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Master's Degree Thesis

Enantiomer Separation of Amino Acid Derivatives Using Polysaccharides Derived Chiral Stationary Phases by High-Performance Liquid Chromatography

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고성능액체크로마토그래피에서다당유도체를기초로한키랄고정상을이용한아미노산유도체의광학분리

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2016 년 2 월 25 일

조선대학교 대학원

약 학 과

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지도교수 이 원 재

이 논문을 약학 석사학위신청 논문으로 제출함

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이스람 포크룰의 석사학위논문을 인준함

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ABSTRACT

Enantiomer Separation of Amino Acid Derivatives Using Polysaccharides Derived Chiral Stationary Phases by High-Performance Liquid Chromatography

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Liquid chromatographic enantiomer separation of α-amino acids and esters as nitrobenzoxadiazole (NBD) and fluorenylmethoxycarbonyl (FMOC) derivatives was performed also a chiral analytical method was developed to determine the purity of α-amino acids and esters as FMOC derivatives using several chiral stationary phases (CSPs) based on polysaccharide derivatives under fluorescence detection. In case of NBD derivatives, the performance of Chiralpak IA was superior for enantiomer resolution of NBD derivatives of several α-amino acid methyl esters. Also this convenient analytical method was applied to determine the optical purity of α-amino acids esters as NBD derivatives. It was investigated that the enantiomeric impurity levels of 0.02-1.73% were found after determination of enantiomeric purities of several commercially available L-amino acid methyl esters as NBD derivatives.

In case of FMOC derivatives, the degree of enantiomer separation of FMOC α-amino acid ester derivatives is better than that of the corresponding acids. Chiralpak ID showed the best enantiomer separation. Several commercially available racemic and L-α-amino acid methyl esters after FMOC derivatization were determined for their chemical and optical purities. It was shown that optical impurities of L-amino acid methyl esters range 0.02-0.55%, while their chemical impurities of the corresponding acids range 0.14-13.26%. The analytical method developed in this study expected to be very useful for liquid chromatographic enantiomer separation of amino acid derivatives using polysaccharides derived chiral stationary phases.

Keywords: Chiral stationary phase, Enantiomer separation, α-Amino acid, Nitrobenzoxadiazole derivative, Fluorenylmethoxycarbonyl derivative

국문초록

고성능액체크로마토그래피에서다당유도체를 기초로한키랄고정상을이용한아미노산유도체 의광학분리

이스람 포크룰

지도교수: 이 원재

약학과

조선대학교 대학원

고성능 액체 크로마토그래피에서 키랄 선택자로 다당 유도체를 기초로 하는 키랄 고정상을 사용하여 여러 종류의 아미노산과 이들 에스테르의 NBD 과 FMOC 유도체의 광학분리를 수행하였다.본 연구에서 개발한 광학분리 방법을 이용하여 형광검출하에서 위 분석물질들의 광학순도를 측정하였다. 아미노산 에스테르의 NBD 유도체의 광학분리의 경우, 여러 키랄 고정상중에서 Chiralpak IA 에서의 아미노산 메틸 에스테르의 광학분리가 가장 우수하게 나타났다. 본 광학분리 방법을 이용하여 시판되고 있는 L-아미노산 메틸 에스테르의 순도를 측정하였고 광학불순물이 0.02-1.73%함유되어 있음을 확인하였다. 또한 아

미노산과 이들 에스테르의 FMOC 유도체의 광학분리의 경우, 아미노산 에스테르 FMOC 유도체의 광학분리가 아미노산 FMOC 유도체 것보다 더 좋게 나타났고 Chiralpak ID 에서 광학분리가 가장 우수하게 나타났다. 본 실험방법을 이용하여 시판되고 있는 L-아미노산 메틸에스테르를 광학분리하였을 때, 이들의 광학불순물은 0.02-0.55% 함유되어 있지만 화학적 불순물의해당 아미노산도 0.14-13.26% 함유되어 있음이 관찰되었다. 본 연구의고성능액체크로마토그래피에서키랄선택자로다당유도체를 기초로 하는 키랄고정상을이용한 분석법이 아미노산 유도체의 광학분리에 매우 유용하게 응용될 수 있으리라 기대한다.

Keywords: Chiral stationary phase, Enantiomer separation, α-Amino acid, Nitrobenzoxadiazole derivative, Fluorenylmethoxycarbonyl derivative

PART 1. Liquid Chromatographic Enantiomer Separation of α-Amino Acid Esters as Nitrobenzoxadiazole Derivatives Using Polysaccharide-Derived Chiral Stationary Phases

1. Abstract

Liquid chromatographic enantiomer separation of α-amino acid esters as nitrobenzoxadiazole (NBD) derivatives was performed using several chiral stationary phases (CSPs) based on polysaccharide derivatives under fluorescence detection. For enantiomer separation by normal HPLC, the non-aqueous derivatization method of α-amino acid esters for NBD analytes was introduced. Among the six CSPs used in this study, the performance of Chiralpak IA was superior for enantiomer resolution of NBD derivatives of several α-amino acid methyl esters. Also the convenient analytical method using polysaccharide-derived CSPs developed in this study was applied to determine the optical purity of α-amino acids esters. It was investigated that the enantiomeric impurity levels of 0.02-1.73% were found after determination of enantiomeric purities of several commercially available L-amino acid methyl esters. It is expected to be quite useful for enantiomer separation of other α-amino acid esters as NBD derivatives by normal HPLC.

Keywords: α-amino Acid Esters, Chiral Stationary Phase, Enantiomer Separation, NBD Derivatives

2. Introduction

In the fields of pharmaceutical industry, α-amino acids and/or esters have been widely used as important chiral building blocks and their enantiomer separation for development of chiral drugs has been of great interest [1]. For the determination of enantiomeric purity of α-amino acids and/or ester derivatives, several analytical methods have been developed [1, 2]. Among them, the liquid chromatographic enantiomer separation on chiral stationary phase (CSP) has been known to be one of the most convenient and versatile methods. Particularly, polysaccharide-derived CSPs have been widely and successfully used for separating a variety of enantiomer compounds [2-4]. Related to this study, we have been reported enantiomer separation of α-amino acids and/or esters as several aromatic moiety derivatives using these polysaccharide-derived CSPs [5-8]. In this study, we focused on nitrobenzoxadiazole (NBD) group which is a fluorescence active derivatizing groupof α-amino acids esters, because NBD fluorescence detection may provide strong advantages of selectivity and sensitivity in enantiomer separation. In previous studies, some analytical results of amino acids as NBD derivatives have been reported [9-12]. In particular, for enantiomer resolution of amino acids as NBD derivatives, Pirkle type chiral columns of Sumochiral OA 2500, Chiralpak QN-AX, and Chiralpak QD-AX were used by Zaitsu group [10-12].

Although they reported pretty good enantiomer separation results under aqueous HPLC conditions, but not high enantioselectivity. In general, for NBD derivatization of α-amino acids, they have used aqueous sodium borate buffer system [9-12]. Here, we are to perform enantiomer separation of α-amino acid esters as NBD derivatives by normal phase chromatography using polysaccharide-derived CSPs. Until now, no previous studies have been reported for enantiomer separation of amino acid esters as NBD derivatives using polysaccharide-derived CSPs by normal phase chromatography.

3. Experimental Section

Chromatographic analysis was carried out using an HPLC system with HP series 1100 with G1310A Iso pump, an automatic sample injector and an HP 1046A programmable fluorescence detector. All covalently immobilized CSPs (Chiralpak IA, Chiralpak IB, Chiralpak IC, Chiralpak ID, Chiralpak IE and Chiralpak IF) derived from polysaccharides were purchased from the Daicel Chemical Company (Tokyo, Japan). HPLC grade hexane, 2-propanol and other solvents were obtained from J. T. Baker. All α-amino acid methyl esters, 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD chloride) and several bases such as sodium bicarbonate were obtained from Aldrich (Milwaukee, WI), Sigma (St. Louis, MO), Advanced ChemTech (Louisville, KY) and Chem-Impex International (Wood Dale, IL). The NBD racemic or L-analytes used in this study were synthesized, as shown on Fig. 1. The NBD derivatives of α-amino acid esters were prepared by stirring 0.25 mmol of α-amino acid ester hydrochloride salts, 0.5 mmol of NBD chloride and 2.5 mmol of sodium bicarbonate in 5 mL ethanol at room temperature for 12 hours. After 12 hours, the reaction mixtures were sonicated at 50oC for 30 to 60 minutes and then filtered to remove sodium bicarbonate and diluted by adding ethanol. And then the resulting solutions were directly injected into the HPLC.

Chromatography was performed at room temperature and a flow rate of 1 mL/ min; 20-40% 2-propanol/hexane (V/V) were used as mobile phases. UV and fluorescence detectors were connected on-line with detection wavelength of UV 337 nm in HP 1100 series of HPLC system and fluorescence excitation 470 nm and emission 530 nm with HP 1046A fluorescence detector [9-12].

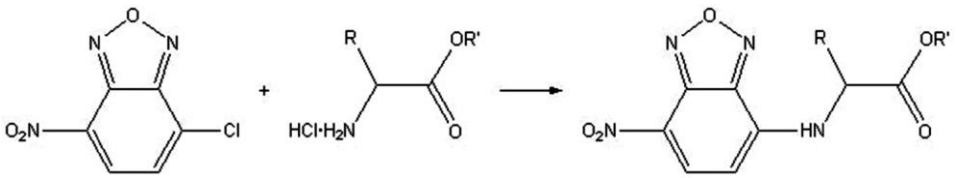
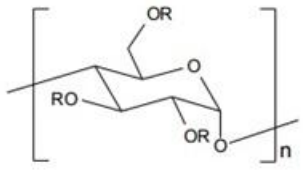
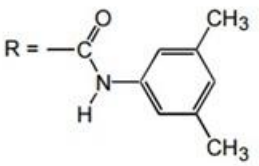


Figure 1. Preparation of α-amino acid esters as NBD derivatives.

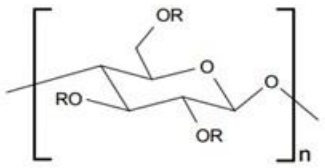


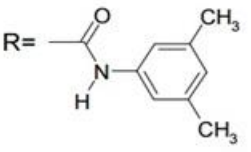


Particle size: 5μm

Chiralpak IA

Amylose tries-(3, 5-dimethylphenylcarbamate)
immobilized to silica gel

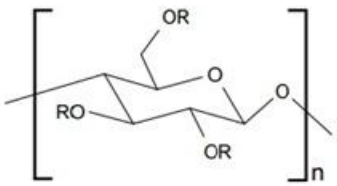


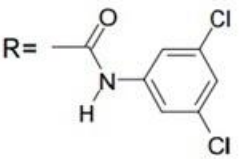


Particle size: 5μm

Chiralpak IB

Cellulose tris-(3, 5-dimethylphenylcarbamate)
immobilized to silica gel



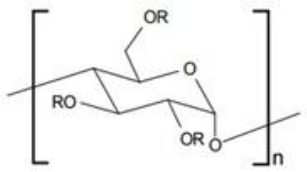


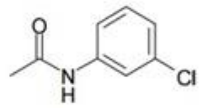
Particle size: 5μm

Chiralpak IC

Cellulose tris-(3, 5-dichlorophenylcarbamate)
immobilized to silica gel

Figure 2. The structure of chiral selector of covalently bonded polysaccharide-derived CSPs (Chiralpak IA, Chiralpak IB and Chiralpak IC)

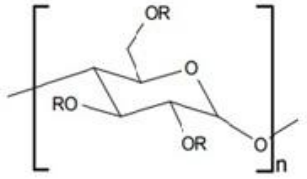


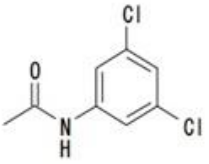
R = 

Particle size: 5μm

Chiralpak ID

Amylose tris-(3-chlorophenylcarbamate)
immobilized to silica gel

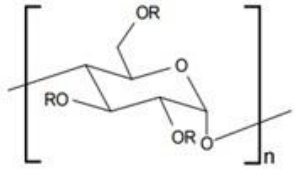


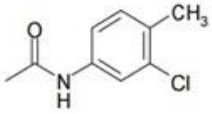
R = 

Particle size: 5μm

Chiralpak IE

Amylose tris-(3, 5-dichlorophenylcarbamate)
immobilized to silica gel



R = 

Particle size: 5μm

Chiralpak IF

Amylose tris-(3-chloro-4-methylphenylcarbamate)
immobilized to silica gel

Figure 2. The structure of chiral selector of covalently bonded polysaccharide-derived CSPs (Chiralpak ID, Chiralpak IE and Chiralpak IF)

4. Results and Discussion

For normal HPLC analytes, a new convenient, nonaqueousderivatization method of α-amino acid esters as NBD derivatives was developed. The NBD derivatives were prepared by reacting NBD chloride and α-amino acid methyl ester HCl with sodium bicarbonate in ethanol at room temperature. For optimized non-aqueous derivatization, several solvents (methanol, ethanol, 2- propanol and acetonitrile) and bases (sodium bicarbonate, sodium carbonate, sodium borate, triethylamine and 1,8-diazabicycloundec-7-ene) were investigated and, finally, sodium bicarbonate in ethanol for reaction condition was used. Although the reaction in methanol was the fastest, it provided the by-product of methoxy NBD. Especially, the use of other bases gave the unexpected impurity during preparation process. After NBD derivatization with sodium bicarbonate in ethanol, the reaction mixture was filtered to remove the solid base and then the resulting solution was used. Tables 1-6 show the chromatographic results for enantiomer separation of several α-amino acid methyl esters as NBD derivatives on six covalently bonded CSPs derived from polysaccharides (Chiralpak IA, Chiralpak IB, Chiralpak IC, Chiralpak ID, Chiralpak IE and Chiralpak IF). Chiralpak IA in Table 1 showed the greatest enantioselectivity, while Chiralpak ID in Table 4 showed the smallest.

In addition to Chiralpak IA,most analytes were separated in Chiralpak IB or Chiralpak IE but the resolution factor and separation factor is not as good as that on Chiralpak IA. The degree of enantioselectivity for six CSPs is as follows; Chiralpak IA >Chiralpak IE >Chiralpak IB >Chiralpak IF ~ Chiralpak IC >Chiralpak ID. Especially, all investigated analytes were base-line separated on Chiralpak IA (α = 1.11-2.21, Rs = 1.54-9.10) and on Chiralpak IE (α = 1.08-1.95, Rs = 1.16-10.43). Consistently, the L-isomers of all investigated analytes are secondly eluted on Chiralpak IA in Table 1 and Chiralpak IB in Table 2, except for phenylalanine methyl ester on Chiralpak IB. On the contrary, the D-isomers of all resolved analytes are secondly eluted on Chiralpak IE in Table 5 and Chiralpak IF in Table 6, except for serine methyl ester on Chiralpak IE. Therefore, the use of Chiralpak IA and Chiralpak IE is complementary to check the trace amount of the enantiomer of the analyte[11], because Chiralpak IA in Table 1 and Chiralpak IE in Table 5 provide good base-line resolution for all analytes with the opposite elution order, except for serine methyl ester. The stability test of optical purity for analytes after NBD derivatization of α-amino acid methyl esters was performed. Table 7 shows stability test results of optical purity for NBD derivatives of L-methionine methyl ester (Sigma-Aldrich) after NBD derivatization in ethanol on Chiralpak IA.

During 20 days of storage at 4°C, its optical purity was almost unaffected, showing the stability of analytes. Furthermore, we applied the developed chromatographic method to determine the enantiomeric purities of several commercially available α-amino acid methyl esters as NBD derivatives. As shown in Table 8, their enantiomeric impurity levels of 0.02-1.73% were found. All investigated a-amino acid methyl esters in this study have over 99% optical purity, except for L-phenylglycine methyl ester. Fig. 2 shows typical chromatograms to determine the enantiomeric purities of L-methionine methyl ester (Sigma-Aldrich) (D:L=0.05:99.95) and L-phenylalanine methyl ester (Advanced ChemTech) (D:L=0.04:99.96) as NBD derivatives on Chiralpak IA under fluorescence detection.

Table 1. Separation of enantiomers of α-amino acid methyl esters as NBD derivatives on Chiralpak IA

Analyte	**α**	**k'$_1$**	**R$_s$**	**Conf.***
Alanine	1.37	2.02	3.62	L
Leucine	1.93	1.07	7.29	L
Methionine	1.66	2.27	6.12	L
Norleucine	2.21	1.18	8.88	L
Norvaline	1.78	1.33	6.47	L
Phenylalanine	1.96	2.31	9.10	L
Phenylglycine	1.11	2.84	1.54	L
Serine	1.39	3.94	4.08	L
Valine	1.49	1.42	5.10	L

Mobile phase: 25% 2-propanol/hexane (V/V); α: Separation factor, k'$_1$: Capacity factor of first eluted enantiomer, R$_s$: Resolution factor.

*indicates the absolute configuration of the second eluted enantiomer.

Table 2. Separation of enantiomers of α-amino acid methyl esters as NBD derivatives on Chiralpak IB

Analyte	**α**	**k'$_1$**	**R$_s$**	**Conf.***
Alanine	1.04	4.49	0.39	L
Leucine	1.08	1.91	0.77	L
Methionine	1.07	4.08	1.09	L
Norleucine	1.07	1.77	0.96	L
Norvaline	1.08	1.81	1.11	L
Phenylalanine	1.11	4.45	1.63	D
Phenylglycine	1.06	4.63	0.85	L
Serine	1.05	7.06	0.37	L
Valine	1.11	2.49	1.32	L

Mobile phase: 25% 2-propanol/hexane (V/V), α: Separation factor, k'$_1$: Capacity factor of first eluted enantiomer, R$_s$: Resolution factor.

*indicates the absolute configuration of the second eluted enantiomer.

Table 3. Separation of enantiomers of α-amino acid methyl esters as NBD derivatives on Chiralpak IC

Analyte	**α**	**k'$_1$**	**R$_s$**	**Conf.***
Alanine	1.10	6.61	1.16	L
Leucine	1.00	4.70	-	-
Methionine	1.14	8.18	1.08	D
Norleucine	1.09	5.55	1.44	L
Norvaline	1.07	5.35	1.00	L
Phenylalanine	1.00	7.05	-	-
Phenylglycine	1.09	7.75	1.56	D
Serine	1.00	3.71	-	-
Valine	1.11	5.56	1.76	L

Mobile phase: 40% 2-propanol/hexane (V/V), α: Separation factor, k'$_1$: Capacity factor of first eluted enantiomer, R$_s$: Resolution factor.

*indicates the absolute configuration of the second eluted enantiomer.

Table 4. Separation of enantiomers of α-amino acid methyl esters as NBD derivatives on Chiralpak ID

Analyte	**α**	**k'$_1$**	**R$_s$**	**Conf.***
Alanine	1.22	8.84	2.04	D
Leucine	1.00	3.83	-	-
Methionine	1.29	8.76	2.40	D
Norleucine	1.00	4.88	-	-
Norvaline	1.06	5.28	0.67	D
Phenylalanine	1.00	6.82	-	-
Phenylglycine	1.17	9.99	2.76	L
Serine	1.06	11.29	1.03	L
Valine	1.00	5.16	-	-

Mobile phase: 20% 2-propanol/hexane (V/V), α: Separation factor, k'$_1$: Capacity factor of first eluted enantiomer, R$_s$: Resolution factor.

*indicates the absolute configuration of the second eluted enantiomer.

Table 5. Separation of enantiomers of α-amino acid methyl esters as NBD derivatives on Chiralpak IE

Analyte	**α**	**k'_1**	**R_s**	**Conf.***
Alanine	1.95	5.81	10.43	D
Leucine	1.38	3.17	3.91	D
Methionine	1.08	6.58	1.16	D
Norleucine	1.17	4.94	2.52	D
Norvaline	1.46	4.48	5.54	D
Phenylglycine	1.32	21.17	5.48	D
Phenylalanine	1.14	5.51	1.99	D
Serine	1.19	4.18	2.02	L
Valine	1.36	5.09	3.78	D

Mobile phase: 40% 2-propanol/hexane (V/V), α: Separation factor, k'_1: Capacity factor of first eluted enantiomer, R_s: Resolution factor.

*indicates the absolute configuration of the second eluted enantiomer.

Table 6. Separation of enantiomers of α-amino acid methyl esters as NBD derivatives on Chiralpak IF

Analyte	**α**	**k'_1**	**R_s**	**Conf.***
Alanine	1.25	8.11	2.63	D
Leucine	1.00	3.92	-	-
Methionine	1.00	10.15	-	-
Norleucine	1.04	4.68	0.33	D
Norvaline	1.08	5.25	1.38	D
Phenylalanine	1.68	7.96	4.50	D
Phenylglycine	1.36	7.72	4.14	D
Serine	1.00	13.28	-	-
Valine	1.15	4.80	1.78	D

Mobile phase: 20% 2-propanol/hexane (V/V), α: Separation factor, k'_1: Capacity factor of first eluted enantiomer, R_s: Resolution factor.

*indicates the absolute configuration of the second eluted enantiomer.

Table 7. Stability test results of optical purity for L-methionine methyl ester (Sigma-Aldrich) as NBD derivative stored at 4°C after NBD derivatization in ethanol on Chiralpak IA

Storage period	D : L ratio[a]	RSD[b]
0 Day	0.05 : 99.95	0.01%
1 Day	0.05 : 99.95	0.02%
2 Day	0.06 : 99.94	0.02%
4 Day	0.06 : 99.94	0.01%
7 Day	0.06 : 99.94	0.01%
10 Day	0.06 : 99.94	0.01%
15 Day	0.08 : 99.92	0.01%
20 Day	0.08 : 99.92	0.02%

Mobile phase: 20% 2-propanol/hexane (V/V).

[a]Average value of three times determined.

[b]Relative standard deviation.

Table 8. Determination of the enantiomeric purity of some commercially available L-Amino acid methyl esters as NBD derivatives

Sample	Company	D : L ratio (Average)[a]
L-Alanine methyl ester	Sigma-Aldrich	0.02 : 99.98
L-Leucine methyl ester	Sigma-Aldrich	0.09 : 99.91
L-Methionine methyl ester	Sigma Aldrich	0.05 : 99.95
L-Norleucine methyl ester	Chem-Impex International	0.66 : 99.34
L-Phenylalanine methyl ester	Advanced Chem Tech	0.04 : 99.96
L-Phenylglycine methyl ester	Sigma-Aldrich	1.73 : 98.27

[a]Average value of three times determined.

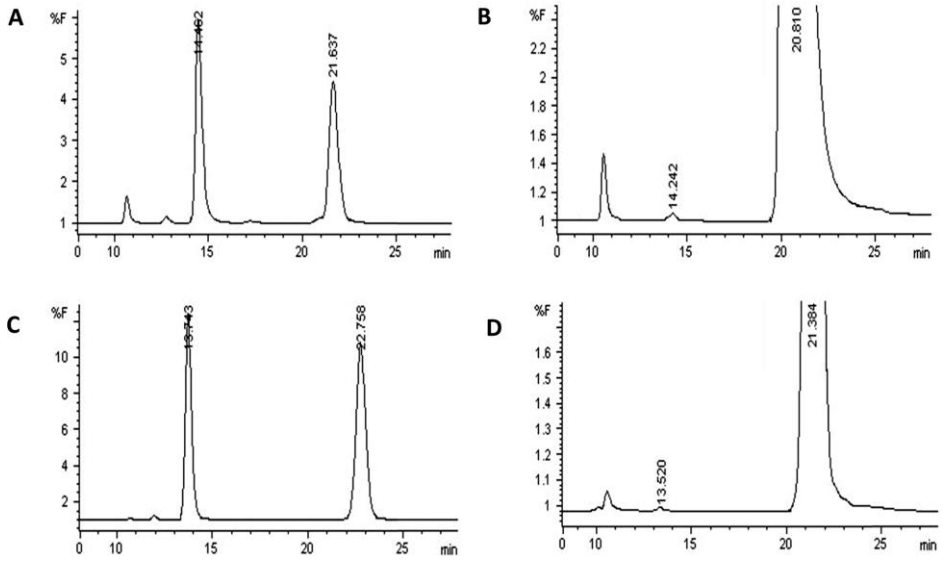


Figure3. Chromatograms of the enantiomeric resolution of the NBD derivatives of (A) racemic methionine methyl ester, (B) L-methionine methyl ester (Sigma-Aldrich) (D:L = 0.05:99.95), (C) racemic phenylalanine methyl ester and (D) L-phenylalanine methyl ester (Advanced ChemTech) (D:L = 0.04:99.96) on Chiralpak IA. Mobile phase: 20% 2-propanol/ hexane (V/V). Flow rate: 1 mL/min. Fluorescence detection excitation: 470 nm, emission: 530 nm.

5. Conclusion

The enantiomer separation of α-amino acid methyl esters as NBD derivatives was performed using several polysaccharide-derived covalently immobilized CSPs under fluorescence detection. A new convenient NBD derivatization method for α-amino acid esters was introduced for normal chiral HPLC analytes. In addition to strong UV detection of NBD derivatives, fluorescence detection used in this study has strong advantages of selectivity and sensitivity in enantiomer separation of α-amino acid esters as NBD derivatives. The performance of Chiralpak IA was the greatest among the other CSPs, showing the base-line separation for all investigated analytes. This analytical method was applied to determine enantiomeric purities of several commercially available α-amino acid methyl esters. It is expected that the convenient analytical method developed in this study will be very useful for enantiomer separation of α-amino acid esters as NBD derivatives on polysaccharide-derived CSPs.

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PART 2. Chromatographic enantiomer separation and determination of chemical and optical purity of α-amino acids and methyl esters as fluorenylmethoxycarbonyl derivativesunder fluorescence detection

1. Abstract

A chiral analytical method to determine the purity of α-amino acid and esters as fluorenylmethoxycarbonyl (FMOC) derivatives was developed and the liquid chromatographic enantiomer separation of FMOC α-amino acids and their methyl esters derivatives was performed on three covalently bonded polysaccharide-derived chiral stationary phases (Chiralpak ID, Chiralpak IE and Chiralpak IF). In general, the degree of enantiomer separation of FMOC α-amino acid ester derivatives is better than that of the corresponding acids. Among three CSPs, Chiralpak ID showed the best enantiomer separation.Several commercially available racemic and L-α-amino acid methyl esters after FMOC derivatization were determined for their chemical and optical purities. It was shown that optical impurities of L-amino acid methyl esters range 0.02-0.55%, while their chemical impurities of the corresponding acids range 0.14-13.26%. In addition to 2-propanol in hexane as a mobile phase, six different mobile phases have been used on Chiralpak ID for determine the effect of mobile phase on the enantiomer separation and it is investigated that the separation factors and retention time on Chiralpak ID are considerably influenced by the nature of mobile phase.

The analytical method developed in this study expected to be very useful for determination of the purity of α-amino acids and their methyl ester as FMOC derivatives on polysaccharide-derived chiral columns.

Keywords: Chiral stationary phase, Enantiomer separation, α-Amino acid, FMOC derivatives.

2. Introduction

A large proportion of therapeutic agents contain chiral molecules that's why the separation of enantiomers is of great interest in the pharmaceutical industries since more than half of pharmaceutical active ingredients are chiral. Chiral compound exist in two enantiomeric forms, two enantiomers shows identical physical and chemical properties and consequently they are discriminated only under chiral environment. Therefore, two enantiomers may show different pharmacological activities in living systems, which are chiral and provide chiral environment. Consequently, only one enantiomer of chiral drugs shows desired activities such as biological activities while the other enantiomer shows undesired effects such as toxicity [1,2]. In this instance, several analytical methods have been developed for determining the enantiomeric composition of chiral compounds, among them HPLC separation of enantiomers on chiral stationary phases (CSPs) has been the method of choice [3-5]. Polysaccharide derived chiral columns have been widely and successfully used as a separating tool for a variety of enantiomers compounds [6-8]. In this study, we have done enantiomeric separation of α-amino acids and their methyl esters as fluorenylmethoxycarbonyl (FMOC) derivatives using three polysaccharide derived CSPs (Chiralpak ID, Chiralpak IE and Chiralpak IF).

These chiral stationary phases manufactured by covalently bonding of the chiral selectors on a silica matrix [7].However, there are no chemical linkages between the chiral selectors and the silica matrix for other coated CSPs like Chiralcel OD and Chiralpak AD. The chiral selectors of polysaccharide derivatives on the coated CSPs maybe washed off from the silica gel if proper solvent not used as mobile phases and therefore, the coated type CSP may be damaged or completely destroyed. Due to their coated nature, these CSPs can only be used with a little range of solvents. Therefore, the solvents such as halogenated solvents, tetrahydrofuran, ethyl acetate and acetone which is partially or totally dissolve the chiral selectors of the polysaccharide derivatives must be avoided as mobile phases for these coated CSPs [6]. α-Amino acids and esters have been widely used as important chiral building blocks in the fields of pharmaceutical chemistry and biochemistry. The fluorenylmethoxycarbonyl (FMOC) group is one of the most commonly used protecting groups for α-amino acids and provides the advantages of high sensitivity in fluorescence detection [9-11] Especially, the FMOC protecting group is very popular, because it is frequently used for amino protecting groups in solid phase synthesis and combinatorial chemistry [12,13]. Related to this study, we also determine the chemical and/or optical purity of several commercially available α-amino acid methyl esters as FMOC derivatives on Chiralpak ID.

3. Materials and methods

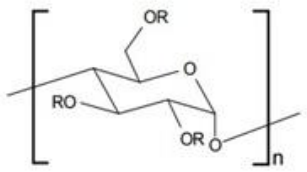
3.1. Apparatus:

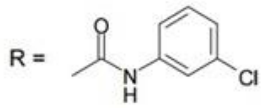
Chromatography was performed with HP series 1100 HPLC system consisting of G1310A Iso pump, an automatic sample injector and an HP 1046A programmable fluorescence detector. The excitation and emission wavelengths were 264nm and 312nm respectively. All enantiomeric separation of α-amino acids and methyl esters as fluorenylmethoxycarbonyl derivatives were carried out at ambient temperature (approximately 25°C) with a flow-rate of 1mL/min. Three covalently bonded type CSPs Chiralpak ID, Chiralpak IE and Chiralpak IF (250 mm x 4.6mm, I.D.) was purchased from Daicel Corporation (Tokyo, Japan).

3.2. Chemicals:

HPLC grade 2-propanol, hexane, ethyl acetate and tetrahydrofuran were obtained from J.T. Baker, HPLC grade acetonitrile obtained from Fisher scientific Korea Ltd, HPLC grade toluene and dichloromethane were obtained from Merck.Trifluoroacetic acid (TFA) was obtained from Acros Organics.Fluorenylmethyloxycarbonyl (FMOC) chloride was obtained from Fluka Company (Switzerland). All α-amino acids and methyl esters used for enantiomer separation were obtained from Sigma-Aldrich. Several commercially available L-amino acid methyl esters used in this study these are: alanine (Sigma-Aldrich), leucine (Sigma-Aldrich), methionine (Sigma-Aldrich), norleucine (Chem-Impex International), norvaline (Chem-Impex International), phenylalanine (Advanced ChemTech), phenylglycine (Sigma-Aldrich), serine (Acros) and valine (Sigma-Aldrich). The racemic and enantiomerically pure FMOC α-amino acids and methyl esters were prepared according to conventional methods [14]. For preparation of FMOC amino acids, racemic or L-amino acids (5 mmol) were dissolved in 10% aqueous sodium carbonate solution (12.5 mmol). Dioxane (7.5 mL) was added and then FMOC chloride (5 mmol) was added slowly then the mixture was stirred for overnight. After overnight stirring at room temperature the reaction mixture was poured into water and extracted with ether.

The aqueous solution was acidified with c-HCl in an ice-bath and it was filtered to get FMOC amino acids as solid form. For the preparation of FMOC amino acid esters, racemic or L-amino acid methyl esters (0.23 mmol) were dissolved in 10% sodium carbonate solution (0.58 mmol), 3 mL of dioxane was added and the mixture was stirred. After five minutes FMOC chloride (0.28 mmol) was added slowly and the reaction mixture was stirred again with a magnetic stirrer for 12 hours at room temperature. The resulting solution was filtered to get the desired sample solution for HPLC injection.

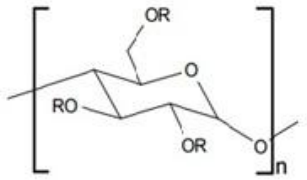


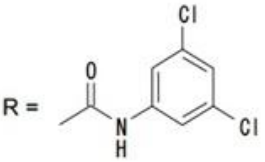


Particle size: 5μm

Chiralpak ID

Amylose tris-(3-chlorophenylcarbamate)
immobilized to silica gel

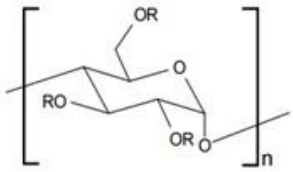


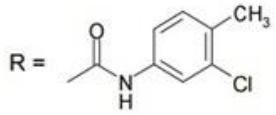


Particle size: 5μm

Chiralpak IE

Amylose tris-(3, 5-dichlorophenylcarbamate)
immobilized to silica gel





Particle size: 5μm

Chiralpak IF

Amylose tris-(3-chloro-4-methylphenylcarbamate)
immobilized to silica gel

Figure 4. The structure of chiral selector of covalently bonded polysaccharide-derived CSPs (Chiralpak ID, Chiralpak IE and Chiralpak IF)

4. Result and discussion

4.1. Result of enantiomer separation of α-amino acids and methyl esters as FMOC derivatives:

Tables 9-11 shows the chromatographic results for enantiomer separation of several α-amino acids and methyl esters as fluorenylmethoxycarbonyl derivatives on three covalently bonded polysaccharide-derived chiral stationary phases (Chiralpak ID, Chiralpak IE and Chiralpak IF).Chiralpak ID in Table 9 showed the greatest enantioselectivity, while Chiralpak IF in Table 11 showed smallest enantioselectivity. In Table 9, we can see that FMOC α-amio acid methyl esters gives good separation factors (α= 1.36-4.03) and resolution factors (R_s= 5.43-20.99) than corresponding acids (separation factors α= 1.00-1.75 and resolution factors R_s= 0.83-9.56). L-Isomers of all investigated analytes (except FMOC valine) are secondly eluted on Chiralpak ID. In Table 10, all analytes of FMOC α-amino acids and their methyl esters were well separated on Chiralpak IE, like Chiralpak ID here also enantiomer separation of FMOC α-amio acid methyl esters is better than that of acids. In Chiralpak IE, D-isomers of all investigated analytes (except FMOC serine and FMOC serine-methyl ester) are secondly eluted. In Table 11, enantiomer separation is not good on Chiralpak IF compare to Chiralpak ID and Chiralpak IE.

In case of FMOC amino acid methyl esters, except valine all analytes is separated but the elution order is not consistent, while two analytes of corresponding acids (serine and valine) is not separated and identical elution order is seen in other analytes. The degree of enantioselectivity for three CSPs is as follows Chiralpak ID >Chiralpak IE >Chiralpak IF. All analytes of FMOC α-amino acid methyl esters were base-line separated on Chiralpak ID and Chiralpak IE, where three analytes of FMOC α-amino acid methyl esters (alanine, methionine and phenylglycine) were partially separated on Chiralpak IF.

4.2. Results of Chemical and Optical purities:

The chromatographic method in this study was used for determination of the chemical purities of commercially available racemic amino acid methyl esters as FMOC derivatives on Chiralpak ID. We can see from Table 12 that racemic norvaline methyl ester (Chem-Impex International) gives highest amount of impurities (17.50 %), where phenylglycine methyl ester (Sigma-Aldrich) gives lowest amount of impurities (0.49%). Figure 5 shows the typical chromatograms of the resolution of FMOC racemic methionine methyl ester (Sigma-Aldrich), FMOC racemic norleucine methyl ester (Chem-Impex International) and FMOC racemic valine methyl ester (Alfa-Aesar). We also used our developed chromatographic method to determined chemical and optical purities of commercially available L-amino acid methyl esters as FMOC derivatives on Chiralpak ID as shown in Table 13. The chemical impurities of 0.14-13.26% and optical impurities of 0.02-0.55% for commercially available L-amino acid methyl esters were determined in the mobile phase of 10% 2-propanol/hexane (v/v) containing 0.1% trifluoroacetic acid. Table 14 shows the results of all chiral impurities of commercially available L-amino acid methyl esters as FMOC derivatives on Chiralpak ID. In Figure 6, we can see the chromatograms of the resolution of commercially available racemic and L-alanine methyl ester (Sigma-Aldrich) as FMOC derivatives including the total chiral impurities.

Four peak ratio for L-alanine methyl as FMOC derivatives is (D-acid: L-acid: D-methyl ester: L-methyl ester = 0.21: 13.42: 0.50: 85.88).

4.3. Effect of mobile phase on the enantiomer separation:

Table 15 shows the comparative results of chromatographic enantiomer separation of five FMOC α-amino acids using six different mobile phases on Chiralpak ID. We can see from the table that the separation factors and retention time on Chiralpak ID are considerably influenced by the nature of mobile phase. Standard mobile phase using 10% 2-propanol/hexane (v/v) containing 0.1% TFA showed fairly good enantiomer separation compare to other two alcohol/hexane mobile phases 3% acetonitrile + 7% 2-propanol/hexane (v/v) containing 0.1% TFA and 3% toluene + 7% 2-propanol/hexane (v/v) containing 0.1% TFA. Among the three halogenated solvents highest enantioselectivity were obtained by using 40% dichloromethane/ hexane (v/v) containing 0.1% TFA (α= 1.04-1.17, R_s= 1.08-2.34).Compared to chromatographic results obtained from alcohol and non-alcohol in hexane with acid additive as mobile phases, the greatest separation factors and resolution factors were observed in mobile phases containing 2-propanol in hexane with acid additive.

Table 9. Separation of the enantiomers of α-amino acids and methyl esters as FMOC derivatives on Chiralpak ID

	FMOC amino acid				**FMOC amino acid methyl ester**			
Analyte	**α**	**k'$_1$**	**R$_s$**	**Conf.**	**α**	**k'$_1$**	**R$_s$**	**Conf.**
Alanine	1.37	2.67	5.18	L	3.68	5.03	20.99	L
Leucine	1.00	2.22	-	-	1.74	3.22	6.50	L
Methionine*	1.75	5.21	9.56	L	4.03	5.84	15.48	L
Norleucine	1.12	2.19	1.50	L	2.85	3.72	12.50	L
Norvaline	1.07	2.49	1.21	L	2.32	4.06	13.02	L
Phenylalanine	1.19	3.33	3.21	L	1.36	6.16	5.43	L
Phenylglycine	1.32	5.80	4.52	L	1.65	7.94	7.36	L
Serine*	1.31	6.06	4.11	L	2.14	5.09	11.19	L
Valine	1.07	2.20	0.83	D	1.61	3.42	6.08	L

Mobile phase: 10% 2-propanol/hexane (v/v) containing 0.1% TFA for FMOC amino acids& 10% 2-propanol/hexane (v/v) for FMOC amino acid methyl esters, Flow rate: 1mL/min, Fluorescence detection (excitation: 264 nm, emission: 312 nm). k'$_1$: Retention factor of the first eluted enantiomer. α: Separation factor. R$_s$: Resolution factor. Conf.: The absolute configuration of the second eluted enantiomer.

*Mobile phase: 20% 2-propanol/hexane (v/v)

Table 10. Separation of the enantiomers of α-amino acids and methyl esters as FMOC derivatives on Chiralpak IE

	FMOC amino acid				**FMOC amino acid methyl ester**			
Analyte	**α**	**k'$_1$**	**R$_s$**	**Conf.**	**α**	**k'$_1$**	**R$_s$**	**Conf.**
Alanine	1.15	3.53	2.15	D	1.14	6.34	2.01	D
Leucine	1.17	3.13	1.90	D	1.08	5.00	1.09	D
Methionine	1.05	5.86	0.79	D	2.03	10.49	12.24	D
Norleucine	1.09	3.08	1.02	D	1.09	5.09	1.18	D
Norvaline	1.07	3.45	0.96	D	1.16	5.56	2.21	D
Phenylalanine	1.11	5.02	1.41	D	2.48	6.10	10.81	D
Phenylglycine	1.02	6.92	0.23	D	1.54	7.75	6.08	D
Serine	1.31	6.78	3.21	L	1.38	12.63	5.16	L
Valine	1.16	3.77	1.85	D	1.14	4.23	1.88	D

Mobile phase: 10% 2-propanol/hexane (v/v) containing 0.1% TFA for FMOC amino acids &10% 2-propanol/hexane (v/v) for FMOC amino acid methyl esters, Flow rate: 1mL/min, Fluorescence detection (excitation: 264 nm, emission: 312 nm).

k'$_1$: Retention factor of the first eluted enantiomer. α: Separation factor.

R$_s$: Resolution factor. Conf.: The absolute configuration of the second eluted enantiomer.

Table 11. Separation of the enantiomers of α-amino acids and methyl esters as FMOC derivatives on Chiralpak IF

	FMOC amino acid				**FMOC amino acid methyl ester**			
Analyte	**α**	**k'$_1$**	**R$_s$**	**Conf.**	**α**	**k'$_1$**	**R$_s$**	**Conf.**
Alanine	1.05	3.12	0.71	L	1.05	5.26	0.53	L
Leucine	1.11	2.31	1.49	L	1.30	3.58	1.74	L
Methionine	1.27	4.74	3.54	L	1.07	10.62	0.67	D
Norleucine	1.07	2.54	0.96	L	1.21	3.85	1.47	L
Norvaline	1.10	2.66	1.40	L	1.14	4.69	1.00	D
Phenylalanine	1.08	4.22	1.10	L	1.47	6.70	3.00	D
Phenylglycine	1.24	5.59	3.60	L	1.04	7.21	0.32	D
Serine	1.00	5.82	-	-	1.33	9.31	2.74	L
Valine	1.00	3.19	-	-	1.00	4.64	-	-

Mobile phase: 10% 2-propanol/hexane (v/v) containing 0.1% TFA for FMOC amino acids &10% 2-propanol/hexane (v/v) for FMOC amino acid methyl esters, Flow rate: 1mL/min, Fluorescence detection (excitation: 264 nm, emission: 312 nm). k'$_1$: Retention factor of the first eluted enantiomer. α: Separation factor. R$_s$: Resolution factor. Conf.: The absolute configuration of the second eluted enantiomer.

Table 12. Chemical purities of commercially available racemic amino acid methyl esters as FMOC derivatives on Chiralpak ID

Analyte	**Company**	**Chemical purity (racemic amino acid : racemic methyl ester)**	**RSD[a]**
Alanine	Sigma-Aldrich	4.06 : 95.94	0.14%
Leucine	Bachem	0.54 : 99.46	0.15%
Methionine	Sigma Aldrich	11.56 : 88.44	0.06%
Norleucine	Chem-Impex International	7.62 : 92.38	0.22%
Norvaline	Chem-Impex International	17.50 : 82.50	0.42%
Phenylalanine	Advanced ChemTech	0.56 : 99.44	0.08%
Phenylglycine	Sigma-Aldrich	0.49 : 99.51	0.07%
Serine*	Acros	0.79 : 99.21	0.04%
Valine	Alfa-Aesar	0.51 : 99.49	0.04%

Mobile phase: 10% 2-propanol/hexane (v/v) containing 0.1% TFA, Flow rate: 1mL/min, Fluorescence detection (excitation: 264 nm, emission: 312 nm), [a]Relative standard deviation.

*Mobile phase: 20% 2-propanol/hexane (v/v) containing 0.1% TFA

Table 13. Chemical and optical purities of commercially available L-amino acid methyl esters as FMOC derivatives on Chiralpak ID

		Chemical purity of L-amino acid methyl esters		**Optical purity of L-amino acid methyl esters**	
Analyte	**Company**	**Peak ratio (racemic acid : racemic methyl ester)**	**RSD[a]**	**Peak ratio (D-methyl ester : L-methyl ester**	**RSD[a]**
Alanine	Sigma-Aldrich	13.26 : 86.74	0.16%	0.55 : 99.45	0.05%
Leucine	Sigma-Aldrich	0.14 : 99.86	0.03%	0.04 : 99.96	0.01%
Methionine*	Sigma-Aldrich	0.90 : 99.10	0.01%	0.02 : 99.98	0.01%
Norleucine	Chem-Impex International	13.02 : 86.98	0.03%	0.07 : 99.93	0.02%
Norvaline	Chem-Impex International	8.60 : 91.40	0.15%	0.28 : 99.72	0.02%
Phenylalanine	Advanced ChemTech	0.56 : 99.44	0.04%	0.03 : 99.97	0.01%
Phenylglycine	Sigma-Aldrich	1.94 : 98.06	0.02%	0.24 : 99.76	0.03%
Serine*	Acros	0.63 : 99.37	0.01%	0.05 : 99.95	0.01%
Valine	Sigma-Aldrich	0.26 : 99.74	0.01%	0.05 : 99.95	0.01%

Mobile phase: 10% 2-propanol/hexane (v/v) containing 0.1% TFA, Flow rate: 1mL/min, Fluorescence detection (excitation: 264nm, emission: 312 nm), storage temperature was 4°C, [a]Relative standard deviation.

*Mobile phase: 20% 2-propanol/hexane (v/v) containing 0.1% TFA.

Table 14. All chiral impurities of commercially available L-amino acid methyl esters as FMOC derivatives on Chiralpak ID

Analyte	**Company**	**Peak ratio (D-acid : L-acid: D-methyl ester : L-methyl ester)**
Alanine	Sigma-Aldrich	0.21 : 13.42 : 0.50 : 85.88
Leucine*	Sigma-Aldrich	0.02 : 0.11 : 0.05 : 99.82
Methionine**	Sigma-Aldrich	0.01 : 0.59 : 0.05 : 99.35
Norleucine	Chem-Impex International	0.04 : 12.99 : 0.06 : 86.91
Norvaline	Chem-Impex International	0.14 : 8.46 : 0.26 : 91.14
Phenylalanine	Advanced ChemTech	0.03 : 0.55 : 0.03 : 99.39
Phenylglycine	Sigma-Aldrich	0.03 : 1.91 : 0.25 : 97.81
Serine**	Acros	0.07 : 0.55 : 0.05 : 99.33
Valine	Sigma-Aldrich	0.04 : 0.22 : 0.05 : 99.69

Mobile phase: 10% 2-propanol/hexane (v/v) containing 0.1% TFA, Flow rate: 1mL/min, Fluorescence detection excitation: 264 nm, emission: 312 nm. Storage temperature was 4°C.

*Chiralpak IE column was used.

**Mobile phase: 20% 2-propanol/hexane (v/v) containing 0.1%TFA

Table 15. Effect of mobile phase on the enantiomer separation of FMOC racemic amino acids on Chiralpak ID

Mobile phase	**10% 2-Propanol/Hexane (v/v) containing 0.1% TFA**				**3% Acetonitrile+7% 2-Propanol/ Hexane (v/v) containing 0.1% TFA**				**3% Toluene+7% 2-Propanol/ Hexane (v/v) containing 0.1% TFA***			
Analyte	**α**	**k'$_1$**	**R$_s$**	**Conf.**	**α**	**k'$_1$**	**R$_s$**	**Conf.**	**α**	**k'$_1$**	**R$_s$**	**Conf.**
Ala	1.37	2.67	5.18	L	1.05	2.14	0.99	L	1.18	3.92	1.93	L
Leu	1.00	2.22	-	-	1.04	1.47	0.64	D	1.00	2.91	-	-
Phe	1.19	3.33	3.21	L	1.00	2.88	-	-	1.36	3.87	5.81	L
PG	1.32	5.80	4.52	L	1.00	4.03	-	-	1.14	7.71	1.74	L
Val	1.07	2.20	0.83	D	1.07	1.59	1.03	D	1.07	3.05	1.14	D

Flow rate: 1 mL/min, TFA: Trifluoroacetic acid, k'1: Retention factor of the first eluted enantiomer. α: Separation factor. Rs: Resolution factor. Conf.: The absolute configuration of the second eluted enantiomer, Fluorescence detection (excitation: 264 nm, emission: 312 nm), * UV 290 nm

Table 15 (Continued). Effect of mobile phase on the enantiomer separation of FMOC racemic amino acids on Chiralpak ID

Mobile phase	**40% Dichloromethane/Hexane (v/v) containing 0.1% TFA**				**20% Ethyl acetate/Hexane (v/v) containing 0.1% TFA**				**15% Tetrahydrofuran/Hexane (v/v) containing 0.1% TFA**			
Analyte	**α**	**k'_1**	**R_s**	**Conf.**	**α**	**k'_1**	**R_s**	**Conf.**	**α**	**k'_1**	**R_s**	**Conf.**
Ala	1.11	5.23	1.26	L	1.07	4.42	1.05	D	1.00	4.28	-	-
Leu	1.04	3.59	1.08	L	1.00	2.91	-	-	1.00	3.22	-	-
Phe	1.09	5.09	1.37	L	1.05	4.3	0.87	L	1.00	5.83	-	-
PG	1.17	8.31	2.34	L	1.11	5.15	1.49	L	1.00	6.87	-	-
Val	1.12	3.85	2.25	L	1.03	2.95	0.38	L	1.07	2.74	1.07	D

Flow rate: 1 mL/min, TFA: Trifluoroacetic acid, k'1: Retention factor of the first eluted enantiomer. α: Separation factor. Rs: Resolution factor. Conf.: The absolute configuration of the second eluted enantiomer, Fluorescence detection (excitation: 264 nm, emission: 312 nm).

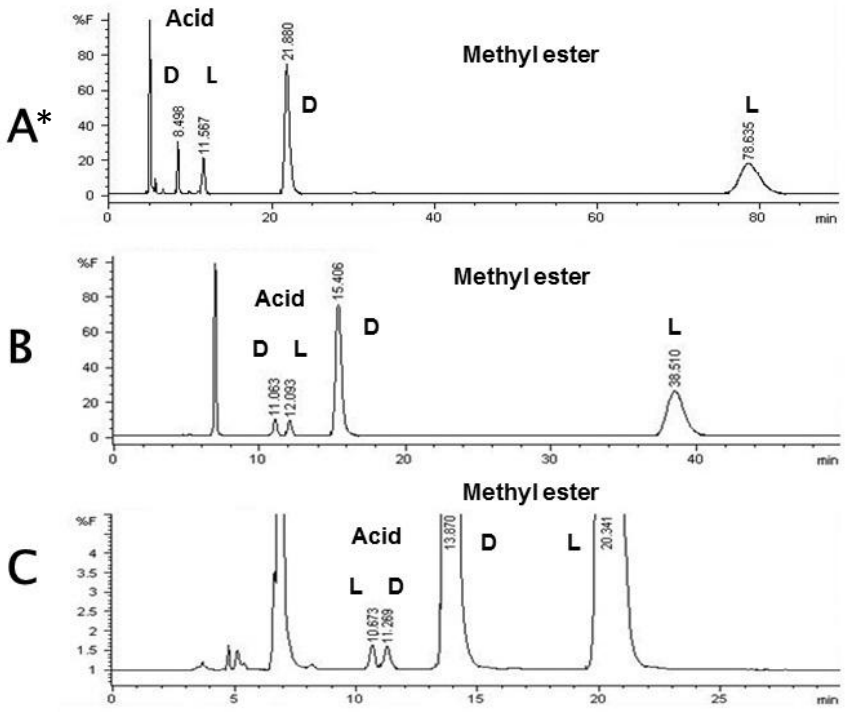


Figure 5. Chromatograms of the resolution of (A) FMOC racemic methionine methyl ester (Sigma Aldrich), amino acid: methyl ester = 11.56: 88.44, (B) FMOC racemicnorleucine methyl ester (Chem-Impex International), amino acid: methyl ester = 7.62: 92.38, (C) FMOC racemic valine methyl ester (Alfa-Aesar), amino acid: methyl ester = 0.51: 99.49. Mobile phase: 10% 2-propanol/hexane (v/v) containing 0.1% TFA, Flow rate: 1mL /min, Fluorescence detection (excitation: 264 nm, emission: 312 nm).

*Mobile phase: 20% 2-propanol/hexane (v/v) containing 0.1% TFA.

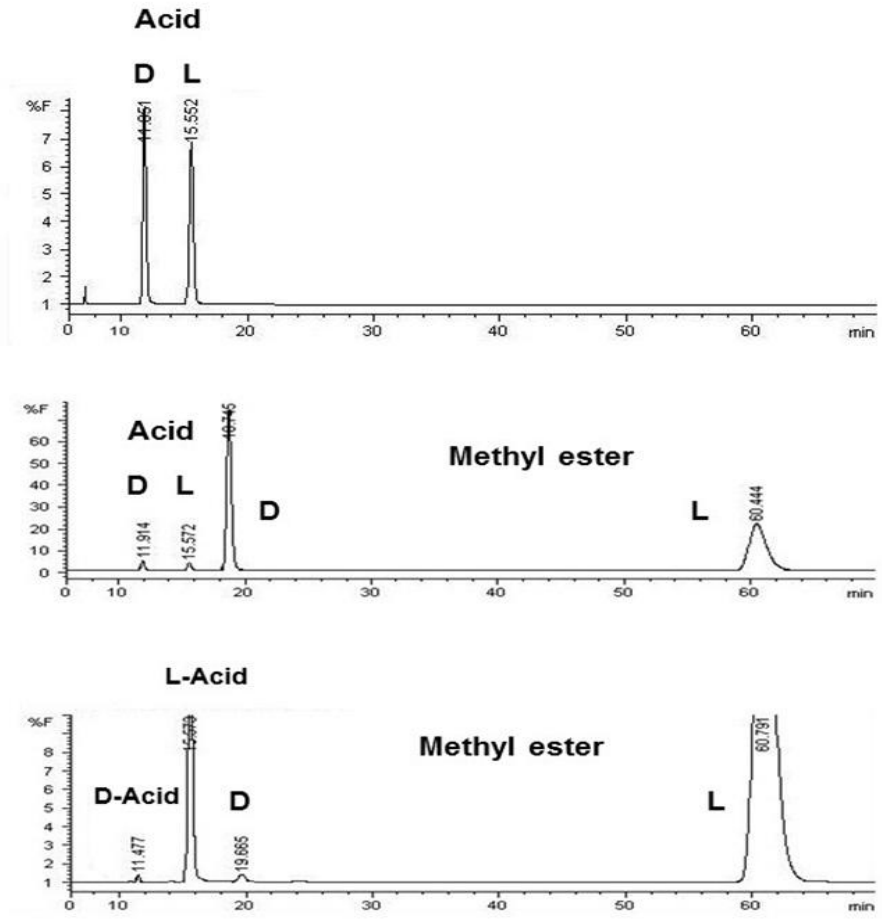


Figure 6. Chromatograms of the resolution of racemic alanine (the top), racemic alanine methyl ester (Sigma-Aldrich) (the middle) and L-alanine methyl ester (Sigma-Aldrich) (the bottom) as FMOC derivatives under fluorescence detection (excitation: 264nm, emission: 312 nm) on Chiralpak ID. Mobile phase: 10% 2-propanol/hexane (v/v) containing 0.1% TFA, Flow rate: 1mL /min.

5. Conclusion

In conclusion, an HPLC fluorescence analysis of enantiomer separation and determination of chemical and/or optical purity of α-amino acids and methyl esters as FMOC derivatives on covalently bonded type CSPs (Chiralpak ID, Chiralpak IE and Chiralpak IF) was performed. In general, the performance of Chiralpak ID is the best among the other CSPs and the degree of enantiomer separation of FMOC α-amino acid ester derivatives is better than that of the corresponding acids. This analytical method was applied to determine chemical and optical purities of several commercially available racemic and L-α-amino acid methyl esters after FMOC derivatization. Also, in addition to 2-propanol in hexane of mobile phase, six different mobile phases have been used on Chiralpak ID for determine the effect of mobile phase on the enantiomer separation. It is expected that the convenient analytical method developed in this study will be very useful for determination of the purity of α-amino acids and their methyl ester as FMOC derivatives on polysaccharide-derived chiral columns.

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