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Journal of Pharmaceutical and Biomedical Analysis 46 (2008) 914–919
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Enantioseparation of *N*-fluorenylmethoxycarbonyl α-amino acids on polysaccharide-derived chiral stationary phases by reverse mode liquid chromatography

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Received 27 January 2007; received in revised form 18 September 2007; accepted 9 January 2008
Available online 17 January 2008

Abstract

The enantioseparation of *N*-protected fluorenylmethoxycarbonyl (*N*-FMOC) α-amino acids was carried out on three polysaccharide-derived chiral stationary phases, such as cellulose tris(3,5-dimethylphenylcarbamate) (Chiralcel OD), amylose tris(3,5-dimethyl-phenylcarbamate) (Chiralpak AD) and cellulose tris(4-methylbenzoate) (Chiralcel OJ), and the influence of acetonitrile composition and pH of the eluents on the enantioseparation in reverse mode chromatography was examined. The best separation of the enantiomers was achieved with 40% acetonitrile in 50 mM phosphate buffer at pH 2. However, increasing the composition of acetonitrile to 50% on Chiralcel OD yielded a considerable decrease of retention time with minimum loss of resolution. The elution order of *N*-FMOC α-amino acid enantiomers on Chiralcel OD and OJ were quite different, indicating that both phases could be used in a complementary manner for the separation of the enantiomers of *N*-FMOC α-amino acids. The positive relationship between the capacity factor of *N*-FMOC α-amino acids and the hydrophobicity of amino acids indicated that hydrophobicity plays an important role on the retention of the *N*-FMOC α-amino acids in the reverse mode.

Keywords: Chiral stationary phase; Enantiomer separation; Reverse mode; *N*-FMOC α-amino acids; Hydrophobicity

1. Introduction

Amino acids are ubiquitous and have diverse roles in living cells. With increasing age of living body, L-amino acids that constitute biological proteins gradually transformed into D-form [1,2]. So, racemization of amino acids can be used to investigate tissue turnover and pathogenesis of typical disease of old age [3]. This observation has led to an increased need for enantioselective analytical methodologies for the analysis of amino acids. There has been a growing interest in the *N*-protected α-amino acids to help in the understanding of the roles played by the amino acids. The fluorenylmethoxycarbonyl (FMOC) protecting group is probably the most important among several amino acid protecting groups in use today [4–8]. The utilization of the FMOC group, instead of the originally used butoxycarbonyl group, has revolutionized solid-phase peptide synthesis. When compared with other *N*-protecting groups for amino acids, the FMOC group provides the advantage of high sensitivity in fluorescence detection [9–11].

The enantioseparation of *N*-FMOC α-amino acids has been carried out mostly by capillary electrophoresis and high-performance liquid chromatography. For the capillary electrophoretic methods, β-cyclodextrin [12,13], γ-cyclodextrin [13,14], δ-cyclodextrin [15] and vancomycin [16,17] were used as chiral selectors and achieved acceptable resolution. Enantioseparations of the *N*-FMOC α-amino acids by liquid chromatographic methods were carried out using the chiral stationary phases derived from polysaccharides [18–22], cyclodextrins [23,24], macrocyclic antibiotics [25], cinchona alkaloids [26,27], quinine and quinidine [28–30], and certain amino acids [31–34]. Other types of chiral stationary

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0731-7085/$ – see front matter © 2008 Published by Elsevier B.V.
doi:10.1016/j.jpba.2008.01.012

Table 1
Separation parameters for the enantiomers of *N*-FMOC α-amino acids on Chiralcel OD, Chiralpak AD and Chiralcel OJ with eluents in different pH

Phase	Analyte[a]	pH 2			pH 4			pH 6		
		k'[b]	Rs	Conf.[c]	k'	Rs	Conf.	k'	Rs	Conf.
OD	Ala	10.1	6.82	D	10.6	5.67	D	4.24	1.44	D
	ABA	13.4	4.20	D	13.9	3.43	D	5.18	1.47	D
	Asn	1.68	2.23	D	2.15	1.04	D		–	
	Asp	2.75	1.97	D	6.75	0.80	D		–	
	Glu	2.91	1.41	D	4.65	0.84	D		–	
	Gln	1.59	1.58	L	2.17	1.58	L		–	
	Ile	29.3	4.71	D		–[d]			–	
	Norleu	48.6	2.92	D	34.2	1.49	D		–	
	PG	49.4	2.02	D	33.2	1.51	D	9.89	0.80	D
	Phe	74.3	2.34	D		–			–	
	Ser	4.02	4.13	D	3.48	2.29	D	1.68	0.74	D
	Thr	4.75	3.41	D	4.05	1.68	D	2.01	0.79	D
	Tyr	18.6	1.34	D		–			–	
	Val	30.5	3.55	D		–			–	
AD	Ala	3.17	1.10	L		–			–	
	ABA	4.12	0.81	L		–			–	
	Leu	7.24	0.32	L	9.47	0.57	L		–	
	Met	5.84	0.90	L	6.17	0.88	L	0.82	0.38	L
	Norleu	8.72	0.81	L	11.3	0.83	L		–	
	Norval	5.62	0.77	L	6.82	0.79	L		–	
	PG	8.36	0.40	D	7.35	0.38	D		–	
	Phe	11.8	1.97	D		–			–	
	Ser	1.23	2.53	D	1.09	0.82	D		–	
	Thr	1.12	1.11	D	1.20	1.04	D		–	
	Tyr	1.86	2.01	D		–			–	
	Val	5.16	0.61	L	6.01	0.61	L		–	
OJ	ABA	2.42	1.12	L	3.15	0.85	L		–	
	Ile	5.51	2.77	L	6.60	2.31	L	1.03	0.87	L
	Leu	4.89	4.73	L	5.90	3.48	L	1.11	1.38	L
	Met	4.43	0.82	L	4.23	0.81	L		–	
	Norleu	5.75	2.10	L	6.50	1.28	L	0.96	0.79	L
	Norval	3.81	1.55	L	4.44	1.60	L	0.72	0.44	L
	PG	8.99	1.06	D	7.32	1.04	D		–	
	Thr	0.92	0.90	L	0.89	0.85	L		–	
	Tyr	2.82	0.75	L	2.73	0.75	L		–	
	Val	3.90	1.49	L	4.78	1.15	L	0.75	0.70	L

Analysis was carried out with 40% acetonitrile in 50 mM phosphate buffer at flow rate of 0.5 ml/min.
[a] *N*-FMOC derivatives of amino acids.
[b] Capacity factor of the first eluted enantiomer.
[c] Absolute configuration of the first eluted enantiomer.
[d] Not separated.

phases, such as protein based [35] and molecularly imprinted monolithic stationary phases [36] were also reported. Among theses phases, the polysaccharide- and cinchona-derived stationary phases and the Pirkle-type phases showed relatively high efficiency on enantioseparation of *N*-FMOC α-amino acids. Polysaccharide-derived phases are most commonly used for the enantioseparation of *N*-FMOC α-amino acids with reasonable resolution results. However, most all of the experiments using polysaccharide-derived stationary phases were carried out in normal mode chromatography, though these phases could be used successfully in reverse mode with polar solvents. Polar solvents may offer the advantages of alternative chiral recognition mechanisms, and a greater solubility of some analytes and fewer environment-related problems [37]. Furthermore, the *N*-FMOC derivatives are often poorly soluble in some organic solvents [38].

To date, there have been no reports on a systematic approach for the enantioseparation of *N*-FMOC α-amino acids on polysaccharide-derived stationary phases in reverse mode chromatography. In this study, we report on the resolution of 18 *N*-FMOC α-amino acids characterized on three different polysaccharide-derived chiral stationary phases, which included cellulose tris(3,5-dimethylphenylcarbamate) (Chiralcel OD), amylose tris(3,5-dimethyl-phenylcarbamate) (Chiralpak AD) and cellulose tris(4-methylbenzoate) (Chiralcel OJ), along with various aqueous eluents. Well understanding of the retention behavior and chiral resolution of the analytes could help to elucidate the separation mechanism of *N*-FMOC α-amino acids.

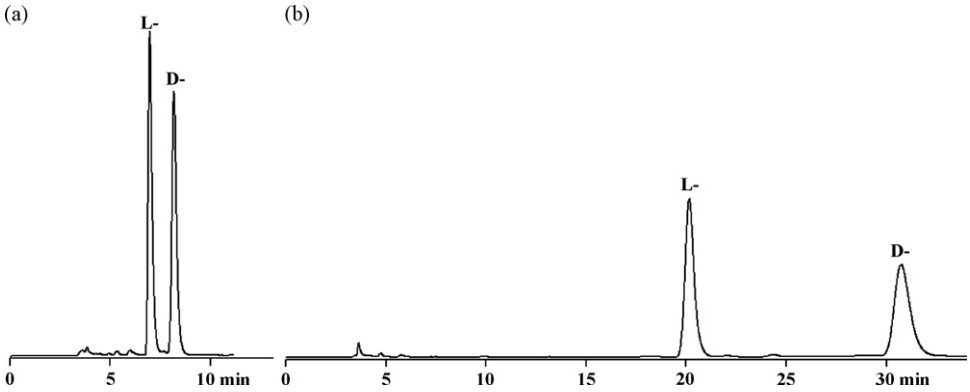

Fig. 1. Representative chromatograms for the separation of enantiomers of *N*-FMOC α-leucine on Chiralcel OJ with the eluents of 40% acetonitrile in 50 mM phosphate buffer at either (a) pH 6 or (b) pH 2.

2. Experimental

2.1. Materials and reagents

All organic solvents were of analytical grade and purchased from J.T. Baker (NJ, USA). 9-FMOC chloride and racemic- and L-amino acids, such as alanine (Ala), aminocaprylic acid (ACA), aminobutyric acid (ABA), asparagine (Asn), aspartic acid (Asp), glutamic acid (Glu), glutamine (Gln), isoleucine (Ile), leucine (Leu), methionine (Met), norleucine (Norleu), Norvaline (Norval), phenylglycine (PG), phenylalanine (Phe), serine (Ser), threonine (Thr), tyrosine (Tyr) and valine (Val) were obtained from Sigma Chemical Co. (MO, USA). Deionized water was purified using a Milli-Q water system (Millipore, MO, USA). Racemic and enantiomerically pure *N*-FMOC α-amino acids were prepared according to conventional methods [39] as follows: amino acid (5 mmol) was dissolved in a 10% aqueous sodium carbonate solution (12.5 mmol), and dioxane (7.5 ml) was then added with stirring in an ice-bath. After 9-FMOC chloride (5 mmol) was slowly added, the reaction mixture was stirred at room temperature for 5 h. The mixture was then poured into water and extracted with ether. The aqueous solution in an ice-bath was acidified with concentrated hydrochloric acid. The filtered *N*-FMOC α-amino acid was then dried under vacuum.

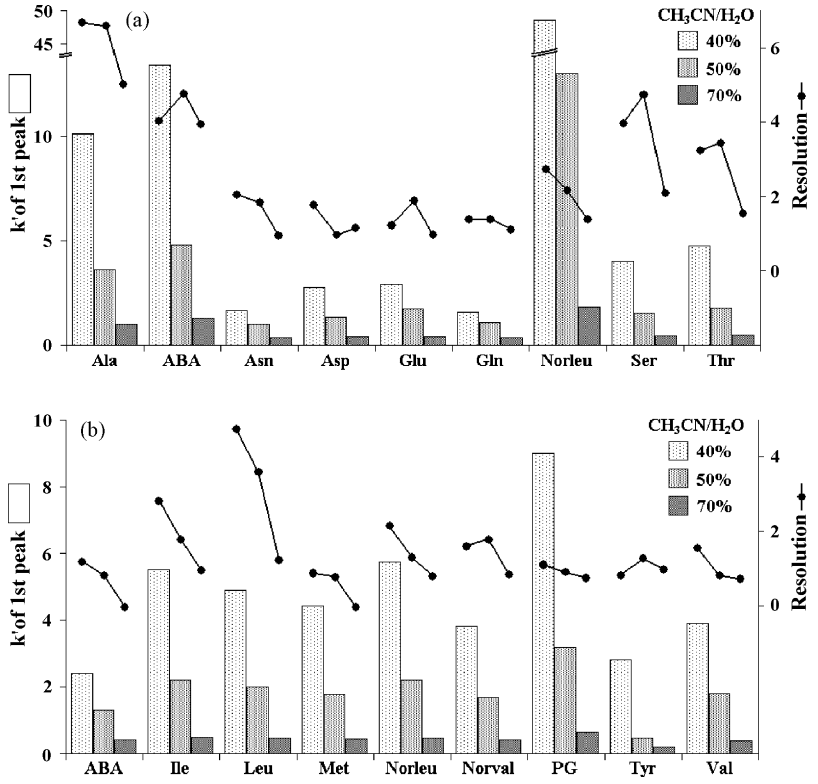

Fig. 2. Effects of the acetonitrile composition of eluents (pH 2) on the capacity factor (bars) and resolution (circles and lines) of *N*-FMOC α-amino acids separated on (a) Chiralcel OD and (b) Chiralcel OJ.

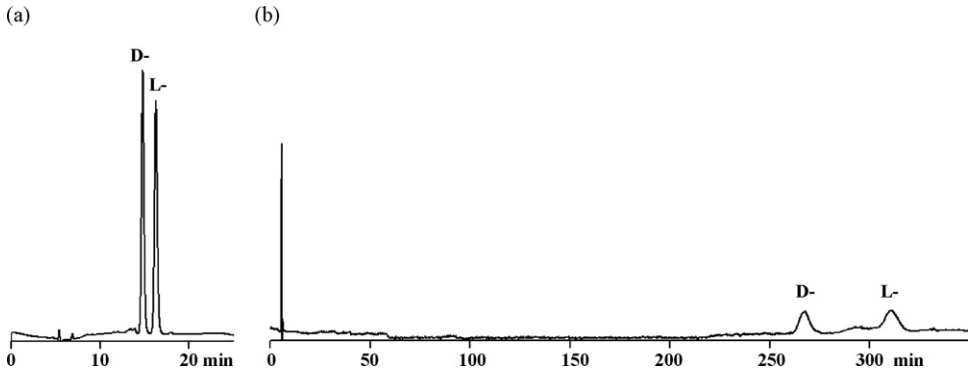

Fig. 3. Representative chromatograms for the separation of enantiomers of *N*-FMOC α-norleucine on Chiralcel OD with the eluents of either (a) 70% or (b) 40% acetonitrile in 50 mM phosphate buffer in pH 2.

2.2. Chromatography

The HPLC system consisted of an SCL-10A system controller, LC-10AD pump, SPD-10AVP diode array detector (Shimadzu, Kyoto, Japan) and Rheodyne 7725 injector. The chiral stationary columns used were Chiralcel OJ (4.6 mm i.d. × 15 cm), Chiralcel OD (4.6 mm i.d. × 25 cm) and Chiralpak AD (4.6 mm i.d. × 15 cm) from Daicel Chemical (Tokyo, Japan). The mobile phase consisted of acetonitrile and 50 mM phosphate buffer at various composition and pH. The mobile phase was titrated with 1 M HCl or NaOH to adjust pH and filtered through a membrane filter (0.22 μm) before application. The flow rate was set at 0.5 ml/min and the elution of analytes was monitored at 254 nm. The column temperature was adjusted to 25 °C. *N*-FMOC α-amino acids were dissolved in either methanol or water at a concentration of 1 mg/ml and injected as a volume of 2 μl. The L-enantiomers were employed to enable identification of the elution order of the enantiomers. When a new mobile phase was applied for analysis, the column was equilibrated by running eluent until a stable baseline signal was obtained. Column void times were determined from the first perturbation of baseline caused by the methanol or ethanol injection.

3. Results and discussion

3.1. Effects of pH of eluents on separation

The liquid chromatographic results for the enantioseparation of *N*-FMOC α-amino acids on Chiralcel OD, Chiralpak AD and Chiralcel OJ for three different eluents (40% acetonitrile in 50 mM phosphate buffer, pH 2, 4 or 6) in reverse mode are presented in Table 1. The separation and retention of the analytes were influenced considerably by the pH of the eluents. By changing the pH of the eluent from 4 to 6, the retention times of the analytes as well as the resolution of the enantiomers decreased significantly on all the three phases (Fig. 1) due to the decrease of hydrophobicity of the analytes by ionization at pH 6. Decreasing the pH of the eluent from 4 to 2 on Chiralpak AD and Chiralcel OJ led some neutral amino acids like ABA, Leu, Ile, Norleu, Val and Norval to decrease their retention times. Chiralcel OD showed either increasing (Norleu, PG, Ser and Thr) or decreasing (Asn, Asp, Glu and Gln) effects of pH change from 4 to 2 on the retention times with significant improvement of the chiral resolution. However, there was no further gain on the chiral resolution of *N*-FMOC α-amino acids as well as low stability of the stationary phases at the pH lower than 2, hence, the best separation of the enantiomers for the experiment was judged to be with an eluent of pH 2.

Among 18 analytes, 14 were baseline separated ($Rs > 1.2$) on Chiralcel OD with the eluent of pH 2, but only three analytes on Chiralpak AD. These results suggested that the enantioseparation of *N*-FMOC α-amino acid on a cellulose-based stationary phase (Chiralcel OD and OJ) was more effective than on an amylose-based stationary phase (Chiralpak AD). Li and Lee [20] have reported that the cellulose-based Chiralcel OD showed a larger separation factor than did the amylose-based Chiralpak AD or AS for the enantioseparation of *N*-FMOC α-amino acid in normal mode chromatography. The D-isomer was eluted prior to L-isomer on Chiralcel OD for all separated amino acids except Gln (L-isomer eluted first). Interestingly, this situation was reversed on Chiralcel OJ with the exception of PG. The reversal of elution order is very advantageous in cases of trace analysis of the unwanted enantiomer or preparative separation of highly retained enantiomers. The elution order of *N*-FMOC α-amino acids in reverse mode chromatography was not consistent with that in normal mode [20], suggesting that chiral recognition mechanisms and retention behavior of the analytes for both of the modes would differ.

When the chromatography was conducted with the eluent at pH 2, Chiralcel OJ could separate the enantiomers of Leu, Met and Norval, which were not separated on Chiralcel OD, an indication that Chiralcel OD and OJ can play the role in a complementary manner for the separation of the enantiomers of *N*-FMOC α-amino acids. Several analytes with a very large capacity factor were observed on Chiralcel OD, such as Phe ($k' = 74.3$), PG ($k' = 49.4$), Norleu ($k' = 48.6$) and Val ($k' = 30.5$). Taken together, the OD phase could be judged as the most effective among the three stationary phases for the separation of enantiomers of *N*-FMOC amino acids in reverse mode chromatography.

3.2. Effects of acetonitrile content on separation

Fig. 2 indicates the variation in capacity factors and the resolution of separated analytes by changes of the eluent acetonitrile composition. The effect of acetonitrile composition in the eluents on the retention of the analytes was significant. Increasing the composition of acetonitrile from 40% to 50% at pH 2 caused nearly a 54% decrease of the capacity factor on Chiralcel OD and 60% on Chiralcel OJ, while the average resolution decrease was 4% and 11% of separated analytes on Chiralcel OD and Chiralcel OJ, respectively. Most analytes separated with 40% acetonitrile on Chiralcel OD were also separated fairly well with 50% acetonitrile in considerably reduced retention times. The separation of Norleu on Chiralcel OD with 40% acetonitrile required about 6 h, while only 20 min was required with 70% acetonitrile demonstrating sufficient resolution for baseline separation (Fig. 3).

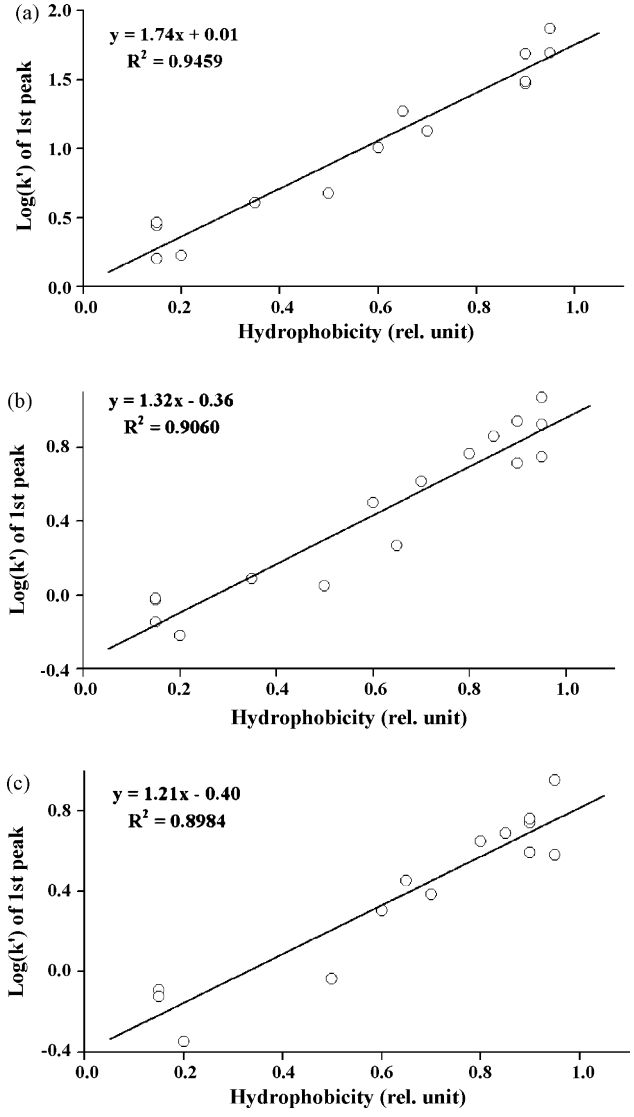

Fig. 4. Effects of the hydrophobicity of amino acids on the capacity factor of *N*-FMOC α-amino acids separated on (a) Chiralcel OD, (b) Chiralpak AD and (c) Chiralcel OJ with 40% acetonitrile in 50 mM phosphate buffer in pH 2. Circles represent the experimental data and solid line the trend.

3.3. Relationship between retention and hydrophobicity

The ratio of mean capacity factors of nonpolar amino acid group (Leu, Ile, Norleu, Val, Norval, Phe and PG) and polar amino acid group (Asp, Asn, Glu and Gln) on Chiralcel OD was about 21, while these values on Chiralpak AD and Chiralcel OJ were about 10 and 8, respectively. This implied that the retention time window for *N*-FMOC α-amino acids on Chiralcel OD would be wider than that on Chiralpak AD or Chiralcel OJ. Fig. 4 indicates the relationship between the capacity factor of *N*-FMOC α-amino acids separated with 40% acetonitrile in 50 mM phosphate buffer at pH 2 and the hydrophobicity of amino acids calculated by proteins with known 3D structures [40,41]. The logarithmic value of the capacity and hydrophobicity showed an excellent linear relationship. The squares of correlation coefficient (R^2) for the analytes separated on Chiralcel OD, Chiralpak AD and Chiralcel OJ were 0.95, 0.91 and 0.90, respectively, showing the best correlation of retention time and hydrophobicity of *N*-FMOC α-amino acids separated on Chiralcel OD. These results indicated that hydrophobicity plays an important role on the retention of the *N*-FMOC amino acids in reverse mode chromatography. However, a very weak relationship between the capacity factor and hydrophobicity was observed in normal mode chromatography [20]. No closer relationship between hydrophobicity and resolution of enantiomers in either normal or reverse mode chromatography was observed.

4. Conclusions

The separation and retention of *N*-FMOC α-amino acids on polysaccharide-derived chiral stationary phases were considerably influenced by the pH of the eluents. The best separation of the enantiomers was seen on Chiralcel OD with the eluent of 40% acetonitrile in 50 mM phosphate buffer (pH 2). However, Chiralcel OD and OJ can still play the role in a complementary manner for the separation of the enantiomers of *N*-FMOC α-amino acids. Increasing the composition of acetonitrile in the eluents caused a dramatic decrease of the capacity factor but with a slight change of resolution on Chiralcel OD, providing easy control of analysis time. The hydrophobicity of amino acids and the capacity factors showed acceptable relationships, supporting that the main retention mechanism of *N*-FMOC α-amino acids on the polysaccharide-derived chiral stationary phases in reverse mode chromatography could be the hydrophobic interactions.

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