injected via a 7125 Rheodyne valve and a 20-μl injection loop. A 250 p.s.i. back pressure regulator (Fisher) was installed before the injection loop to help maintain a constant flow-rate (1 p.s.i.=6894.76 Pa).

To saturate the C_{18} column with Leu–Leu–Leu peptide, buffer A containing 2 m*M* Leu–Leu–Leu was applied at a flow-rate of 1 ml/min. Saturation of the resin was detected by monitoring the breakthrough curve of Leu–Leu–Leu at 214 nm. After changing to an appropriate eluent, 10-μl samples of 2.4 m*M* racemic tryptophan were applied to the column over a 3-h period. D- And L-tryptophan were also injected independently, as well as in a dose–response manner, to determine the elution order. Experiments were isocratic, with a constant flow-rate of 0.6 ml/min. Before each experiment, the column was equilibrated with buffer A and re-saturated with Leu–Leu–Leu. When not in use, the C_{18} resin was stored in methanol–water (50:50). Column efficiency was estimated with the Dynamax data acquisition module using the peak width at half peak height [10], and 45 μ*M* uracil, monitored at 254 nm, was injected into a methanol–water (63:37) mobile phase to approximate t_0.

As a control, racemic tryptophan was injected into the C_{18} column without prior Leu–Leu–Leu saturation. Buffer A, as well as buffer A containing 1 *M* NaCl, were employed as mobile phases.

The derivatized TosoHaas resins were rinsed sequentially with several column volumes of 50% methanol in dI water, dI water, 2% acetic acid in dI water, and dI water, then equilibrated with buffer A before each experiment. Running buffer was applied isocratically at flow-rates of 0.6 and 0.1 ml/min.

Chromatography on the polystyrene affinity supports was performed on an Ultrafast Microprotein Analyzer (Michrom BioResources, Pleasanton, CA, USA) in conjunction with a Multiposition Electric Actuator (VICI Valco Instruments, Houston, TX, USA) and an EZChrom Chromatography Data System (Scientific Software, San Ramon, CA, USA). Samples (20 μl) were injected onto the columns via a Gilson 231-401 Sampling Injector (Gilson, Middleton, WI, USA) and a 20-μl sample loop. Buffer A containing 0.1 m*M* Leu–Leu–Leu was applied isocratically at a flow-rate of 0.6 ml/min. The columns were washed with over 13 column volumes of methanol–buffer A (70:30), then re-equilibrated with buffer A between each experiment. In each column, five injections were made: phosphate buffer (blank), two injections of 48 m*M* D-Trp in buffer A, and two injections of 48 m*M* L-Trp in buffer A.

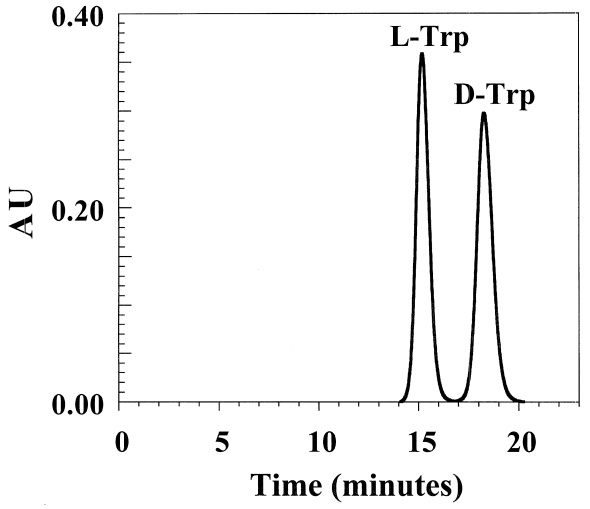

Fig. 1. Chiral HPLC separation of tryptophan enantiomers in the Leu–Leu–Leu–C_{18} column 203 min following saturation. R_s= 2.12; α=1.23. Mobile phase: 1 m*M* phosphate buffer (pH 6.3) containing 0.1 m*M* Leu–Leu–Leu. Conditions: flow-rate 0.6 ml/min; detection at 280 nm; 10-μl injection of 2.4 m*M* racemic tryptophan in 1 m*M* phosphate buffer, pH 6.3.

3. Results and discussion

3.1. Effects of mobile phase composition

A shown in Fig. 1, tryptophan resolution is easily reproduced with D-Trp as the more-retained isomer. Enantiomers are not resolved until the C_{18} column is saturated with approximately 1 μmol Leu–Leu–Leu per m^2 of resin. Furthermore, the isomers are retained in the C_{18} column more than twice as long as in the Leu–Leu–Leu-saturated C_{18} column (data not shown). As illustrated in Fig. 2, capacity factors (k') in the peptide-saturated column increase with time. These observations imply, as stated by Ravichandran and Rogers, that Leu–Leu–Leu gradually desorbs from the surface. Even in running buffer containing 0.1 m*M* peptide, equilibrium between mobile phase and adsorbed peptide is not achieved. As peptide molecules desorb from the C_{18} surface, the hydrophobicity of the stationary phase increases. Retention times of the enantiomers gradually approach that on the pure C_{18} stationary phase.

We further noted that peak efficiency (N) values doubled to ~2500 in the 3 h following saturation (data not shown). This increase, as well as the higher N value (4300) achieved in a non-saturated C_{18} column, suggest the interactions between tryptophan and the adsorbed peptide contribute to peak broadening. As Leu–Leu–Leu desorbs, the interactions decrease and efficiency increases.

In eluent containing Leu–Leu–Leu, chiral discrimination may occur at the C_{18} surface or in the bulk solution. Repeating the experiment using only aqueous phosphate buffer, we found that D- and L-tryptophan are still separated in the absence of mobile phase Leu–Leu–Leu; hence we can conclude that resolution occurs at the surface. A gradual stripping of adsorbed peptide therefore parallels a decrease in enantioselectivity over time (Fig. 2). As peptide desorbs, less Trp–Leu–Leu–Leu complex on the support can be formed, and selectivity decreases. Selectivity is improved by employing a running buffer containing peptide: the mobile phase peptide, adsorbing to the surface, shifts the surface equilibrium in the direction of forming more Trp–Leu–Leu–Leu complex. This promotes better separation than when using no mobile phase peptide. Hence in the 3-h time frame of the experiment, enantioselectivities decreased from 1.44 to 1.25 in the presence of mobile phase LLL, while enantioselectivities decreased from 1.40 to 1.23 in the absence of mobile phase LLL.

It should be noted that Ravichandran and Rogers observed a constant and lower enantioselectivity value (α=1.19) using 0.1 m*M* Leu–Leu–Leu in buffer A at 2–24 h following column saturation. The higher enantioselectivity values we observed using the same 0.1 m*M* LLL mobile phase can be explained by a higher peptide density achieved when saturating the C_{18} support (1 μmol peptide per m^2 resin versus 0.65 μmol peptide per m^2 resin achieved by Ravichandran and Rogers). Ravichandran and Rogers' observation of constant enantioselectivity, however, implies a stable stationary phase, i.e., one in which the peptide desorbing from the C_{18} surface is replenished by peptide in the running buffer. This contradicts their observation of increasing retention time with time following saturation. As a result, we are unable to explain the observation of constant enantioselectivity at this time.

Tryptophan retention also increases in eluent containing 1 *M* NaCl (Fig. 2). Salt promotes hydrophobic interactions between tryptophan and the

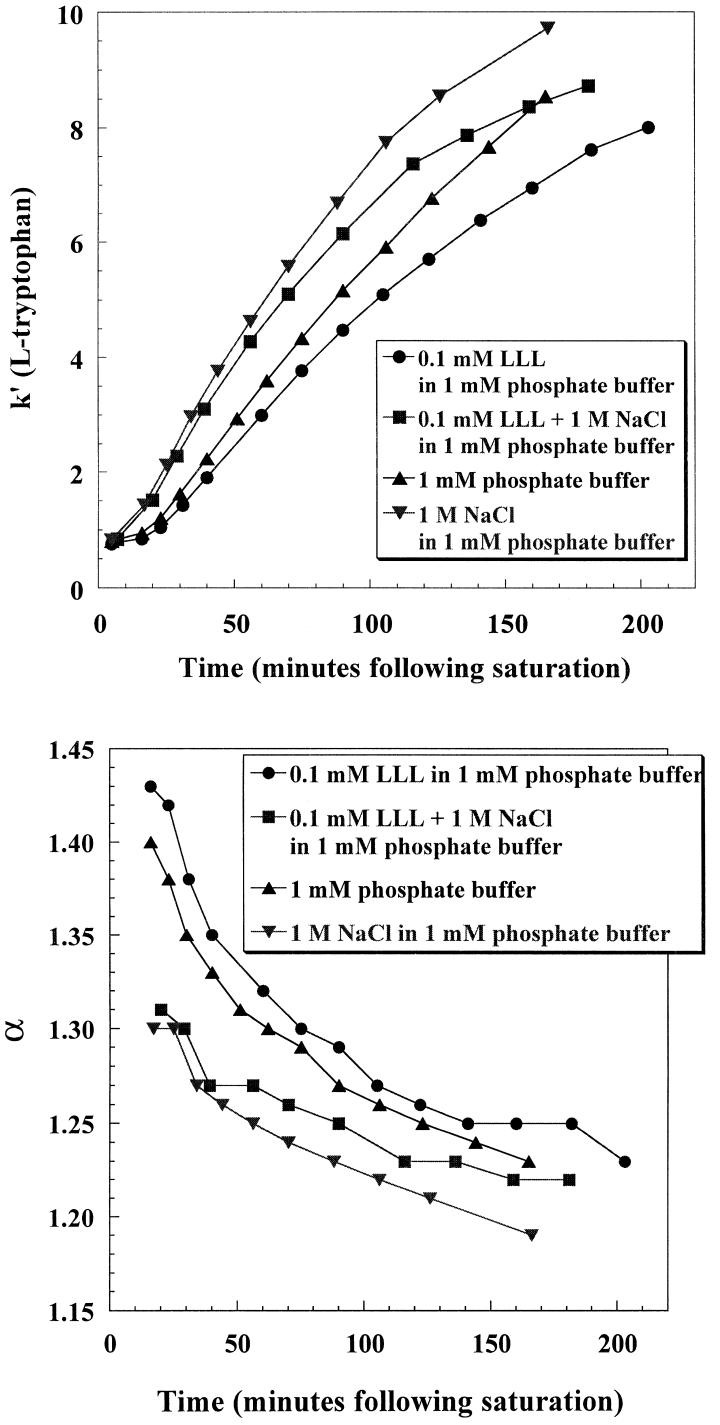

Fig. 2. (Top) Dependence of k' (L-tryptophan) with time. Conditions as in Fig. 1. The same trends are found for D-tryptophan. (Bottom) Decrease in enantioselectivity with time. Conditions as in Fig. 1.

stationary phase; e.g., even in a non-saturated C_{18} column, the capacity factor of tryptophan increases 43% when using 1 *M* NaCl in the phosphate solution. Enantiomeric resolution, however, decreases. (Fig. 2). These results support the hypothesis of quadrupolar ion paring: Na^+ and Cl^- ions compete with tryptophan to pair with Leu–Leu–Leu; hence α decreases.

3.2. Effect of the nature of the affinity support

Although peptide densities greater than or equal to those on the Leu–Leu–Leu–C_{18} support were achieved, D- and L-tryptophan are not resolved when Leu–Leu–Leu or related peptide ligands are bonded to the stationary phase. Tryptophan enantiomers are retained in these columns, with capacity factors in the Leu–Leu–Leu-type TosoHaas columns roughly an order of magnitude lower than in the Leu–Leu–Leu–C_{18} columns ($k'=0.21$–0.29 at 0.6 ml/min; $k'=0.26$–0.36 at 0.1 ml/min). The enantiomers are retained longer in the hydrophobic Leu–Leu–Leu-type columns than in the TosoHaas Leu–Leu–Leu-type columns: capacity factors average 1.4, 1.8, 1.8 and 10.7 in the LLLELL, LLELLL, LLDLLL and Leu–Leu–Leu polystyrene columns, respectively. With no hydrophilic Asp or Glu residues, Leu–Leu–Leu peptide immobilized to polystyrene bound Trp most tightly. These hydrophobic stationary phases concentrate tryptophan enantiomers at the surface; therefore, chiral recognition appears to be a function of ligand structure and/or conformation. Only on a C_{18} stationary phase to which Leu–Leu–Leu peptide is physically adsorbed will Trp enantiomers be resolved. Covalently attaching the peptide to either hydrophobic or hydrophilic supports disrupts the chiral-specific interactions.

In summary, because separation of D,L-tryptophan is achieved when the mobile phase contains no Leu–Leu–Leu, α values decrease as Leu–Leu–Leu desorbs from the C_{18} surface, and no separation is achieved with a non-Leu–Leu–Leu-saturated column, we conclude that resolution of D,L-tryptophan with Leu–Leu–Leu occurs at the C_{18} surface. Decreased enantioselectivity values in 1 *M* NaCl support the quadrupolar ion-pairing theory of Knox and Jurand. Bonded Leu–Leu–Leu peptide, which is unable to quadrupolar ion-pair, does not separate the enantiomers. Furthermore, adding ion-pairing sites to the bonded peptide does not promote separation. Differences in peptide structure between bonded LLDLLL, for example, and adsorbed LLL could account for the differences in enantioselectivity. Whether the nature of the C_{18} stationary phase also contributes to selectivity – by adsorbing the Leu–Leu–Leu peptide in a conformation that discriminates between the enantiomers – has not be determined.

This work illustrates that the affinity ligand, the stationary phase, and the means by which the ligand is attached to the stationary phase affect enantiomer association constants and the degree to which the enantiomers are resolved. Before designing or scaling-up columns for industrial enantiomeric separations, the roles of each must be defined.

Acknowledgements

We gratefully acknowledge financial support and equipment use from Bayer Corporation.

References

[1] S. Stinson, Chem. Eng. News 74 (1995) 44.
[2] C.E. Dalgliesh, J. Chem. Soc. 137 (1952) 3940.
[3] J. Knox, J. Jurand, J. Chromatogr. 234 (1982) 222.
[4] K. Ravichandran, L. Rogers, J. Chromatogr. 402 (1987) 49.
[5] J. Knox, J. Jurand, J. Chromatogr. 218 (1981) 341.
[6] T. Ihara, Y. Sugimoto, M. Asada, T. Nakagama, T. Hobo, J. Chromatogr. A 694 (1995) 49.
[7] R. Baczuk, G. Landram, R. Dubois, H. Dehm, J. Chromatogr. 60 (1971) 351.
[8] J. Buettner, C. Dadd, G. Baumbach, B. Masecar, D. Hammond, Int. J. Peptide Protein Res. 47 (1996) 70.
[9] C.G. Miller, Methods: Companion Methods Enzymol. 6 (1994) 315.
[10] P.C. Wankat, Rate-Controlled Separations, Blackie, New York, 1994.