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Investigation on enantiomeric separations of fluorenylmethoxycarbonyl amino acids and peptides by high-performance liquid chromatography using native cyclodextrins as chiral stationary phases

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Abstract

A systematic study was carried out to investigate enantiomeric separations of fluorenylmethoxycarbonyl (FMOC) amino acids and their peptides. Twenty amino acids were derivatized by 9-fluorenylmethyl chloroformate (FMOC-Cl) and its analogues, FMOC-glycyl-Cl and FMOC-β-alanyl-Cl. All derivatives were chromatographed on native β- and γ-cyclodextrin columns using acetonitrile as the main mobile phase component. The results indicated that glycyl and β-alanyl groups between FMOC and amino acid moieties enhanced chiral selectivities of amino acid derivatives. The addition of modifiers, triethylamine, acetic acid and methanol, into the mobile phase caused alterations in retention, enantiorecognition and elution order. The structures of amino acids and the type of chiral stationary phase employed exhibited significant impacts on chiral resolutions. It is also found that the number and position of glycyl moieties affect the retentions and enantioselectivities of FMOC derivatized glycyl containing peptides.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Cyclodextrin-based stationary phases; Amino acids, FMOC derivatives

1. Introduction

Amino acids are known as the building blocks of all living organisms. The protein and enzymes used in biochemistry, pharmacology and medical science are believed to be made from the L-enantiomers of the amino acids. It is therefore important to have an efficient, low cost means of separating amino acids into their enantiomers. One method used has been the conversion of amino acids into diastereomers prior to analysis by HPLC on an achiral stationary phases [1]. Another has been the use of a chiral stationary phase (CSP) [2–5] or a mobile phase containing a chiral additive [6]. Cyclodextrin (CD) based chiral stationary phases are currently among the most useful CSPs. Enantiomers of aromatic amino acids and their analogues [7] and derivatized amino acid enantiomers [2] have been resolved on cyclodextrin stationary phases. Extensive work has been done by S.H. Lee and coworkers [5] in which

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several amino acid derivatization methods and β-CD based CSPs were used to obtain a better understanding of the chiral recognition mechanism for amino acids. Recently, Chang et al. [2] reported the separation of N-*tert.*-butoxycarbonyl amino acids on a hydroxypropyl derivatized β-CD column in reversed-phase mode. It was found in that work that acetonitrile, a nonaqueous polar organic solvent, as a mobile phase with triethylamine and acetic acid as modifiers can enhance chiral selectivities. Several other studies, carried out to evaluate this type of mobile phase, have shown that this mobile phase has a unique effect on enantioselectivity compared to conventional normal- and reversed-phase modes [8–14]. The proposed chiral resolution mechanism for nonaqueous polar organic mobile phase modes is believed to be due to the formation of hydrogen bonds between the secondary hydroxyl groups of cyclodextrins and hydrogen bonding functionalities of the analytes [8–14].

The current study was conducted to discern the effect of 9-fluorenylmethyl chloroformate and its analogues, FMOC-glycyl and FMOC-β-alanyl, as derivatizing reagent for amino acids on chiral resolutions. Twenty amino acids were investigated on native β- and γ-cyclodextrin stationary phases using a polar organic mobile phase. The importance of glycyl and β-alanyl groups between the FMOC moiety and amino acids in chiral resolutions, the effect of the number and linkage sequence of the glycyl group in FMOC-peptides on chiral selectivity and elution order of derivatized enantiomers, and the influence of the different chiral stationary phases and different mobile phase compositions on enantioresolutions are examined.

2. Experimental

2.1. Chemicals

9-Fluorenylmethyl chloroformate (FMOC-Cl), FMOC-glycine, FMOC-β-alanine and amino acids were purchased from ICN (Irvine, CA, USA), Sigma Chemical Company (St. Louis, MO, USA), Aldrich (Milwaukee, WI, USA) and Novobiochem (La Jolla, CA, USA). Acetonitrile, methanol (both are OmniSolv grade), acetic acid (HPLC grade) and triethylamine (99+% pure) were obtained from EM Science (Gibbstown, NJ, USA) and Fisher Scientific (Pittsburgh, PA, USA). Other materials were purchased from Aldrich or Sigma Chemical Company.

2.2. Preparation of FMOC-glycyl-Cl and FMOC-β-alanyl-Cl derivatizing agents

Since commercial availability of glycyl and β-alanyl-amino acids is limited, the method described by Carpino [15] was used to convert FMOC-glycine and FMOC-β-alanine into their acid chlorides. A 1.0-g amount of FMOC-glycyl-OH or 0.5 g FMOC-β-alanyl-OH was placed in a 50-ml rounded bottom flask and was dissolved in 15 ml CH_2Cl_2. To this flask, 3.4 ml (or 1.2 ml in the case of FMOC-β-alanyl-Cl reaction) of $SOCl_2$ was added and the mixture was allowed to reflux for 4 h. After the reaction was finished, the solvent and excess of $SOCl_2$ were removed by a rotary evaporator. The residue was redissolved in 1 ml of CH_2Cl_2. A 10-ml volume of hexane was then added to precipitate FMOC-glycyl-Cl or FMOC-β-alanyl-Cl. After filtration the product was dried in vacuo at room temperature for 2 h. The product was always kept in a desiccator after each use. The product can be used for about two months without apparent hydrolyzation that was indicated by the formation of small yellowish chunks. The purities of these chlorinated products were determined by following the HPLC analysis procedure provided in the same paper [15]. Both acid chlorides were found to be greater than 95% pure.

2.3. Sample preparation

About 1 mg of an amino acid was placed in a vial and 250 μl of distilled water and 250 μl of borate buffer (40 m*M*, pH 7.7) were added to the vial to dissolve the amino acid. After dissolution, 400 μl of acetonitrile (MeCN) was then added into the vial, followed by about 3 mg of FMOC-Cl or FMOC-glycyl-Cl. The mixture was vortexed, allowed to stand for about 30 min and then extracted with about 3 ml ethyl acetate. The top layer was then collected and ready for analysis.

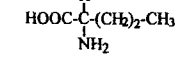
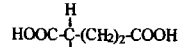
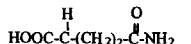
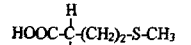
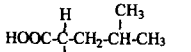
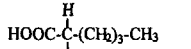
FMOC-β-alanyl-Cl hydrolyzed into FMOC-β-alanyl-OH upon contacting aqueous solution and therefore, the derivatizing procedure was modified as follows. About 1 mg of an amino acid was placed into a vial and 0.5 ml of HPLC grade MeCN was

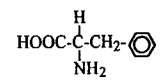
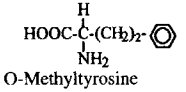
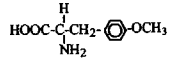
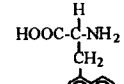
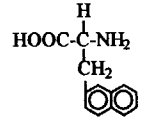
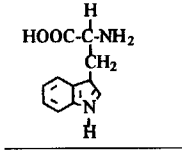
Table 1
Summary of HPLC separations of amino acid derivatives using FMOC-Cl and analogues as the derivatizing reagents and native β-CD and γ-CD as the stationary phase

Amino acid Name & structure	Mobile phase[a]	β-CD as the stationary phase						γ-CD as the stationary phase					
		Derivatizing reagents						Derivatizing reagents					
		FMOC-Cl		FMOC-GLY-Cl		FMOC-β-ALA-Cl		FMOC-Cl		FMOC-GLY-Cl		FMOC-β-ALA-Cl	
		k_1'[b]	α[c]	k_1'	α	k_1'	α	k_1'	α	k_1'	α	k_1'	α
Alanine													
	A	2.0	1.00	3.4	1.00	2.8	1.00	1.8	1.00	3.4	1.07(L)[d]	2.8	1.00
	B	6.6	1.00	9.7	1.00	9.7	[e]	4.8	1.00	10.0	1.05(L)	10.2	1.02(D)
	C	1.7	1.00	2.9	1.00	2.6	1.00	1.4	1.00	2.1	1.05(L)	2.4	1.00
β-Amino-isobutyri acid													
	A	2.2	1.00	3.5	1.00			2.1	1.00	4.5	1.00		
	B	7.3	1.03[f]	15.2	1.00			6.2	1.03[f]	13.3	1.00		
	C	2.2	1.00	3.9	1.00			1.8	1.04[f]	3.1	1.00		
α-Amino-n-butyric acid													
	A	1.6	1.00	2.8	1.00			1.6	1.00	3.0	1.06(L)		
	B	3.2	1.00	8.1	1.00			4.8	1.00	9.1	1.05(L)		
	C	1.7	1.00	2.7	1.00			1.4	1.00	2.1	1.01(L)		
Aspartic acid													
	A	6.6	1.00	12.0	1.22(D)			7.3	1.03(L)	13.0	1.06(D)		
	B	14.7	1.00	29.0	1.25(D)			15.5	1.05(L)	25.1	1.06(D)		
	C	18.4	1.04(L)	34.6	1.04(D)			11.9	1.05(L)	24.2	1.16(D)		
Asparagine													
	A	4.7	1.03(L)	7.5	1.04(L)			4.3	1.02(L)	8.3	1.17(L)		
	B	11.2	1.00	25.1	1.04(L)			7.0	1.00	21.2	1.10(L)		
	C	3.9	1.00	6.1	1.00			2.6	1.00	6.1	1.07(L)		
Valine													
	A	1.7	1.04(D)	3.6	1.05(D)	2.5	1.11(D)[g]	1.6	1.05(L)	3.0	1.00	2.7	1.03(D)[h]
	B	4.8	1.04(D)	11.0	1.04(D)	8.2	1.12(D)[h]	4.3	1.00	7.5	1.00	8.1	1.04(D)
	C	2.0	1.00	3.7	1.00	2.9	1.06(D)	2.0	1.00	3.7	1.00	2.9	1.00

(Continued on p. 264)

Table 1 (*continued*)

Amino Acid Name & Structure	Mobile Phase[a]	β-CD as the stationary phase						γ-CD as the stationary phase					
		Derivatizing reagents						Derivatizing reagents					
		FMOC-Cl		FMOC-GLY-Cl		FMOC-β-ALA-Cl		FMOC-Cl		FMOC-GLY-Cl		FMOC-β-ALA-Cl	
		k_1'[b]	α[c]	k_1'	α	k_1'	α	k_1'	α	k_1'	α	k_1'	α
Norvaline													
	A	1.6	1.00	2.8	1.00			1.7	1.00	3.3	1.09(L)		
	B	6.7	1.00	15.1	1.00			4.3	1.00	9.3	1.07(L)		
	C	2.0	1.00	3.4	1.04(L)			1.3	1.00	2.2	1.06(L)		
	A	20.6	1.08(L)	39.7	1.09(L)			20.3	1.09(L)	37.4	1.21(L)		
	B	[i]						[i]					
	C	31.8	1.12(L)	70.9	1.15(L)			26.3	1.14(L)	43.5	1.28(L)		
Glutamine													
	A	4.8	1.00	10.1	1.05(L)			5.0	1.03(L)	8.66	1.26(L)		
	B	13.7	1.00	26.4	1.07(L)			14.1	1.02(L)	24.2	1.21(L)		
	C	3.0	1.00	6.8	1.05(L)			2.8	1.00	4.2	1.17(L)		
Methionine													
	A	1.8	1.00	3.6	1.03(L)	2.4	1.15(D)[h]	1.5	1.04(L)	2.9	1.10(L)	2.3	1.00
	B	4.5	1.00	11.6	1.04(L)	8.2	1.07(D)	5.2	1.00	7.5	1.08(L)	7.0	1.02(D)
	C	1.3	1.00	3.1	1.09(L)	3.0	1.00	1.3	1.00	2.1	1.07(L)	2.4	1.00
Leucine													
	A	2.3	1.07(D)	6.6	1.07(D)	3.8	1.22(D)	1.7	1.00	3.6	1.15(L)	2.6	1.00
	B	6.2	1.08(D)	19.6	1.03(D)	10.2	1.25(D)	6.3	1.00	9.9	1.12(L)	8.6	1.05(D)
	C	2.7	1.00	5.9	1.09(L)	3.6	10.8(D)	1.4	1.00	2.4	1.10(L)	2.6	[e]
Norleucine													
	A	1.9	1.00	5.6	1.06(D)			1.6	1.00	3.1	1.07(L)		
	B	5.1	1.03(D)	15.0	1.05(D)			4.7	1.00	8.5	1.06(L)		
	C	1.7	1.00	3.9	1.09(L)			1.5	1.00	2.2	1.08(L)		

Phenylalanine													
	A	2.0	1.04(D)	4.3	1.30(D)	3.2	1.25(D)	2.0	1.00	3.4	1.11(D)	2.4	1.00
	B	8.0	1.09(D)	16.8	1.28(D)	10.7	1.25(D)	5.0	1.00	9.5	1.11(D)	7.9	1.12(D)[g]
	C	2.7	1.00	5.4	1.00	4.2	1.08(D)	1.1	1.00	2.7	1.00	2.7	1.06(D)
Homophenylalanine													
	A	2.5	1.05(D)	8.6	1.05(D)	4.3	1.10(D)	1.7	1.00	4.1	1.11(L)	3.3	1.00
	B	6.8	1.05(D)	27.4	1.02(L)	14.8	1.05(D)	5.1	1.00	11.5	1.05(L)	10.2	1.04(D)
	C	2.4	1.00	5.8	1.16(L)	5.2	1.07(L)	1.16	1.00	3.1	1.05(L)	3.1	1.00
O-Methyltyrosine	A	1.7	1.00	5.0	1.00			1.4	1.05(L)	3.1	1.10(L)		
	B	5.0	1.00	11.3	1.03(L)			4.2	1.00	8.3	1.04(L)		
	C	1.5	1.00	3.3	1.04(L)			1.1	1.00	2.3	1.06(L)		
3-(1-Naphthyl)-alanine													
	A	2.0	1.00	3.2	1.06(L)	3.1	1.02(L)	2.2	1.06(L)	6.0	1.13(L)	4.2	1.06(D)
	B	5.0	1.00	9.3	1.07(L)	10.4	1.04(L)	6.2	1.00	16.0	1.21(L)	11.6	1.06(D)
	C	2.1	1.00	3.4	1.06(L)	3.6	1.03(L)	2.0	1.00	5.1	1.18(L)	4.3	1.00
3-(2-Naphthyl)-alanine													
	A	1.8	1.00	3.3	1.00			1.9	1.00	3.9	1.00		
	B	5.5	1.00	9.9	1.02(L)			4.7	1.00	9.6	1.02(D)		
	C	2.4	1.00	3.6	1.06(L)			1.7	1.00	2.9	1.06(L)		
Tryptophan													
	A	4.2	1.00	7.1	1.08(L)	6.0	1.06(L)	4.1	1.00	12.1	1.29(D)	8.5	1.05(D)
	B	9.4	1.00	21.6	1.07(L)	12.9	1.05(L)	9.1	1.00	29.0	1.29(D)	22.1	1.08(D)
	C	3.2	1.00	5.0	1.06(L)	4.2	1.04(L)	2.4	1.00	5.0	1.00	4.7	1.06(D)

[a] A, B and C represent three different mobile phase compositions. A is MeCN–TEA–HOAc (1000:12:3); B is MeCN–TEA–HOAc (1000:7:0.5); C is MeOH–MeCN–TEA–HOAc (100:900:7:0.5) (all by volume ratio).

[b] k_1' is the capacity factor for the first eluted enantiomer.

[c] α is the ratio of the capacity for the second eluted enantiomer to that of the first eluted enantiomer.

[d] When separation occurs, the first eluted enantiomer is shown in parentheses.

[e] Enantiomer peak(s) coeluted with FMOC-β-alanyl-OH peak.

[f] Elution order could not be determined due to the lack of pure enantiomer standard.

[g] D-enantiomer coeluted with FMOC-β-alanyl-OH peak before L-enantiomer.

[h] L-enantiomer coeluted with FMOC-β-alanyl-OH peak after D-enantiomer.

[i] Under this mobile phase condition, the enantiomer peak(s) did not elute in 80 min.

added, followed by the addition of 3 mg of FMOC-β-alanyl-Cl. After vortexing for about 1 min, the vial was left to stand for 15 to 30 min. The supernatant was then collected as the sample solution. Once the derivatization was completed, the solution was hydrolytically stable. The sample solution was diluted with MeCN if necessary before injection.

2.4. Apparatus and HPLC methods

A Shimadzu Model LC-6A solvent delivery module, SPD-6A UV detector and CR2AX Chromatopac recorder were used. The detection wavelength was set at 265 nm. All separations were carried out at room temperature at a flow-rate of 1.0 ml/min. The β-cyclodextrin (β-CD) bonded phase column (Cyclobond I) and γ-cyclodextrin (γ-CD) column (Cyclobond II) (both 250×4.6 mm I.D., 5μm particle diameter, supplied by Astec, Whipney, NJ, USA) were used as CSPs. The mobile phases were prepared in volume ratio and are indicated in Tables 1–3 and Figs. 1–4. The solvent peak of the chromatogram was used as the hold-up time (t_0).

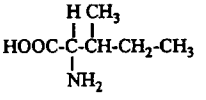
3. Results and discussion

3.1. Chromatographic results

The structures of 9-fluorenylmethyl chloroformate, 9-fluorenylmethoxycarbonyl glycyl chloride and 9-fluorenylmethoxycarbonyl β-alanyl chloride and their reactions with amino acids are shown in Fig. 1. As can be seen from this figure, the difference in

Table 2
Summary of HPLC separations of isoleucine derivatives using FMOC-Cl and analogues as the derivatizing reagents and native β-CD and γ-CD as the stationary phases

The structure of isoleucine:



Mobile phase[a]	Derivatizing reagent	Peaks obtained	Elution order	D and L		D-allo and L-allo	
				k_1'[b]	α[c]	k_1'[b]	α[c]
β-Cyclodextrin was used as the stationary phase							
A	FMOC-Cl	1		1.43	1.00	1.43	1.00
	FMOC-GLY-Cl	4	D-allo, D, L-allo, L	6.38	1.16	6.04	1.13
	FMOC-β-ALA-Cl	[d]					
B	FMOC-Cl	4	D-allo, D, L-allo, L	4.96	1.09	4.83	1.05
	FMOC-GLY-Cl	3	D-allo, D+L-allo, L	14.80	1.09	13.56	1.09
	FMOC-β-ALA-Cl	4	D-allo, D, L-allo, L	12.31	1.29	11.17	1.27
C	FMOC-Cl	2	D-allo+L-allo, D+L	2.58	1.00	2.40	1.00
	FMOC-GLY-Cl	2	D-allo+L-allo, D+L	5.59	1.00	4.71	1.00
	FMOC-β-ALA-Cl	4	D-allo, L-allo, D, L	4.76	1.12	4.72	1.11
γ-Cyclodextrin was used as the stationary phase							
A	FMOC-Cl	1		1.45	1.00	1.45	1.00
	FMOC-GLY-Cl	2	D+L, D-allo+L-allo	2.87	1.00	3.04	1.00
	FMOC-β-ALA-Cl	2	D-allo+D, L-allo+L	2.30	1.05	2.30	1.05
B	FMOC-Cl	1		3.78	1.00	3.78	1.00
	FMOC-GLY-Cl	2	D+L, D-allo+L-allo	7.91	1.00	8.49	1.00
	FMOC-β-ALA-Cl	3	D-allo+D, L, L-allo	8.13	1.06	8.13	1.07
C	FMOC-Cl	1		1.47	1.00	1.47	1.00
	FMOC-GLY-Cl	1		2.23	1.00	2.23	1.00
	FMOC-β-ALA-Cl	All peaks coeluted with derivatizing reagent peak.					

[a,b,c] The sample annotations as in Table 1.

[d] D and D-allo peaks coeluted with derivatizing reagent peak. The capacity factor for this derivatizing reagent peak is 3.85. Only L-allo and L peaks could be seen. $k'_{\text{L-allo}}=4.81$, $k'_{\text{L}}=5.25$.

Table 3
Effect of glycyl (GLY) moiety on enantiomeric separations of FMOC derivatized alanine (ALA) and leucine (LEU)[a]

FMOC-derivatives	β-CD		γ-CD	
	k' [b]	α [c]	k' [b]	α [c]
DL-Alanine	1.98	1.00	1.76	1.00
GLY-DL-ALA	3.35	1.00	3.36	1.07(L)[d]
DL-ALA-GLY	3.51	1.00	3.56	1.06(D)
GLY-GLY-DL-ALA	9.54	1.00	7.95	1.08(L)
DL-ALA-GLY-GLY	10.15	1.06(D)	9.96	1.06(D)
GLY-DL-ALA-GLY	8.86	1.04(L)	7.25	1.00
DL-Leucine	2.31	1.07(D)	1.69	1.00
GLY-DL-LEU	6.61	1.07(D)	3.60	1.15(L)
DL-LEU-GLY	3.32	1.00	2.56	1.08(D)
GLY-GLY-DL-LEU	18.02	1.18(D)	8.21	1.12(L)
DL-LEU-GLY-GLY	9.48	1.00	7.92	1.03(D)
GLY-DL-LEU-GLY	10.40	1.06(L)	7.03	1.00
β-ALA-DL-LEU	3.80	1.22(D)	2.54	1.00
β-ALA-DL-LEU-GLY	4.67	1.05(D)	3.91	1.00
β-ALA-GLY-DL-LEU	9.82	1.05(L)	3.72	1.00
β-ALA-DL-LEU-GLY-GLY	11.23	1.000	8.14	1.00

[a] The mobile phase for this experiment was MeCN–TEA–HOAc (1000:12:3).
[b,c,d] The same annotations as in Table 1.

FMOC-glycyl-Cl from FMOC-Cl is an extra glycyl moiety and the difference of FMOC-β-alanyl-Cl from FMOC-glycyl-Cl is one additional methylene group. More hydrogen bonding sites (carbonyl and amino groups) are introduced onto FMOC-glycyl and FMOC-β-alanyl derivatives as compared to FMOC derivatives. This change enhanced chiral selectivity of amino acids as will be described later. Twenty amino acids, including aliphatic amino acids, aromatic amino acids, acidic amino acids and their amides and D,L-isoleucine which is composed of two stereogenic centers were examined. Only native β- and γ-cyclodextrin columns were used as stationary phases since it has been previously shown that the use of an α-CD column resulted in unreasonably long retention times [12]. The major component in the mobile phase was the nonaqueous organic polar solvent, acetonitrile (MeCN). Triethylamine (TEA), acetic acid (HOAc) and methanol (MeOH) were employed as mobile phase modifiers. In this system, it is believed that the cavity of native cyclodextrin is filled with acetonitrile molecules [10–14]. The de-

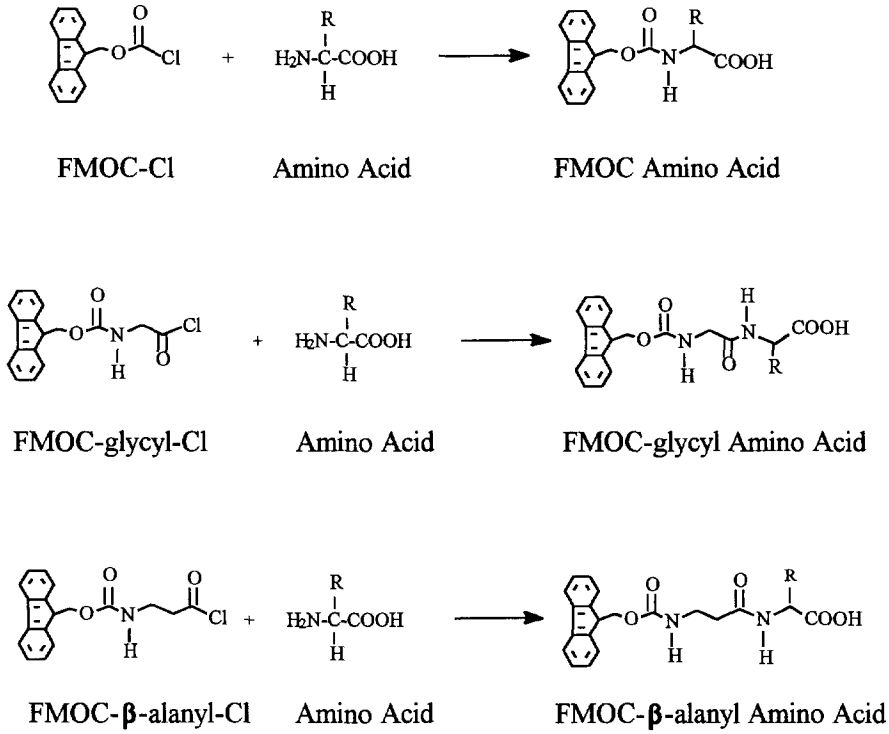


Fig. 1. The structures of derivatizing reagents used in this study and their reactions with amino acids.

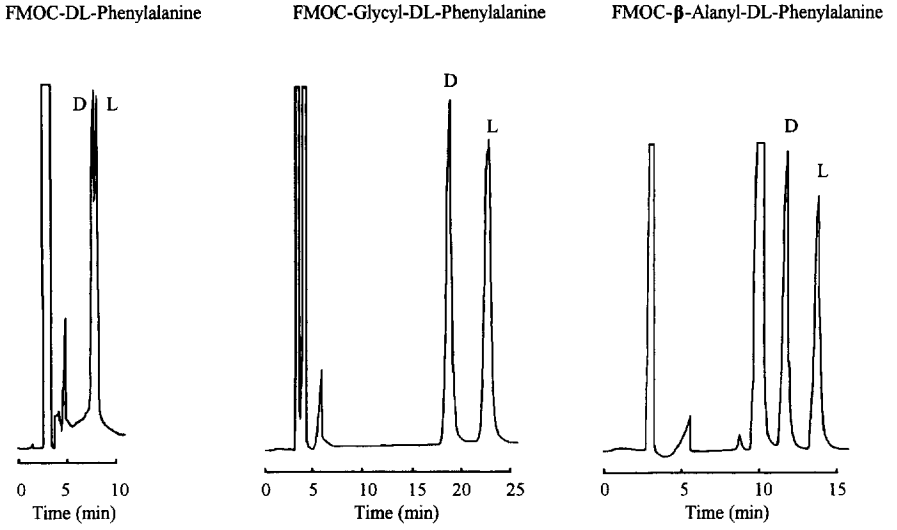


Fig. 2. Effect of derivatizing reagent on chiral separation of DL-phenylalanine. Stationary phase: native β-CD column. Mobile phase: MeCN–TEA–HOAc (1000:12:3).

rivatized amino acids are thought to stretch out on the top of cyclodextrin like a lid to allow optimal interactions between the functionalities of cyclodextrin (the primary and secondary hydroxyl groups) and the derivatized amino acid (peptide bond, carbamate bond and carboxylic acid group) [11,12]. Thus, the structures of cyclodextrins and amino acid derivatives and the diameter of the cyclodextrin cavity play a significant role in retention and enantioselectivity. From Table 1, it can be seen that in general, FMOC-glycyl amino acids were better resolved on a γ-CD column and FMOC-β-alanyl amino acids on a β-CD column. Also, with only a few exceptions, FMOC-glycyl- and FMOC-β-alanyl amino acids were better resolved than FMOC-amino acid enantiomers. This is probably due to the fact that the bulky structure of the fluorene residue hindered the effective interaction between the functionalities on stereogenic center of analyte molecules and CSP, resulting in a short retention and weak enantioselective interaction. The extension of chain length and the introduction of peptide bonds resulting from the presence of glycyl or β-alanyl groups reduced the hindrance and provided more active sites for hydrogen bonding interactions. An example is shown in Fig. 2 in which phenylalanine was only partially separated when it was derivatized with FMOC-Cl reagent. However, when it was converted into the FMOC-glycyl- and FMOC-β-alanyl derivatives, more than baseline separation was accomplished. It can also be noticed from Table 1 that different derivatizing reagent, i.e., FMOC-Cl, FMOC-glycyl-Cl or FMOC-β-alanyl-Cl, can sometimes reverse the elution order of derivatized amino acid enantiomers.

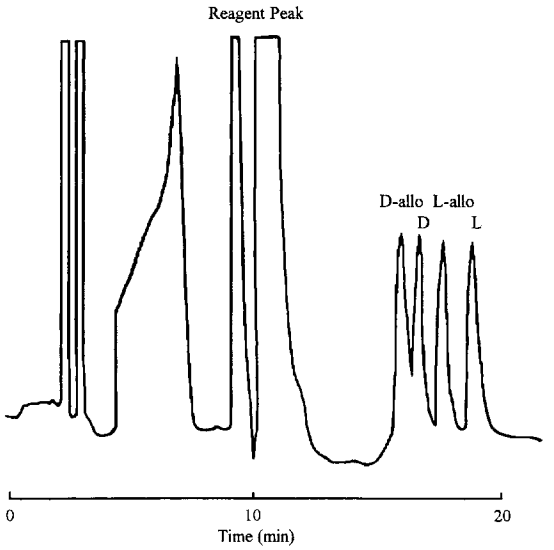


Fig. 3. Separation of FMOC-glycyl isoleucine on a native β-CD column. Mobile phase: MeCN–TEA–HOAc (1000:12:3).

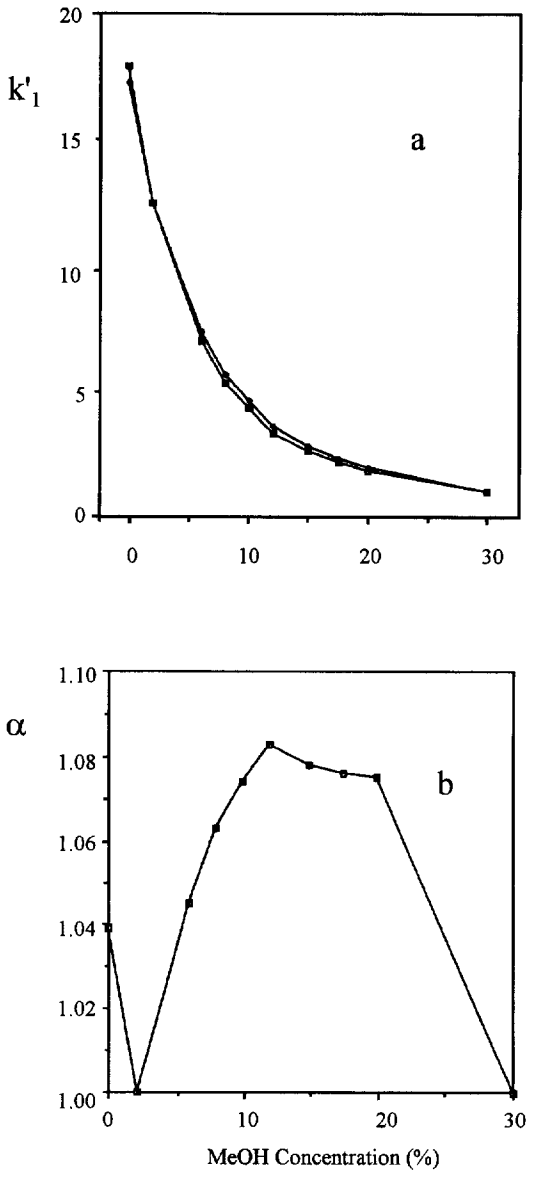


Fig. 4. Influence of methanol concentration in the mobile phase compositions on (a): capacity factors (k') of FMOC-glycyl-D-norleucine (□) and FMOC-glycyl-L-norleucine (●), and (b): enantioselectivity (α) of FMOC-glycyl-DL-norleucine. The stationary phase was a native β-CD column. The mobile phase composition started at MeCN–TEA–HOAc (1000:7:0.5). Then the ratio of TEA and HOAc was kept constant while changing the ratio of MeCN to MeOH.

More examples were observed on a γ-CD column [alanine, aspartic acid, valine, methionine, leucine, homophenylalanine and 3-(1-naphthyl)-alanine] than on a β-CD column (only aspartic acid, methionine, leucine and homophenylalanine).

As expected, the R group of an amino acid has a significant effect on chiral recognition. For instance, longer retention and better resolution were achieved for basic amino acids and acidic amino acids and their amides as they offer extra amino, carboxylic acid or amide functionalities which allow for stronger interactions between analytes and CSPs. FMOC-glycyl acidic amino acids showed higher retention and better resolution than their amide derivatives on a β-CD CSP, while better resolution was not always observed on a γ-CD CSP. Sometimes, the structural influence on enantioselectivity was subtle. For instance, phenylalanine, homophenylalanine and O-methyltyrosine have structural similarities. However, FMOC-glycyl-phenylalanine was significantly better resolved on a β-CD CSP while similar enantioselectivities were obtained on a γ-CD CSP for all three FMOC-glycyl-derivatives. Moreover, FMOC-glycyl-3-(1-naphthyl) alanine was much better resolved than its isomer, FMOC-glycyl-3-(2-naphthyl)alanine, on both stationary phases. Chiral separation of isoleucine which contains two stereogenic centers is summarized in Table 2. It can be seen from Table 2 that better resolutions between enantiomers and diastereomers of derivatized DL-isoleucine were obtained on a β-CD column. The best enantiomeric separation of derivatized isoleucine is shown in Fig. 3.

3.2. Effect of mobile phase composition

Three different mobile phase compositions were employed, MeCN–TEA–HOAc (1000:12:3), MeCN–TEA–HOAc (1000:7:0.5), and MeOH–MeCN–TEA–HOAc (100:900:7:0.5) (all by volume ratios). From the data in Tables 1 and 2 it can be seen that all analytes were retained 2 to 6 times longer due to the increase of the ratio of TEA to HOAc (12:3 versus 7:0.5). This increase in retention may be attributed to the formation of strong complex between carboxylic acid of a derivatized amino acid and TEA in mobile phase. Also noticed is that different chiral selectivities were observed for most of the derivatized amino acids when the ratio of TEA to HOAc was changed.

Interesting results were observed when the mobile phase contained 10% MeOH. First of all, retention was significantly reduced for most derivatized amino acids. This reduction may be due to the competition of methanol with the analytes for interactions with the hydroxyl groups of the cyclodextrin stationary phase. Secondly, the enantioselectivities for most amino acid derivatives were reduced. However, in the case of FMOC-glycyl-aspartic acid on a γ-CD CSP, an exceptionally better separation was obtained with MeOH in the mobile phase. Lastly, the elution orders for some enantiomers were reversed in the presence of 10% MeOH in mobile phase. More reversals were observed on a β-CD CSP than on a γ-CD CSP [FMOC-glycyl-DL-leucine, FMOC-glycyl-DL-norleucine and FMOC-β-alanyl-DL-homophenylalanine on β-CD and only FMOC-glycyl-DL-3-(2-naphthylalanine) on γ-CD].

The methanol effect on retention and enantioselectivity was further examined and the results are depicted in Fig. 4. It can be seen from Fig. 4 that initial addition of methanol dramatically reduced the retention of the FMOC-glycyl-DL-norleucine. However, beyond the point of 30% methanol, little change in retention was observed (not shown in Fig. 4.) Also, the reduction in retention for FMOC-glycyl-D-norleucine is faster than that for FMOC-glycyl-L-norleucine during the initial addition of MeOH. As a result, at a concentration of 2% methanol, the two enantiomers coeluted and no separation was obtained. As the concentration of methanol exceeded 2%, enantioseparation re-appeared but the elution order was reversed. The enantiomers coeluted again and retentions for both enantiomers were approaching void volume when 30% or more methanol was added. The elution order reversal caused by the addition of methanol to the mobile phase suggests that two different enantiorecognition mechanisms may exist. When the concentration of methanol is less than 2%, one mechanism dominates the enantioselectivity while after it reaches 2%, the other mechanism controls the enantioseparation.

3.3. Effect of the glycyl moiety in peptides derivatized with FMOC

Table 3 gives the retention, enantioselectivity and elution order on β-CD and γ-CD CSPs of FMOC-derivatized DL-alanine, DL-leucine or their peptides containing glycyl moieties at various positions. As can be seen from this table the number and sequence of glycyl moieties in the peptides play a key role in enantioselectivities and elution orders of DL-alanine and DL-leucine. On a γ-CD CSP, enantioseparation was achieved only when a glycyl moiety was introduced into the structure. However, this effect was negated when a β-alanyl moiety was sandwiched between an FMOC-moiety and the existing peptide. When one or two glycyl moieties were introduced between an FMOC moiety and DL-alanine or DL-leucine, the L-enantiomer eluted first and enantioselectivity was improved. If the sequence of glycyl moieties and DL-amino acids was changed, the elution order was reversed and enantioselectivity was reduced. If the DL-amino acid moiety was sandwiched between two glycyl moieties, enantioselectivity was completely lost.

On a β-CD CSP, a different trend was observed. The chiral recognition of FMOC-β-alanyl-DL-leucine was significantly lessened by the introduction of a glycyl moiety into the structure even though the derivatives were still effectively retained. However, the separation of FMOC DL-alanine was achieved when two glycyl moieties are present in the structure and both carboxylic acid and amino groups of alanine are converted into peptide bonds. Enantioseparation of partially separated FMOC-DL-leucine was improved when the DL-leucine moiety was linked with a glycyl dipeptide moiety through its amino group. The elution order of the derivatized enantiomers can still be controlled by the position of glycyl moiety in the structures of derivatives.

The significance of the number and position of glycyl moieties discussed above suggests that the chain length and position of the stereogenic center of a derivatized amino acid have an important and subtle impact on stereoselective interactions between a chiral stationary phase and analyte molecules. It seems that the spatial orientations of different derivatives result in different arrangements on the top of cyclodextrin and thus the chiral recognition process is altered. The study reveals that by regulating the number and position of the glycyl moieties in FMOC-DL-amino acids, it is possible to obtain desirable chiral separations and elution orders.

In summary, precolumn derivatization of amino acids with an FMOC-based derivatizing agent pro-

vides an effective means for enantiomeric separation of amino acids. The combination of native cyclodextrin bonded CSPs and a nonaqueous polar mobile phase offers a wide range of possibilities to optimize chiral separations. The proposed chiral separation mechanism suggests that hydrogen bonding interactions between hydroxyl groups of cyclodextrin and the functionalities of amino acid derivatives is responsible for the retention and chiral recognition. The introduction of glycyl and β-alanyl moieties extends the length of the analyte and provides more active sites for hydrogen bonding interaction, which enhances and somehow alters chiral interactions and may cause reversals of elution orders. Mobile phase compositions also affect the retention and chiral recognition. The shorter retention and different enantioselectivity influenced by the addition of methanol to the mobile phase is probably due to the competition of methanol with the analyte for the hydroxyl groups of cyclodextrin. The enantioresolution and elution order of FMOC-amino acid derivatives can be effectively manipulated by the number of glycyl moieties inserted into the structure of the derivative and the linkage sequence of the resulting glycyl containing peptides.

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