Scientia Chromatographica 2019; 11(2):64-70
Instituto Internacional de Cromatografia
http://dx.doi.org/10.5935/sc.2019.008
ISSN 1984-4433

Enantioseparation of underivatized amino acids by capillary liquid chromatography. 2. (N, S-dioctyl-(D)-penicillamine) ligand exchange chiral stationary phase

Fernanda A. Daher, Natalia Gabrielly Pereira dos Santos, Edvaldo Vasconcelos Soares Maciel, Fernando Mauro Lanças*

University of São Paulo, São Carlos, Institute of Chemistry of São Carlos, 13560-970, SP, Brazil.

*Corresponding author. Tel: +(55) 16 3373 9983; Fax: +(55) 16 3373 9984. E-mail address: flancas@iqsc.usp.br

Abstract

Chiral capillary liquid chromatography of underivatized amino acids was investigated by using a chiral ligand exchange stationary phase (N, S-dioctyl-(D)-penicillamine) and a mobile phase containing Cu (II) ion as a complexion agent. The direct separation of four racemic α-amino acids was achieved and the influence of experimental variables such as the mobile phase composition, copper (II) concentration, pH on the retention, resolution, separation factor (α), and retention factor (k), was studied. The amino acids were isocratically separated using 2 mmol/L Cu(II) (pH 5.3)/methanol/water (15:85, v/v) as the mobile phase at a flow rate of 8μL/min, and determined by UV detection at 254 nm. The results showed that capillary liquid chromatography using a chiral stationary phase and Cu(II) in the mobile phase is an up-and-coming alternative technique for the enantiomeric separation of amino acids.

Keywords: Enantiomeric separation, capillary liquid chromatography; chiral ligand exchange stationary phase; underivatized amino acids, Cu(II) complexes, (N, S-dioctyl-(D)-penicillamine).

Resumo

A cromatografia líquida quiral capilar de aminoácidos intactos (não derivatizados) foi investigada usando uma fase estacionária a qual atua através da troca de ligantes (N, S-dioctyl-(D)-penicillamine), e uma fase móvel contendo íons Cu(II) como agente complexante. Uma separação direta de quatro α-amino ácidos racêmicos foi obtida, e a influência das variáveis experimentais foi investigada. A separação ocorreu no modo isocrático a um fluxo de 8μL/min, com detecção UV em 254 nm. Os resultados mostraram que a cromatografia líquida capilar empregando Cu(II) na fase móvel é uma boa alternative para a separação enantiomérica de amino ácidos quirais intatos.

Palavras chaves: Separação enantiomérica, cromatografia líquida capilar, fase estacionária quiral, troca de ligantes, aminoácidos, complexos de Cobre(II).

1. Introduction

The growing interest in analyzing minute samples in various fields as environmental, clinical, forensic, pharmaceutical chemistry and biotechnology, is one of the driving forces for the rapid development of miniaturized analytical techniques such as capillary liquid chromatography (capillary LC) [1].

Capillary LC is usually carried out on packed fused silica columns of 50-500 μm i.d. and flow rates of 4-10 μL/min. It offers some advantages as reduced consumption of mobile and stationary phases, increased mass sensitivity with concentration sensitive detectors, high separation efficiencies, minimum sample requirements, ease of interfacing with mass spectrometers, and the convenience of use in multidimensional chromatography and unified chromatography [2,3].

An area where the use of capillary LC columns is of particular interest is the separation of enantiomers. Much research has been focused on chiral separations. Although most of the work on enantiomeric separations has been done using conventional LC, miniaturization of the chromatographic system makes it possible to explore new stationary phases that are too valuable to use in larger diameter columns [4]. The reduced consumption of solvents also makes stereoselective additives in the mobile phase less costly [4,5]. Further, due to increased efficiency, it is possible to separate challenging stereoisomers having small chromatographic separation factors (α) [6,7].

Amino acids are an essential class of biological substances, and the separation of amino acid enantiomers has been widely investigated [8]. Chiral ligand exchange chromatography has proven to be a useful means for separating enantiomers of racemic α-amino acids [8]. Cu (II) complexes of optically active amino acids and their derivatives have been employed in resolving various racemic amino acids as chiral mobile phase additives or chiral stationary phases after binding covalently or hydrophobically to solid column support [8–10]. Noteworthy, Davankov et al. [11] was the first to present a chiral-phase system for the enantiomeric separation of amino acids composed by a reversed-phase packing coated with an appropriate resolving agent and a hydro-organic eluent containing the complex metal ion. Chiral coating agents such as N-alkylhydroxyproline (where alkyl is *n*-C_7H_{15}, *n*-$C_{10}H_{21,}$ and *n*-$C_{16}H_{33}$) produce excellent enantioselectivity, but the separation of some amino acids was still not good enough [12,13].

This study aimed to examine the suitability of capillary liquid chromatography in stereoselective separations of underivatized amino acids on a chiral ligand exchange phase (N, S-dioctyl-(D)-penicillamine) employing Cu^{+2} as the complexing agent.

2. Materials and Methods

2.1. Reagents

The four amino acids [valine (Val), isoleucine (Ile), tyrosine (Tyr), phenylalanine (Phe)] were all from Sigma (St. Louis, MO, USA). All other chemicals used were of analytical reagent-grade, and the solvents were of HPLC grade. Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). The mobile phase was prepared by dissolving a specified amount of CuSO4 in deionized water containing methanol as an organic modifier. Before use, the mobile phase was filtered through a 0.45 μm Millipore filter (Millipore, Bedford, MA, USA) and degassed by purging with helium for 30 min.

2.2. Apparatus and Conditions

The capillary LC equipment consisted of a Phoenix 20 syringe pump (Fisons, Beverly, MA, USA) connected to a packed in-house capillary LC column (200 mm x 0.53 mm i.d. x 5μm). A UV-VIS detector (Fisons, Beverly, MA, USA) operated at 254 nm, equipped with a 35 nL, u-shaped detection cell (Fisons, Beverly, MA,

USA). A microinjector made the injections with a 60 nL loop (Valco, Houston, TX, USA). The column was slurry-packed [12] with the N, S-dioctyl-(D)-penicillamine stationary phase (Phenomenex, Torrance, CA, USA). The analysis was carried out in the isocratic mode using 2 mM $CuSO_4.5H_2O$ /MeOH/H_2O (15:85, v/v) as the mobile phase. The data were recorded and integrated with a Chrom-Card data acquisition system (Carlo Erba, Rodano, MI, Italy).

2.3. Results and Discussion

The direct separation of the four amino acids investigated (valine, isoleucine, tyrosine, phenylalanine) was accomplished by ligand exchange capillary liquid chromatography using N, S-(D)-dioctyl-penicillamine as stationary phase (Figure 1) and a hydro-organic mobile phase containing Cu^{+2} as the complexing agent.

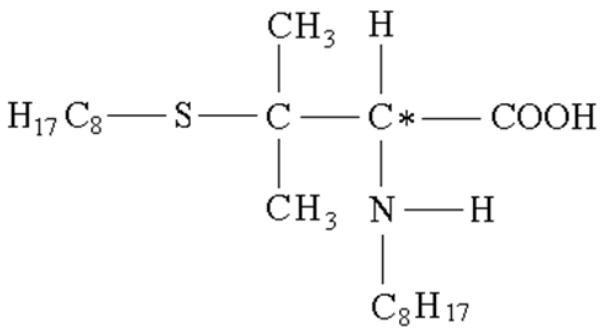

Figure 1. Chemical Structure of N,S-dioctyl-(D)-penicillamine.

Figure 2 shows the chromatogram of the four amino acids' enantiomers separated on N, S-(D)-dioctyl-penicillamine.

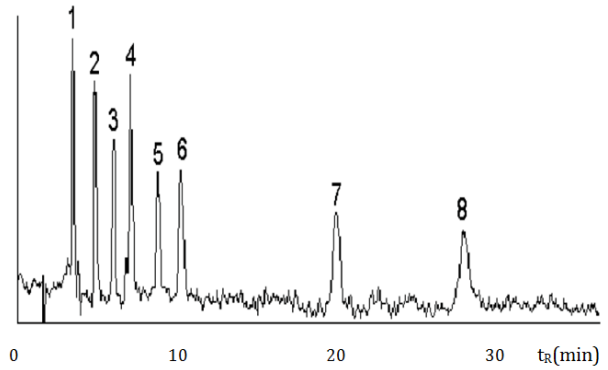

Figure 2. Enantiomeric Separation of the selected amino acids on a chiral capillary LC column. Column: CSP packed on a fused sílica tubing (200 mm x 0.53 mm x 5µm). Mobile phase: Cu^{+2} 2mM; methanol/water (15:85, v/v); pH 5.3; Flow = 8µL/min. 1. L-Val; 2. D-Val; 3. L-Ile; 4. L-Tyr; 5. D-Ile; 6. D-Tyr; 7. L-Phe; 8. D-Phe

The effect of the variation of Cu^{+2} concentration in a mobile phase of constant composition [MeOH/H_2O (15:85, v/v)] on the retention factor (k), resolution (R), and separation factor (α) is summarized in Figures 3-5.

As shown in Figure 3, the retention of the enantiomers increases as the Cu^{+2} concentration increase until a maximum is reached ([Cu^{+2}]=1 mM). From this point, the retention of the enantiomers decreases.

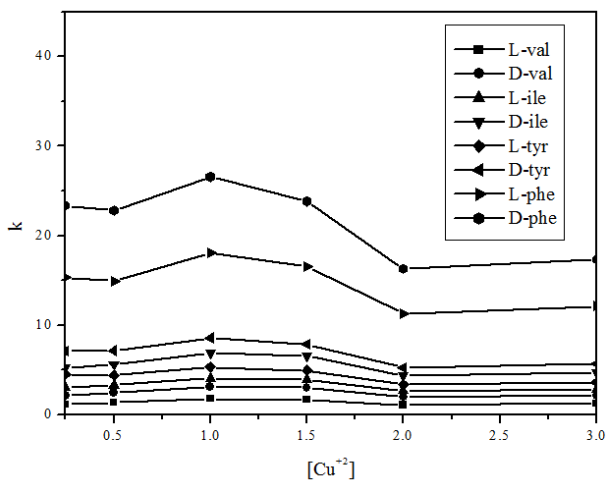

Figure 3. Influence of [Cu^{+2}] on the resolution factor (k) of selected chiral amino acids.

This behavior can also be observed in Figure 4, which shows the effect of the increase of Cu^{+2} concentration on the resolution.

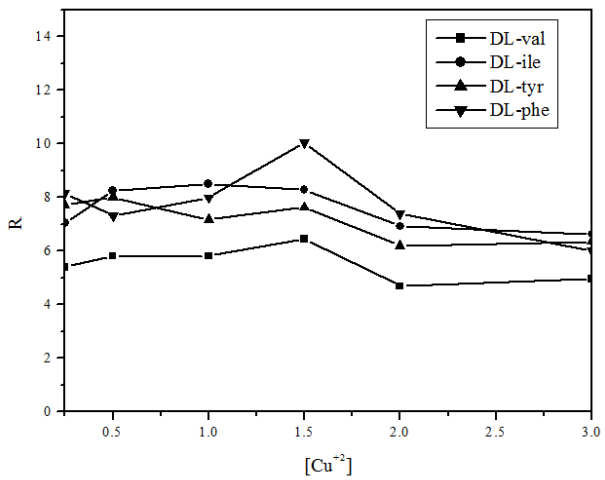

Figure 4. Influence of Cu^{+2} on the resolution (R) of selected chiral amino acids.

The enantioselectivity denoted by the separation factors α does not show any noticeable tendency (Figure 5).

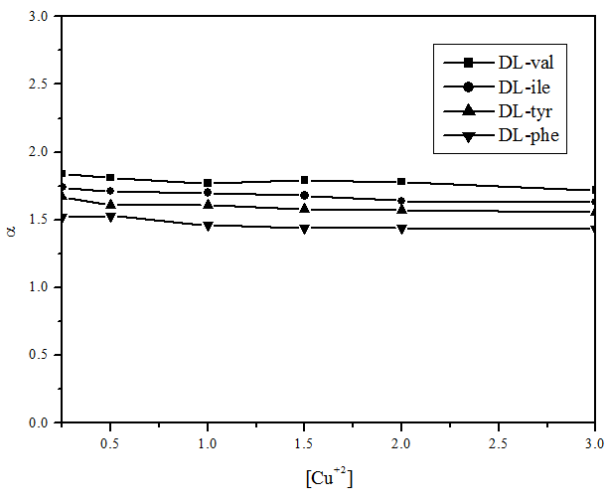

Figure 5. Influence of [Cu^{+2}] on the separation factor (α) of selected chiral amino

The diminution in the retention of the enantiomers at higher concentrations of Cu^{+2} in the mobile phase may be explained by the fact that increasing the Cu^{+2} concentration enhances the formation of the transient complex between Cu^{+2} and α-amino acids. As the stereoselectivity of the isomers of amino acids depends on their complexation with the Cu^{+2} in the mobile phase, this could explain the high-resolution values found between 1mM-2mM. These high-resolution values confirm that N, S-(D)-dioctyl-penicillamine is adequate to the enantiomeric separation of valine, isoleucine, tyrosine, and phenylalanine.

The effect of the mobile phase composition on the retention factor (k), resolution (R), and separation factor (α) is shown in Figures 6-8.

Figure 6 shows that an increase in the organic modifier content in the aqueous mobile phase diminishes the retention of the enantiomers as denoted by the retention factors (k). However, the retention of the more retained enantiomers (phenylalanine) is diminished significantly compared to that of the less retained enantiomer (valine). It confirms that lipophilic interaction between the α-alkyl substituent of the more retained enantiomer and the octadecyl chains of silica gel is expected to decrease as the polarity of the mobile phase decreases by increasing the content of organic modifier in the mobile phase (9). The reduction in the lipophilic interaction would be more significant with a less polar organic modifier (e. g., acetonitrile). In contrast, the organic modifier does not notably affect the retention of the less retained enantiomers in the aqueous mobile phase.

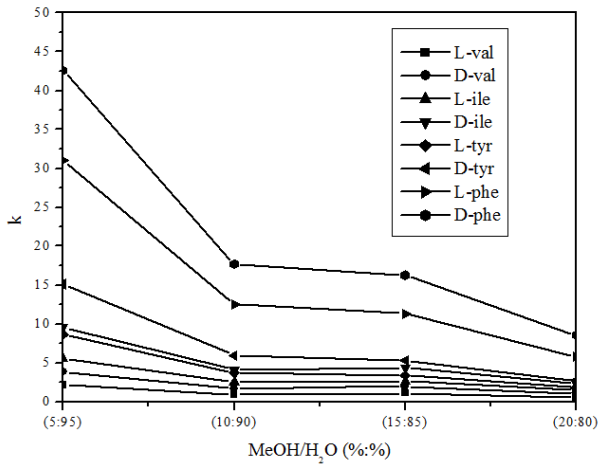

Figure 6. Influence of the methanol content of the mobile phase on the separation factor (k) of selected chiral amino acids.

Figure 7 shows the effect of methanol concentration on the resolution. It can be observed that the lipophilic interaction between α-alkyl substituent of the amino acids and the octadecyl chains of silica gel contributes to the enantiomeric separation of the amino acids as resolution decreases significantly with the increase of methanol concentration.

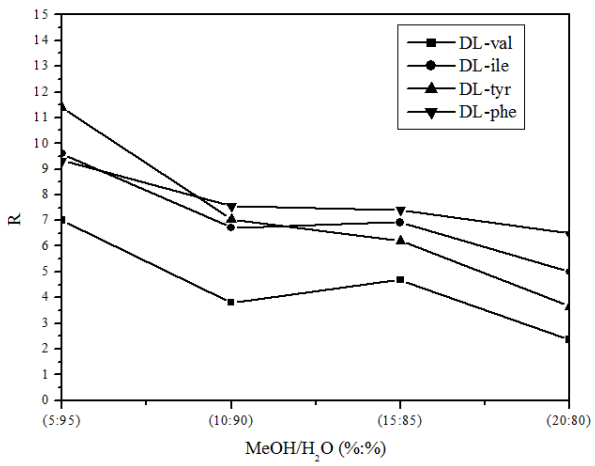

Figure 7. Influence of the methanol content of the mobile phase on the resolution (R) of selected chiral amino acids.

It can be seen in Figure 8 that separations factors α are slightly affected by the variation of methanol (MeOH) in the mobile phase. The separation factors would be modified if less polar organic modifiers were used.

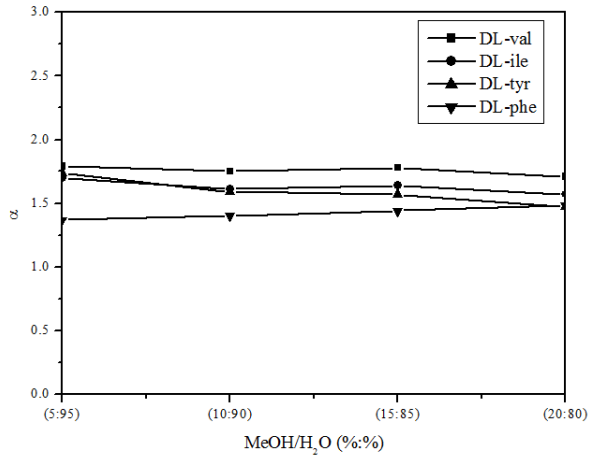

Figure 8. Influence of the methanol content of the mobile phase on the separation factor of selected chiral amino acids.

Tables 1 and 2 show the effect of the mobile phase's pH on the retention and separation factors of the amino acids.

Table 1. Influence of pH on k for selected chital amino acids

pH	L-val	D-val	L-ile	D-ile	L-tyr	D-tyr	L-phe	D-phe
4.20	0.44	0.62	1.13	1.66	1.56	2.22	4.73	6.6
5.30	1.10	1.96	2.67	4.39	3.36	5.28	11.3	16.28
6.04	1.90	2.15	2.97	5.22	3.84	6.56	-	-
7.00	8.39	13.87	19.5	-	-	-	-	-

Table 2. Influence of pH on the resolution (R) of selected chiral amino acids

pH	DL-val	DL-ile	DL-tyr	DL-phe
4.20	1.42	3.6	2.72	-
5.30	4.69	6.93	6.20	5.41
6.04	4.00	8.40	6.66	7.40
7.00	8.00	-	-	

It can be observed that pH influenced the separation factors slightly but had a noticeable effect on retention, which increased with higher pH values. This increase on the retention factors affected the resolution (Table 3) since the maximum resolution of isoleucine and tyrosine was achieved at pH 6.04; for valine, the optimum value of resolution was found at pH 7.0 and for phenylalanine, pH 5.3 (above this pH it has been impossible to get data with phenylalanine).

Table 3. Influence of pH on α of selected chiral amino acids

pH	DL-val	DL-ile	DL-tyr	DL-phe
4.20	1.42	1.47	1.42	-
5.30	1.78	1.64	1.57	1.39
6.04	1.80	1.76	1.70	1.44
7.00	1.65	-	-	

Therefore, the pH of the mobile phase was set at 5.3.

In general, it could be observed that the [Cu^{+2}], the mobile phase composition, and pH affected the resolution of the enantiomers of valine, isoleucine, tyrosine, and phenylalanine. The variation of these parameters showed that the interaction with the stationary phase through hydrophobic interactions was decisive to the resolution of the enantiomers.

3. Conclusion

In conclusion, capillary liquid chromatography on N, S-dioctyl-(D)-penicillamine column demonstrated to be an excellent technique for the enantiomeric separation of amino acids since it was possible both to separate them without a derivatization step and to obtain excellent resolution. Capillary liquid chromatography saves stationary and mobile phases; the columns can be home-made, and fewer samples are required; these advantages make it a promising technique in chiral separations.

Acknowledgments

The authors are grateful to São Paulo Research Foundation, FAPESP (Grants 2017/02147-0, 2015/15462-5, and 2014/07347-9) and Conselho Nacional de Desenvolvimento Cientifico e Tecnológico, CNPq (Grant 307293/2014-9) for the financial support provided. This research project was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

References

[1] Maciel EVS, de Toffoli AL, Lanças FM. Current status and future trends on automated multidimensional separation techniques employing sorbent-based extraction columns. J Sep Sci 2019;42:258–72. https://doi.org/10.1002/jssc.201800824.

[2] Vargas Medina DA, Maciel EVS, de Toffoli AL, Lanças FM. Miniaturization of liquid chromatography coupled to mass spectrometry. TrAC Trends Anal Chem 2020;128:115910. https://doi.org/10.1016/j.trac.2020.115910.

[3] Maciel EVS, de Toffoli AL, Sobieski E, Domingues Nazário CE, Lanças FM. Miniaturized liquid chromatography focusing on analytical columns and mass spectrometry: A review. Anal Chim Acta 2020;1103:11–31. https://doi.org/10.1016/j.aca.2019.12.064.

[4] D'Orazio G. Chiral analysis by nano-liquid chromatography. TrAC Trends Anal Chem 2020;125:115832. https://doi.org/10.1016/j.trac.2020.115832.

[5] Wilson SR, Berg HE, Roberg-Larsen H, Lundanes E. Hyphenations of one-dimensional capillary liquid chromatography with mass spectrometry. Hyphenations Capill. Chromatogr. with Mass Spectrom., Elsevier; 2020, p. 319–67. https://doi.org/10.1016/B978-0-12-809638-3.00009-0.

[6] Fanali S. Nano-liquid chromatography applied to enantiomers separation. J Chromatogr A 2017;1486:20–34. https://doi.org/10.1016/j.chroma.2016.10.028.

[7] Fanali S, D'Orazio G, Farkas T, Chankvetadze B. Comparative performance of capillary columns made with totally porous and core–shell particles coated with a polysaccharide-based chiral selector in nano-liquid chromatography and capillary electrochromatography. J Chromatogr A 2012;1269:136–42. https://doi.org/10.1016/j.chroma.2012.06.021.

[8] Sharaf El-Din MMK, Attia KAM, Nassar MWI, Kaddah MMY. Development and validation of a copper ligand-exchange chromatographic method for the estimation of D-lactic acid in Ringer-lactate solution. Talanta 2018;189:86–91. https://doi.org/10.1016/j.talanta.2018.06.068.

[9] Alizadeh T, Shamkhali AN. Chiral resolution of salbutamol in plasma sample by a new chiral ligand-exchange chromatography method after its extraction with nano-sized imprinted polymer. J Chromatogr B 2016;1009–1010:96–106. https://doi.org/10.1016/j.jchromb.2015.12.021.

[10] Kumar R, Bhushan R. Indirect chiral ligand exchange chromatography for enantioseparation: a modification of conventional techniques. RSC Adv 2014;4:50130–6. https://doi.org/10.1039/C4RA07061E.

[11] Davankov VA, Bochkov AS, Kurganov AA, Roumeliotis P, Unger KK. Separation of unmodified α-amino acid enantiomers by reverse phase HPLC. Chromatographia 1980;13:677–85. https://doi.org/10.1007/BF02303437.

[12] Mangelings D, Eeltink S, Vander Heyden Y. Recent developments in liquid and supercritical fluid chromatographic enantioseparations, 2020, p. 453–521. https://doi.org/10.1016/B978-0-444-64070-3.00009-6.

[13] Ianni F, Pucciarini L, Carotti A, Natalini S, Raskildina GZ, Sardella R, et al. Last ten years (2008-2018) of chiral ligand-exchange chromatography in HPLC: An updated review. J Sep Sci 2019;42:21–37. https://doi.org/10.1002/jssc.201800724.