2014년 2월

석사학위논문

HPLC에서 다당 유도체를 기초로 한 키랄 컬럼을 이용한 거울상 이성질체의 광학분리

조선대학교 대학원

약 학 과

윤 원 남

HPLC에서 다당 유도체를 기초로 한 키랄 컬럼을 이용한 거울상 이성질체의 광학분리

The Enantiomer Separation Using Chiral Columns Based on Polysaccharides by HPLC

2014년 2월 25일

조선대학교 대학원

약 학 과

윤 원 남

HPLC에서 다당 유도체를 기초로 한 키랄 컬럼을 이용한 거울상 이성질체의 광학분리

지도교수 이 원 재

이 논문을 약학 석사학위신청 논문으로 제출함

2013년 10월

조선대학교 대학원

약 학 과

윤 원 남

윤원남의 석사학위논문을 인준함

위원장 홍 준 희 인

위 원 김 은 애 인

위 원 이 원 재 인

2013년 11월

조선대학교 대학원

CONTENTS

PART 1. Liquid Chromatographic Resolution of α-Amino Acid Esters as Benzophenone Imine Derivatives

PART 2. Liquid Chromatographic Enantiomer Separation of α-Amino Acid Esters as NBD derivatives

LIST OF TABLES

LIST OF FIGURES

국문초록

HPLC에서 다당 유도체를 기초로 한 키랄 컬럼을 이용한 거울상 이성질체의 광학분리

윤 원남
지도교수: 이 원재
약학과
조선대학교 대학원

Part 1에서는 고성능 액체 크로마토그래피에서 키랄 선택자로 다당 유도체가 코팅된 키랄 컬럼과 공유결합 된 키랄 컬럼을 사용하여 여러 종류의 α-amino acid ester의 benzophenone imine의 광학분리를 수행하였다. Benzophenone imine 유도체 시료는 α-amino acid ester HCl, benzophenone imine Schiff base, magnesium sulfate anhydrous를 2-propanol 용매에 넣어 상온에서 반응시켜 준비하였다. 일반적으로 Chiralpak IC가 다른 컬럼들에 비해서 상당히 좋은 광학분리 결과를 보여주었다.

Part 2에서는 고성능 액체 크로마토그래피에서 다당 유도체가 공유결합 된 키랄 컬럼을 사용하여 다양한 α-amino acid ester를 NBD-Cl로 유도체화 한 화합물의 광학분리를 수행하였다. 이를 위한 NBD 유도체를 얻기 위해 α-amino acid ester HCl, NBD-Cl, sodium bicarbonate를 ethanol 용매에 반응시키는 매우 편리한 유도체화 방법을 개발하였다. NBD 유도체 물질들을 광학분리 한 결과, Chiralpak IA를 사용했던 경우 매우 훌륭한 광학분리 결과를 보여주었다. 또한 α-amino acid ester의 NBD 유도체의 분석은 UV detection보다 fluorescence detection에서 더 선택적이고 뛰어난 감도를 나타내는 것을 확인하였다.

Keywords: Chiral stationary phase, Enantiomer separation, Chiral column, α-Amino acid ester, Benzophenone imine derivative, NBD derivative

PART 1. Liquid Chromatographic Resolution of α-Amino Acid Esters as Benzophenone Imine Derivatives

Abstract

A convenient liquid chromatographic method for the separation of α-amino acid esters as benzophenone Schiff base derivatives on coated chiral stationary phases (CSPs) (Chiralcel OD, Chiralcel OD-H, Chiralpak AD, Chiralpak AD-H, and Chiralpak AS) or covalently immobilized CSPs (Chiralpak IA, Chiralpak IB, and Chiralpak IC) derived from polysaccharide derivatives is described. Benzophenone imine derivatives of α-amino acid esters were readily prepared by stirring benzophenone imine and the hydrochloride salts of α-amino acid esters in 2-propanol. The chromatographic separations were conducted at a flow rate 1.0 mL/min and a detection wavelength of 254 nm; 0.5% 2-propanol/hexane (v/v) was used on CSPs. In general, the resolution of Chiralpak IC was superior to those of the other CSPs. In addition, the resolutions of other arylimine derivatives of α-amino acid esters and the effects of different mobile phases on the enantiomeric separation of α-amino acid esters as benzophenone imine derivatives on Chiralpak IC were investigated.

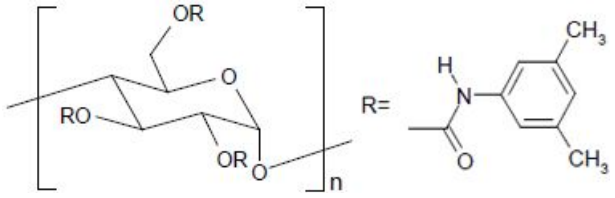
Key words: Chiral stationary phase, Benzophenone imine derivative, Enantiomer separation

1. Introduction

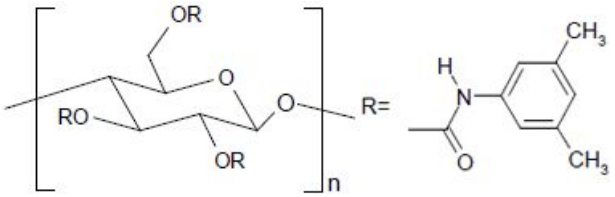
Several methods have been developed to determine the enantiomeric purities and/or configurations of α-amino acids in the pharmaceutical field [1,2]. Of these techniques, liquid chromatographic enantiomer separation on chiral stationary phase (CSPs) has been known to be one of the most convenient and versatile methods. In a previous study, a liquid chromatographic method was described for the separation of enantiomers of α-amino acid esters and chiral amines as 9-anthraldimine Schiff base derivatives on polysaccharide-derived CSPs [3]. Of the CSPs studied, Chiralcel OD (OD-H) provided greatest resolution of the enantiomers of several α-amino acid esters and chiral amines as 9-anthraldimine derivatives. Subsequently, I attempted to develop a convenient method for the derivatization of α-amino acid esters using aromatic Schiff base imines for enantiomer separation, and this work resulted in the selection of the benzophenone imine moiety used as an amine protecting group [4,5]. The benzophenone imine group is expected to function as an aromatic auxiliary group for enantiomer resolution by the chiral selectors of CSPs [6]. Benzophenone Schiff base derivatives have been previously used for the enantioselective synthesis of α-amino acid esters by phase-transfer alkylation [4]. Especially, the benzophenone imine derivatives of α-amino acid esters are readily prepared by simply stirring benzophenone imine and the hydrochloride salts of α-amino acid esters in 2-propanol at room temperature [4,5]. In this study, I describe a convenient chromatographic separation of the enantiomers of α-amino acid esters as their benzophenone imine derivatives on coated and on covalently bonded polysaccharide-derived CSPs.

2. Materials and Methods

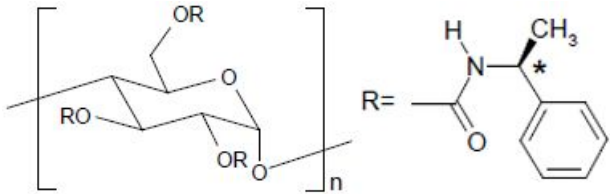
Chromatography was performed at room temperature using an HPLC Breeze system (Waters) equipped with a Waters model 1525 binary pump, an autosampler, and a dual absorbance detector (Waters 2487 detector). HPLC-grade hexane and 2-propanol were obtained from J. T. Baker. Benzophenone imine and all α-amino acid esters were obtained from Aldrich or Sigma or Advanced ChemTech. All polysaccharide-derived CSPs, that is, Chiralcel OD, Chiralpak AD and Chiralpak AS (250 mm L × 4.6 mm I.D., 10 μm), and Chiralcel OD-H, Chiralpak AD-H, Chiralpak IA, Chiralpak IB, and Chiralpak IC (250 mm L × 4.6 mm I.D., 5 μm) were purchased from the Daicel Chemical Company (Fig. 1). Chromatography was performed using a flow rate 1 mL/min, a detection wavelength of UV 254 nm, and 0.5% 2-propanol/hexane (v/v) as mobile phase for all CSPs. In addition, other solvents, such as, tetrahydrofuran, ethyl acetate, and dichloromethane in hexane were added when the covalently immobilized CSP, Chiralpak IC was examined. The racemic and D- or L-analytes used were prepared, as shown in Fig. 2, by stirring 0.5 mmol of α-amino acid ester hydrochloride and an equimolar amount of benzophenone imine in 10 mL of 2-propanol at room temperature for 12 h. Reaction mixtures were filtered to remove ammonium chloride and resulting solutions were directly injected into the chromatograph.



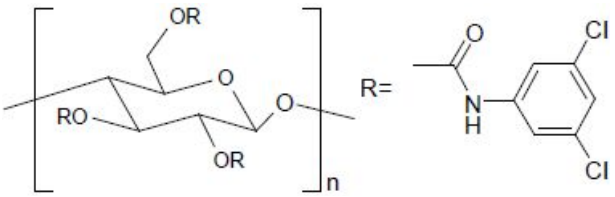
Chiralpak AD/AD-H and Chiralpak IA



Chiralcel OD/OD-H and Chiralpak IB



Chiralpak AS



Chiralpak IC

Figure 1. The structure of chiral selector of coated polysaccharide-derived CSPs (Chiralcel OD, Chiralcel OD-H, Chiralpak AD, Chiralpak AD-H and Chiralpak AS) and covalently bonded polysaccharide-derived CSPs (Chiralpak IA, Chiralpak IB, and Chiralpak IC)

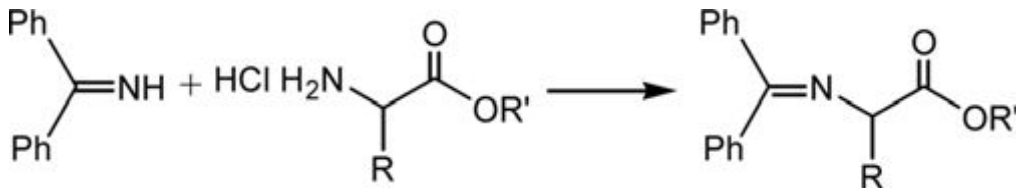


Figure 2. Preparation of the benzophenone imine derivatives of α-amino acid esters.

3. Results and Discussion

3.1. Enantiomeric separation of α-amino acid esters as benzophenone imine Schiff base derivatives

Tables 1-5 summarize chromatographic data for the separation of the enantiomers of several α-amino acid esters as benzophenone imine Schiff base derivatives on coated polysaccharide-derived CSPs (Chiralcel OD, Chiralcel OD-H, Chiralpak AD, Chiralpak AD-H and Chiralpak AS) and on covalently bonded polysaccharide-derived CSPs (Chiralpak IA, Chiralpak IB, and Chiralpak IC) using 0.5% 2-propanol/hexane (v/v) as mobile phase. Of the commonly used coated type CSPs (Chiralcel OD, Chiralcel OD-H, Chiralpak AD, Chiralpak AD-H, and Chiralpak AS) listed in Table 1 and 2, Chiralcel OD-H showed greatest enantioselectivity, and Chiralpak AD the least. Also, it is natural that the separation factors and resolution factors on Chiralcel OD-H and Chiralpak AD-H with the silica particle size 5 μm showed higher than those on Chiralcel OD and Chiralpak AD with the silica particle size 10 μm, respectively. Of the CSPs examined (Tables 1-5), Chiralpak IC provided best enantioseparation, although it did not resolve the benzophenone imine derivatives of two polar analytes (asparagine and aspartic acid). It was interesting to find that the performances of Chiralpak IB and Chiralpak IC were complementary, for whereas Chiralpak IC failed to separate these two polar analytes, they were well separated by Chiralpak IB. Orders of enantioselectivity were OD-H > AD-H > AS~OD > AD for the coated CSPs and IC > IB~IA for the covalently bonded CSPs. In a previous study on the enantiomer separation of 9-anthraldehyde Schiff base derivatives of α-amino acid esters, it was found that among the CSPs investigated, Chiralcel OD (or OD-H) had greatest enantioselectivity, and Chiralpak IC least [3]. The degree of enantiomer separation of benzophenone imine derivatives on Chiralpak IC in this study was lower than that of their

9-anthraldimine derivatives on Chiralcel OD (or OD-H) [3]. However, the derivatization process required to produce benzophenone imine derivatives of amino acid esters is much more straightforward. Typical chromatograms of the benzophenone imine derivative of commercially available L-leucine methyl ester on Chiralpak IC are presented in Fig. 3.

Table 1. Separation of the enantiomers of α-amino acid methyl esters as benzophenone imine derivatives on Chiralcel OD, Chiralcel OD-H

Analyte	Chiralcel OD				Chiralcel OD-H			
	α^a	$k'_1{}^b$	Rs^c	$Conf.^d$	α^a	$k'_1{}^b$	Rs^c	$Conf.^d$
Alanine	1.00	2.98	-		1.07	2.45	0.87	D
Asparagine	1.00	14.50	-		1.00	16.38	-	
Aspartic acid	1.37	10.60	3.61	L	1.47	11.31	6.40	L
Leucine	1.00	1.43	-		1.00	1.42	-	
Phenylalanine	3.65	3.96	9.70	L	3.79	3.32	19.29	L
Phenylglycine	1.42	3.09	2.77	L	1.38	3.24	4.78	L
Valine	1.00	1.18	-		1.00	1.25	-	

Mobile phase: 0.5% 2-propanol/hexane (v/v); Flow rate = 1.0 mL/min; Detection UV 254 nm. [a]Separation factor; [b]Capacity factor for the first enantiomer eluted; [c]Resolution factor; [d]Absolute configuration of the second enantiomer eluted.

Table 2. Separation of the enantiomers of α-amino acid methyl esters as benzophenone imine derivatives on Chiralpak AD, Chiralpak AD-H, and Chiralpak AS

Analyte	Chiralpak AD				Chiralpak AD-H				Chiralpak AS			
	α^a	$k'_1{}^b$	Rs^c	Conf.[d]	α^a	$k'_1{}^b$	Rs^c	Conf.[d]	α^a	$k'_1{}^b$	Rs^c	Conf.[d]
Alanine	1.00	1.53	-		1.12	2.33	1.08	D	1.00	2.69	-	
Asparagine	1.00	6.83	-		1.00	9.59	-		1.00	4.71	-	
Aspartic acid	2.27	3.05	5.24	D	1.31	8.30	5.78	D	1.00	5.50	-	
Leucine	1.00	1.16	-		1.00	1.32	-		1.87	0.78	0.96	D
Phenylalanine	1.00	2.25	-		1.23	3.16	1.25	D	1.83	2.14	1.63	D
Phenylglycine	1.16	3.27	0.91	D	1.35	4.71	2.23	D	1.39	2.79	1.25	D
Valine	1.00	1.14	-		1.00	1.52	-		1.79	0.75	0.97	D

Mobile phase: 0.5% 2-propanol/hexane (v/v); Flow rate = 1.0 mL/min; Detection UV 254 nm. [a]Separation factor; [b]Capacity factor for the first enantiomer eluted; [c]Resolution factor; [d]Absolute configuration of the second enantiomer eluted.

Table 3. Separation of the enantiomers of α-amino acid esters as benzophenone imine derivatives on Chiralpak IA

Analyte	α[a]	k'1[b]	Rs[c]	Conf.[d]
Alanine methyl ester	1.00	1.36	-	–
Asparagine methyl ester	1.16	5.95	3.01	D
Aspartic acid dimethyl ester	1.12	4.99	2.20	D
Leucine methyl ester	1.00	1.16	-	–
Methionine methyl ester	1.00	3.83	-	–
Norleucine methyl ester	1.00	4.04	-	–
Norvaline methyl ester	1.00	1.31	-	–
Phenylalanine methyl ester	1.00	2.05	-	–
Phenylglycine methyl ester	1.23	3.32	2.16	D
Valine methyl ester	1.06	0.99	0.63	L
Alanine ethyl ester	1.00	1.24	-	–
Aspartic acid diethyl ester	1.08	4.12	1.55	D
Isoleucine ethyl ester	1.00	0.99	-	–
Leucine ethyl ester	1.00	1.00	-	–
Phenylglycine ethyl ester	1.12	2.52	1.68	D
Valine ethyl ester	1.04	1.06	0.57	L

Mobile phase: 0.5% 2-propanol/hexane (v/v); Flow rate = 1.0 mL/min; Detection UV 254 nm. [a]Separation factor; [b]Capacity factor for the first enantiomer eluted; [c]Resolution factor; [d]Absolute configuration of the second enantiomer eluted.

Table 4. Separation of the enantiomers of α-amino acid esters as benzophenone imine derivatives on Chiralpak IB

Analyte	α[a]	k'_1[b]	Rs[c]	Conf.[d]
Alanine methyl ester	1.00	1.91	-	–
Asparagine methyl ester	1.55	4.40	6.19	L
Aspartic acid dimethyl ester	1.49	4.23	5.70	L
Leucine methyl ester	1.00	0.93	-	–
Methionine methyl ester	1.05	2.38	0.30	L
Norleucine methyl ester	1.00	1.17	-	–
Norvaline methyl ester	1.00	1.20	-	–
Phenylalanine methyl ester	2.62	1.58	8.39	L
Phenylglycine methyl ester	1.17	1.92	0.69	L
Valine methyl ester	1.00	1.32	-	–
Alanine ethyl ester	1.00	1.61	-	–
Aspartic acid diethyl ester	1.35	3.90	3.38	L
Isoleucine ethyl ester	1.00	0.59	-	–
Leucine ethyl ester	1.00	0.84	-	–
Phenylglycine ethyl ester	1.16	1.69	0.55	L
Valine ethyl ester	1.00	1.28	-	–

Mobile phase: 0.5% 2-propanol/hexane (v/v); Flow rate = 1.0 mL/min; Detection UV 254 nm. [a]Separation factor; [b]Capacity factor for the first enantiomer eluted; [c]Resolution factor; [d]Absolute configuration of the second enantiomer eluted.

Table 5. Separation of the enantiomers of α-amino acid esters as benzophenone imine derivatives on Chiralpak IC

Analyte	α[a]	k'1[b]	Rs[c]	Conf.[d]
Alanine methyl ester	1.48	7.32	6.67	L
Asparagine methyl ester	1.48	7.23*	8.47	L
Aspartic acid dimethyl ester	1.47	7.57*	7.48	L
Leucine methyl ester	1.72	3.91	6.68	L
Methionine methyl ester	2.09	15.61	11.84	L
Norleucine methyl ester	1.80	4.36	7.06	L
Norvaline methyl ester	1.73	4.95	6.78	L
Phenylalanine methyl ester	1.12	8.48	2.08	L
Phenylglycine methyl ester	1.98	9.78	9.59	D
Valine methyl ester	2.12	3.33	12.94	L
Alanine ethyl ester	1.43	6.74	5.90	L
Aspartic acid diethyl ester	1.42	6.97*	7.01	L
Isoleucine ethyl ester	1.86	3.12	4.07	L
Leucine ethyl ester	1.61	3.68	5.59	L
Phenylglycine ethyl ester	1.60	9.66	8.08	D
Valine ethyl ester	1.85	3.11	10.11	L

Mobile phase: 0.5% 2-propanol/hexane (v/v); Flow rate = 1.0 mL/min; Detection UV 254 nm. [a]Separation factor; [b]Capacity factor for the first enantiomer eluted; [c]Resolution factor; [d]Absolute configuration of the second enantiomer eluted; *5% 2-propanol/hexane (V/V).

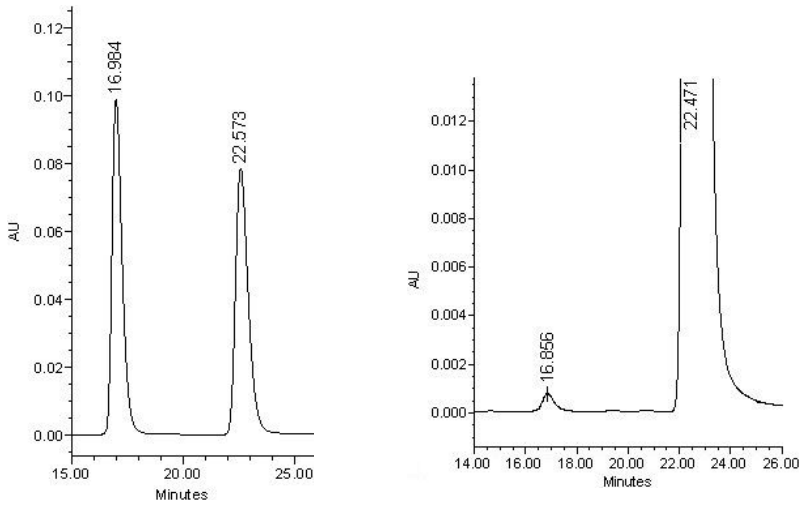
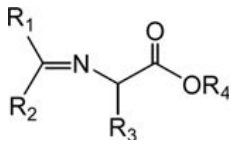


Figure 3. Chromatograms of the enantiomer resolution of the benzophenone imine derivative of racemic leucine methyl ester **(the left)** and L-leucine methyl ester (Aldrich reagent) **(the right)** (D: L= <0.1: >99.9) on Chiralpak IC. Mobile phase : 0.5% 2-propanol/hexane(V/V) ; flow rate = 1 ml/min ; detection wavelength : UV 254 nm ; injection amount 4-5μg.

Table 6 contains the chromatographic data of different aromatic imine derivatives of several α-amino acid esters on Chiralpak IC. Benzophenone imine derivatives were better enantioseparated than benzaldimine, 1-naphthaldimine, and 9-anthraldimine derivatives. Also, as the analyte is sterically hindered, the enantioselectivity increases (entries 1-3 and 8-10). The resolution of benzophenone imine derivatives of amino acid methyl esters was greater than that of the corresponding amino acid ethyl esters. In addition, the elution orders of all benzaldimine and 1-naphthaldimine derivatives were consistent with those of the corresponding benzophenone imine derivatives. However, the observed reversal of the elution orders of π-basic 9-anthraldimine derivative implies the different chiral recognition processes (entries 7 and 14).

Table 6. Separation of the enantiomers of α-amino acid esters as arylimine derivatives on Chiralpak IC



Entry	R_1	R_2	R_3	R_4	α[a]	k'_1[b]	Rs[c]	Conf.[d]
1	Ph	Ph	Me	Me	1.32	3.03	3.67	L
2	Ph	Ph	i-Bu	Me	1.40	1.55	4.18	L
3	Ph	Ph	i-Pr	Me	1.47	1.56	4.80	L
4	Ph	H	i-Pr	Me	1.37	2.61	6.12	L
5	4-MeO-Ph	H	i-Pr	Me	1.17	5.40	3.32	L
6	1-Naphthyl	H	i-Pr	Me	1.10	3.28	1.51	L
7	9-Anthryl	H	i-Pr	Me	1.23	4.07	2.89	D
8	Ph	Ph	Me	Et	1.29	3.01	1.75	L
9	Ph	Ph	i-Bu	Et	1.38	1.45	2.13	L
10	Ph	Ph	i-Pr	Et	1.43	1.52	4.20	L
11	Ph	H	i-Pr	Et	1.29	2.25	3.45	L
12	4-MeO-Ph	H	i-Pr	Et	1.15	5.34	1.81	L
13	1-Naphthyl	H	i-Pr	Et	1.05	3.05	0.44	L
14	9-Anthryl	H	i-Pr	Et	1.22	3.40	3.09	D

Mobile phase = 1% 2-propanol/hexane (v/v); Flow rate = 1.0 mL/min; Detection UV 254 nm. [a]Separation factor; [b]Capacity factor for the first enantiomer eluted; [c]Resolution factor; [d]Absolute configuration of the second enantiomer eluted.

3.2. Effects of mobile phase on the separation of α-amino acid esters as benzophenone imine derivatives

In normal phase, coated type CSPs are not compatible with all solvents and for example, the use of ethyl acetate, tetrahydrofuran, and halogenated solvents as mobile phases or solvents for analytes is prohibited [7-10]. On the other hand, the solvent versatility of covalently bonded Chiralpak IC for the separation of α-amino acid methyl esters as benzophenone imine derivatives was examined (Table 7). Enantioselectivities and resolutions were found to be greatly influenced by the nature of the mobile phase [7,8]. Of the mobile phases investigated, 0.5% 2-propanol/ hexane (v/v) best resolved the benzophenone imine derivatives of α-amino acid methyl esters, whereas the resolution provided by 30% dichloromethane/hexane (v/v) was poorest.

Table 7. Effect of mobile phase on the enantiomeric separation of the benzophenone imine derivatives of α-amino acid methyl esters on Chiralpak IC

Mobile phase	1% tetrahydrofuran/hexane				2% ethyl acetate/hexane				30% dichloromethane/hexane			
Analyte	α^a	$k'_1{}^b$	Rs^c	Conf.[d]	α^a	$k'_1{}^b$	Rs^c	Conf.[d]	α^a	$k'_1{}^b$	Rs^c	Conf.[d]
Alanine	1.40	4.60	2.73	L	1.27	4.70	4.89	L	1.25	2.17	2.68	L
Asparagine	1.00	8.52	-		1.00	5.60	-		1.00	2.39	-	
Aspartic acid	1.00	8.76	-		1.00	5.63	-		1.00	2.41	-	
Leucine	1.50	2.42	5.92	L	1.44	3.11	2.93	L	1.33	1.58	2.91	L
Phenylalanine	1.11	16.25	1.33	L	1.10	10.12	0.88	L	1.10	3.51	1.20	D
Phenylglycine	1.64	6.64	5.01	D	1.51	7.35	3.71	D	1.04	2.14	0.47	D
Valine	1.68	2.56	7.36	L	1.51	3.32	6.10	L	1.19	1.60	3.07	L

Chromatographic conditions; Flow rate = 1.0 mL/min; Detection UV 254 nm. [a]Separation factor; [b]Capacity factor for the first enantiomer eluted; [c]Resolution factor; [d]Absolute configuration of the second enantiomer eluted.

4. Conclusion

A convenient liquid chromatographic method was developed for the resolution α-amino acid esters as their benzophenone imine Schiff base derivatives using several coated and covalently bonded polysaccharide-derived CSPs. Of the CSPs studied, in general, the covalently immobilized CSP, Chiralpak IC, provided excellent resolution of the benzophenone imine derivatives of α-amino acid esters, and greater solvent choice. The developed methods were also used to measure the enantiomeric purities of several commercially available α-amino acid methyl esters. It is expected that the described liquid chromatographic method will provide a useful means of resolving α-amino acid esters.

5. References

1. Subramanian, G. (Ed.) (2001) *Chiral Separation Techniques: A practical approach* second revised ed., VCH, Weinheim.
2. Francotte, E. and W. Lindner (Ed.) (2006) *Chirality in Drug Research*, Wiley-VCH, Weinheim.
3. Huang, H., J. Y. Jin, and W. Lee (2011) Chromatographic enantiomer separation and determination of optical purity for α-amino acid esters as 9-anthraldimine derivatives using polysaccharide based chiral columns, *Kor. Soc. Biotech. Bioeng. J.* 26: 139-142.
4. O'Donnell, M. J., and Polt, R. L. (1982) A mild and efficient route to Schiff base derivatives of amino acids, *J. Org. Chem.* 47: 2663-2666.
5. Greene, T. W. and P. G. M. Wuts (1999) *Protective Groups in Organic Synthesis*, 3rd ed., John Wiley & Sons, New York.
6. Duchateau, A. L. L., J. J. Guns, R. G. R. Kubben, and A. F. P. van Tilburg (1994) High-performance liquid chromatography of diamine enantiomers as Schiff bases on a chiral stationary phase, J. *Chromatogr. A.* 664: 169-176.
7. Jin, J. Y., W. Lee, J. H. Park, and J. J. Ryoo (2006) Covalently bonded and coated chiral stationary phases derived from polysaccharide derivatives for Enantiomer Separation of N-fluorenylmethoxycarbonyl α-amino acids with fluorescence detection, *J. Liq. Chrom. & Rel. Tech.* 29: 1793-1801.
8. Jin, J. Y., S. K, Bae, and W. Lee (2009) Comparative studies between covalently immobilized and coated chiral stationary phases based on polysaccharide derivatives for enantiomer separation of N-protected α-amino acids and their ester derivatives, *Chirality*, 21: 871-877.
9. Thunberg, L., J. Hashemi, and S. Andersson (2008) Comparative

study of coated and immobilized polysaccharide-based chiral stationary phases and their applicability in the resolution of enantiomers. *J. Chromatogr. B*, 875, 72-80.

10. Zhang, T., D. Nguyen, and P. Franco (2008) Enantiomer resolution screening strategy using multiple immobilized polysaccharide-based chiral stationary phases. *J. Chromatogr. A*, 1191, 214-222.
11. Jin, J. Y., W. Lee, J. H. Park, and J. J. Ryoo (2007) Liquid chromatographic enantiomer separation of N-phthaloyl protected α-amino acids on coated and immobilized chiral stationary phases derived from polysaccharide derivatives. *J. Liq. Chromatogr. Relat. Technol.*, 30, 1-9.
12. Jin, J. Y., and W. Lee (2008) Enantiomer separation of N-protected α-amino acids on covalently immobilized cellulose tris(3,5-chlorophenylcarbamate) chiral stationary phase in HPLC, *Bull. Kor. Chem. Soc.* 29: 491-493.
13. Xu, W. J., J. Y. Jin, and W. Lee (2011) Enantiomer separation of chiral amino alcohols as 9-anthraldimine derivatives on coated and covalently bonded chiral stationary phases based on polysaccharide derivatives by high performance liquid chromatography, *Kor. Soc. Biotech. Bioeng. J.* 26: 323-327.

PART 2. Liquid Chromatographic Enantiomer Separation of α-Amino Acid Esters as NBD derivatives

Abstract

A new convenient derivatization method of α-amino acid esters as nitrobenzoxadiazole (NBD) derivatives for chiral resolution was introduced and the enantiomer separation of α-amino acid esters as NBD derivatives was performed by normal HPLC using chiral columns based on polysaccharide derivatives. The NBD derivatives were readily prepared by stirring NBD-Cl and α-amino acid methyl ester HCl with sodium bicarbonate in ethanol. The performance of Chiralpak IA was superior to the other CSPs for enantiomer resolution of NBD derivatives of several α-amino acid methyl esters. Owing to fluorescence detection as well as strong UV absorption, 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 chiral columns.

Keywords: Enantiomer separation, Amino acid ester, NBD derivative, Chiral column

1. Introduction

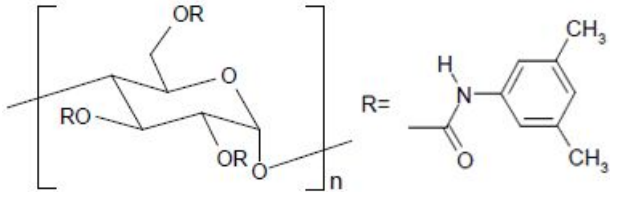
Recently, in the laboratory, it was reported the enantiomer separation results of α-amino acid known as an important material for developing a chiral drugs and these derivatives. For the efficient enantiomer separation, enantiomer analysis of commonly used α-amino acid and these derivatives which amino group is protected in pharmaceutical development was performed [1-3]. In particular, in the case of α-amino acid ester, a new analytical method of optical resolution with chiral stationary phases was announced, after derivatized 9-anthraldimine and benzophenone imine Schiff base [4-6]. In the last studies, the role of the 9-anthraldimine and benzophenone imine groups that was used for optical resolution of α-amino acid esters was considered from two aspects [4,6]. First, 9-anthraldimine and benzophenone imine derivatives had very strong ultraviolet absorption than α-amino acid ester compounds. And second, the aromatic group of 9-anthraldimine and benzophenone imine derivatives as the chiral recognition interaction site was expected. So these were used to easy detective analytes and good optical resolution results [4-6]. In this study, related to studies of the enantiomer separation, we focused nitrobenzoxadiazole (NBD) group which can play the previous two roles as new derivative material of α-amino acid esters. NBD group of planar structure was expected to be able to function the chiral recognition interaction site as the structural property of the hydrophobic aromatic character, and when it is applied to the enantiomer resolution of this study, there are many advantages of a very useful.

On the other hand, in the previous studies, separation and analysis results of the amino acid and amine as NBD derivatives using the NBD-F and NBD-Cl have been reported [7-13]. In particular, Sumochiral OA 2500S, Chiralpak QN-AX, and teicoplanin chiral columns were used in optical resolution of amino acid as NBD derivatives, but the results were

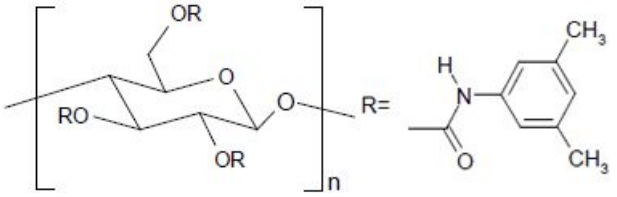
unsatisfactory [9-13]. The amino acid as NBD derivatives were prepared by stirring with NBD-F and sodium borate buffer, and reaction mixture were quenching and analyzed and the derivatization method of amine as NBD derivatives with methyl alcohol and sodium bicarbonate was reported [7,11,12]. Because all of existing analysis methods were performed at reverse-phase chromatography as enantiomer separation condition, the derivatization conditions were also progressed with aqueous or buffer solution. However, for using normal-phase chromatography as optical separation and analysis conditions of the present study, derivatization method that adapted for normal phase and more convenient at non-aqueous condition was required. Up to now, it has not been derivatized to analyze NBD derivatives of amino acid ester and the studies of enantiomer separation using normal-phase chromatography have not been reported. Therefore, in this study, the new derivatization method that adapted for normal-phase chromatography analysis and different from the conventional methods was developed. And using this developed derivatization method, enantiomer separation was performed on the several chiral stationary phase base on the polysaccharide derivatives [4,5].

2. Materials and Methods

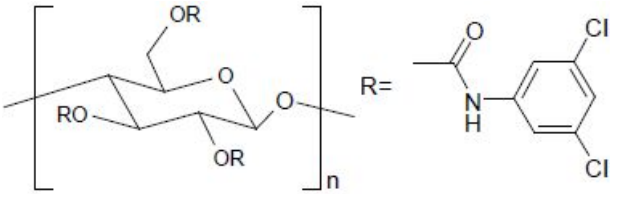
High-performance liquid chromatography was performed at room temperature using 1100 series HPLC equipment of Agilent. All polysaccharide-derived CSPs, that is, Chiralpak IA, Chiralpak IB, and Chiralpak IC (250 mm L × 4.6 mm I.D., 5 μm) were purchased from the Daicel Chemical Company (Fig. 4). HPLC-grade hexane, 2-propanol and other solvents were obtained from J. T. Baker. All α-amino acid methyl esters, NBD-chloride(4-chloro-7-nitro-2,1,3-benzoxadiazole), and several bases such as sodium bicarbonate were obtained from Aldrich (Milwaukee, WI), Sigma (St. Louis, MO), Advanced ChemTech (Louisville, KY). Chromatography was performed using a flow rate 1 mL/min, 20-30% 2-propanol/hexane (v/v) as mobile phase, a detection wavelength of UV 337 nm, detection wavelength of fluorescence excitation 470nm and emission 530nm as UV detector and fluorescence were connected on-line. Used racemic and D- or L-analytes were prepared, as shown in Fig. 5, 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 of ethanol at room temperature for 12 h. Reaction mixtures were filtered to remove sodium bicarbonate and diluted as 1/10 by adding 2-propanol. Resulting solutions were directly injected into the chromatograph.



Chiralapk IA



Chiralapk IB



Chiralpak IC

Figure 4. The structure of Chiral selector of Chiralpak IA, Chiralpak IB and Chiralpak IC

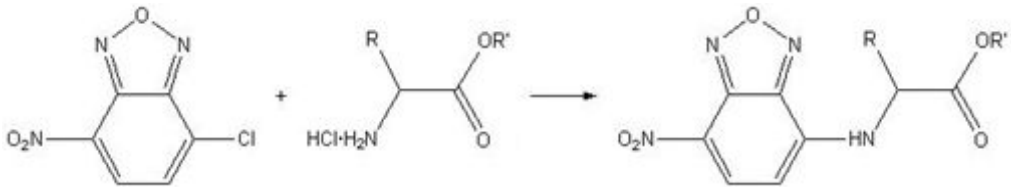


Figure. 5. Preparation of α-amino acid esters as NBD derivatives. NBD derivatives were prepared by stirring NBD-Cl and α-amino acid methyl ester HCl with sodium bicarbonate in ethanol.

3. Results and Discussion

3.1. The effects of solvents and bases on the separation of α-amino acid esters as NBD derivatives

First in this study, followed experiments were progressed for optimizing NBD derivatization method of α-amino acid esters. So derivatization method used NBD-Cl for amine analysis was changed and applied [7]. NBD derivative of racemic leucine methyl ester was synthesized by stirring racemic leucine methyl ester HCl with sodium bicarbonate as base in reaction solvent. Fig. 6 was shown the results of derivatization over the reaction time in various solvents. As shown in the Fig. 6, the reaction was fastest in methyl alcohol used Hao group [7], the next were acetonitrile (ACN) and ethyl alcohol, and isopropyl alcohol (IPA) was slowly reacted. But the case of using methyl alcohol and acetonitrile, instead derivatization reaction was fast, impurity materials such as methoxy NBD were also produced. Even though the reaction was slow compared with methyl alcohol when using ethyl alcohol, impurity materials were not generated relatively. So ethyl alcohol to use as derivatization solvent was adopted.

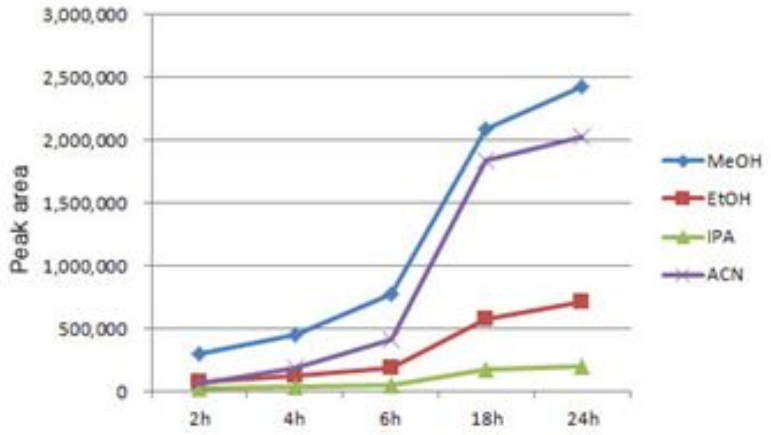


Figure. 6. Solvent effect on synthesis of racemic leucine methyl ester as NBD derivative in sodium bicarbonate. Column: Chiralpak IA; Mobile phase: 20% 2-propanol/hexane (V/V); Flow rate: 1 mL/min; Detection UV 337 nm.

And the next, several kinds of base were used for optimizing NBD derivatization method of α-amino acid esters. So not just sodium bicarbonate that was used to derivatization by Hao group, many kinds of base, such as sodium carbonate, triethylamine, DBU, and etc., were used [6,7]. However, when using triethylamine and DBU than the others of base, were excluded from the results because accurate measurement is difficult for the unexpected various kinds of impurity in initial reaction. As shown in Fig. 7, the reaction was fastest when using sodium carbonate, and sodium bicarbonate was slowly reacted. But using sodium carbonate and sodium borate was increased not only NBD derivative but unexpected impurity materials as increase reaction time. It was expected that using base strong basicity is generated more impurity in derivatization. On the other hand, sodium bicarbonate, one of the mild base, though slowly reaction, was not generated impurity relatively. So the sodium bicarbonate as base was chosen.

Finally, for synthesizing α-amino acid esters as NBD derivatives which analytes of this study, ethyl alcohol and sodium carbonate were used.

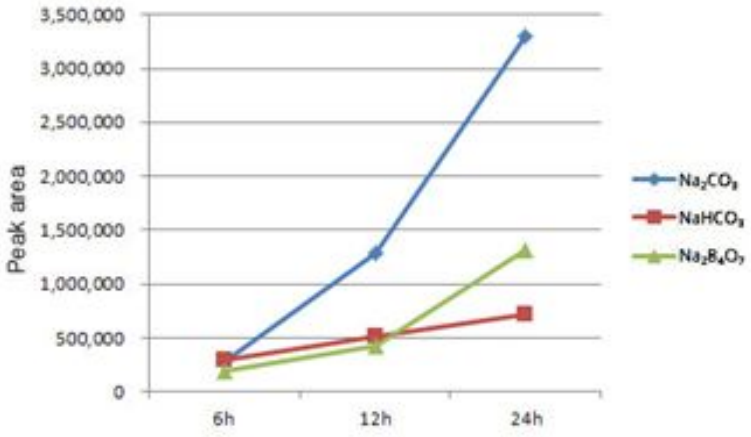


Figure. 7. Base effect on synthesis of racemic leucine methyl ester as NBD derivative in ethanol. Column: Chiralpak IA; Mobile phase: 20% 2-propanol/hexane (V/V); Flow rate: 1 mL/min; Detection UV 337 nm.

3.2. The results of enantiomer separation of α-amino acid methyl esters as NBD derivatives

The α-amino acid methyl esters as NBD derivatives were synthesized by developed derivatization method in this study, and the separation of NBD derivatives was performed using chiral column base on polysaccharides. Tables 8-10 summarize chromatographic data for the enantiomer separation of several α-amino acid esters as NBD derivatives on covalently bonded polysaccharide-derived CSPs (Chiralpak IA, Chiralpak IB, and Chiralpak IC) using 20-30% 2-propanol/hexane (v/v) as mobile phase. The enantiomer separation was attempted to analysis detection with UV 337 nm and fluorescence detection excitation 470nm and emission 530nm in same time. As shown in Table 8, the performance of Chiralpak IA was superior to the other CSPs for enantiomer resolution of several α-amino acid methyl esters as NBD derivatives (α=1.34~2.28, Rs=4.62~12.61). These optical separation results was superior to analysis results of Hamase group using Sumochiral 2500S and Chiralpak QN-AX column and the results of Liu group using teicoplanin column [11-13]. On the other hand, when compared with the results of using a Chiralpak IA chiral column in Table8, using a Chiralpak IB and Chiralpak IV from Table 9, 10, showed the optical resolution results generally low. In particular, in Table 10 using Chiralpak IC chiral column, the two samples did not separated at all, but the optical analysis of the other samples showed a generally good resolution. When performing the enantiomer resolution of α-amino acid ester as NBD derivatives in Chiralpak IA and Chiralpak IB of Table8, 9, it was used 20% 2-propanol/hexane(V/V), but in the Chiralpak IC chiral column of Table10, it was used 30% 2-propanol/hexane(V/V) for efficient analysis.

In addition, the experiments about the order of elution of α-amino acid ester as NBD derivative were conducted. In Table 8 of the Chiralpak IA, L-isomer of all analytes is second eluted. The results of these experiments

were meant that chiral recognition mechanism between chiral stationary phase of Chiralpak IA and NBD derivative of α-amino acid esters is not affected by the chemical structure and consistently acted. The study results of using Sumochiral 2500S, a Chiralpak QN-AX column as in the this study, L-isomer of the amino acid ester as NBD derivatives were eluted second, but the study using the teicoplanin column, D-isomer were secondly eluted [11-13]. In Chiralpak IB of Table 9, the L-isomer of analytes were generally second eluted likely Chiralpak IA of Table 8, but exceptionally, phenylalanine derivative showed the reversed elution order. And the Chiralpak IC of Table 10, the elution order was irregular depending on the analytes.

Table 8. Separation of the enantiomers of α-amino acid methyl esters as NBD derivatives on Chiralpak IA

Analyte	α	k'1	Rs	Conf.*
Alanine	1.37	2.99	5.64	L
Leucine	2.00	1.71	11.01	L
Methionine	1.66	3.39	9.92	L
Norleucine	2.28	1.70	12.61	L
Norvaline	1.72	1.82	7.98	L
Phenylalanine	1.94	3.10	11.77	L
Phenylglycine	1.37	6.10	4.32	L
Serine	1.34	6.38	4.62	L
Valine	1.45	2.08	7.41	L

Mobile phase: 20% 2-propanol/hexane (V/V); Flow rate: 1 mL/min; *indicates the absolute configuration of the second eluted enantiomer. See experimental for detection conditions.

Table 9. Separation of the enantiomers of α-amino acid methyl esters as NBD derivatives on Chiralpak IB

Analyte	α	k'1	Rs	Conf.*
Alanine	1.04	5.92	0.54	L
Leucine	1.08	5.07	1.17	L
Methionine	1.02	2.76	0.29	L
Norleucine	1.04	8.82	0.49	L
Norvaline	1.08	6.32**	1.24	L
Phenylalanine	1.12	6.42	1.81	D
Phenylglycine	1.05	10.68	0.44	L
Serine	1.06	11.12	1.22	L
Valine	1.11	7.68**	1.67	L

Mobile phase: 20% 2-propanol/hexane (V/V); Flow rate: 1 mL/min; *indicates the absolute configuration of the second eluted enantiomer.; **10% 2-propanol/hexane (V/V). See experimental for detection conditions.

Table 10. Separation of the enantiomers of α-amino acid methyl esters as NBD derivatives on Chiralpak IC

Analyte	α	k'1	Rs	Conf.*
Alanine	1.10	9.75	1.92	L
Leucine	1.00	6.75	-	-
Methionine	1.16	13.12	3.92	D
Norleucine	1.10	8.56	1.61	L
Norvaline	1.09	9.75	1.73	L
Phenylalanine	1.00	12.19	-	-
Phenylglycine	1.10	12.52	2.51	D
Serine	1.04	5.98	0.45	D
Valine	1.14	9.91	2.38	L

Mobile phase: 30% 2-propanol/hexane (V/V); Flow rate: 1 mL/min; *indicates the absolute configuration of the second eluted enantiomer. See experimental for detection conditions.

Figure 8 is typical chromatograms of racemic leucine methyl ester and L-leucine methyl ester as NBD derivative performed enantiomer separation on Chiralpak IA at simultaneous on-line UV and fluorescence detection. Detection wavelength is UV 337 nm, fluorescence excitation 470nm and emission 530nm. As shown in Fig. 8, it is easy to find the advantage of more sensitivity in fluorescence detection than UV 337nm. The NBD derivative of leucine methyl ester, un-reacted NBD-Cl, and some impurity peaks were observed under UV 337nm. But under fluorescence detection, un-reacted NBD-Cl is not detected at all, almost no impurity, and leucine methyl ester NBD derivative peak is only detected selectively. So it is expected that we can efficiently utilize the advantages of fluorescence detection by applying the various enantiomer separation analysis of α-amino acid ester as NBD derivatives.

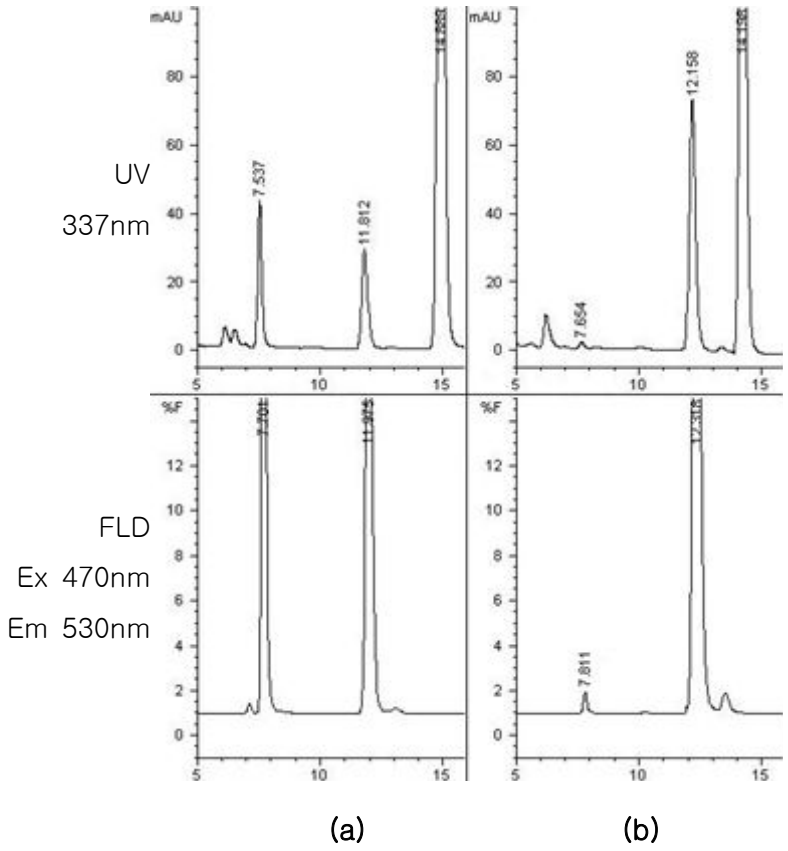


Figure. 8. Chromatograms of the resolution of NBD derivative on Chiralpak IA at simultaneous on-line UV and fluorescence detection. **(a)** racemic leucine methyl ester, **(b)** L-leucine methyl ester (D: L= 1.5: 98.5). The unreacted NBD-Cl peak is shown about 15min on the top chromatograms observed under UV 337nm. See experimental for chromatographic conditions.

4. Conclusion

The enantiomer separation of α-amino acid esters as NBD derivatives was performed using a new convenient derivatization method and chiral columns based on polysaccharide derivatives. To react derivatization analytes for normal HPLC, the NBD derivatives were readily prepared by stirring NBD-Cl and α-amino acid methyl ester HCl with sodium bicarbonate in ethanol. The performance of Chiralpak IA was superior to the other CSPs for enantiomer resolution of NBD derivatives of several α-amino acid methyl esters. In addition, not only UV detection but fluorescence detection were showed that these equipments have strong selectivity and sensitivity in analysis of α -amino acid esters as NBD derivatives. 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 chiral columns.

5. References

1. Jin, J. Y., S. K, Bae and W. Lee (2009) Comparative studies between covalently immobilized and coated chiral stationary phases based on polysaccharide derivatives for enantiomer separation of N-protected α-amino acids and their ester derivatives, *Chirality*, 21: 871-877.
2. Jin, J. Y., W. Lee, J. H. Park, and J. J. Ryoo (2007) Liquid chromatographic enantiomer separation of N-phthaloyl protected α-amino acids on coated and immobilized chiral stationary phases derived from polysaccharide derivatives. *J. Liq. Chrom. & Rel. Tech.* 30: 1-9.
3. Li, Y. H., and W. Lee (2005) Liquid chromatographic enantiomer resolution of N-fluorenylmethoxycarbonyl α-amino acids and their ester derivatives on polysaccharide-derived chiral stationary phases. *J. Sep. Sci.* 28: 2057-2060.
4. Huang, H., W. J. Xu, J. Y. Jin, J. H. Hong, H.-J. Shin and W. Lee (2012) A convenient method for the enantiomeric separation of α-amino acid esters as benzophenone imine Schiff base derivatives. *Arch. Pharm. Res.* 35: 1015-1019.
5. Huang, H., J. Y. Jin, and W. Lee (2012) Liquid chromatographic resolution of α-amino acid esters as benzophenone imine derivatives. *Kor. Soc. Biotech. Bioeng. J.* 27: 167-171.
6. Huang, H., J. Y. Jin, and W. Lee (2011) Chromatographic enantiomer separation and determination of optical purity for α-amino acid esters as 9-anthraldimine derivatives using polysaccharide based chiral columns. *Kor. Soc. Biotech. Bioeng. J.* 26: 139-142.
7. Hao, F., T. Lwin, W. J. Bruckard, and J. T. Woodcock (2004) Determination of aliphatic amines in mineral flotation liquors and reagents by high-performance liquid chromatography after derivatization

with 4-chloro-7-nitrobenzofurazan, *J. Chromatogr. A.* 1055: 77-85.

8. Breda, M., P. Marrari, E. Pianezzola, and M. S. Benedetti (1996) Determination of ethambutol in human plasma and urine by high-performance liquid chromatography with fluorescence detection, *J. Chromatogr. A.* 729: 301-307.

9. Hamase, K., H. Homma, Y. Takigawa, T. Fukushima, T. santa, and K. Imai (1997) Regional distribution and postnatal changes of D-amino acids in rat brain, *Biochim. Biophys. Acta.* 1334: 214-222.

10. Inoue, T., K. Hamase, A. Morikawa, and K. Zaitsu (2000) Determination of minute amounts of D-leucine in various brain regions of rat and mouse using column-switching high-performance liquid chromatography, *J. Chromatogr. B.* 744: 213-219.

11. Hamase, K., A. Morikawa, T. Ohgusu, W. Lindner, and K. Zaitsu (2007) Comprehensive analysis of branched aliphatic D-amino acids in mammals using an integrated multi-loop two-dimensional column-switching high-performance liquid chromatographic system combining reversed-phase and enantioselective columns, *J. Chromatogr. A.* 1143: 105-111.

12. Hamase, K., Y. Miyoshi, K. Ueno, H. Han, J. Hirano, A. Morikawa, M. Mita, T. Kaneko, W. Lindner, and K. Zaitsu (2010) Simultaneius determination of hydrophilic amino acid enantiomers in mammalian tissues and physiological fluids applying a fully automated micro-two-dimensional high-performance liquid chromatographic concept, *J. Chromatogr. A.* 1217: 1056-1062.

13. Song, Y., M. Shenwu, S. Ahao, D. Hou and Y.-M. Liu (2005) Enantiomeric separation of amino acids derivatized with 7-fluoro-4-nitrobenzoxadiazole by capillary liquid chromatography/tandem mass spectrometry. *J. Chromatogr. A.* 1091: 102-109.

ABSTRACT

The enantiomer separation using chiral columns based on polysaccharides by HPLC

Wonnam Yun
Advisor: Prof. Wonjae lee, Ph. D.
College of Pharmacy
Graduate School of Chosun University

In part 1, using several coated and covalently bonded chiral stationary phases (CSPs) from polysaccharide derivatives as chiral selector, the separation of α-amino acid esters as benzophenone imine derivatives is performed by HPLC. The benzophenone imine derivatives were prepared by stirring α-amino acid ester hydrochloride salts, benzophenone imine Schiff base, and magnesium sulfate anhydrous in 2-propanol. In general, the resolution of Chiralpak IC was superior to those of the other CSPs.

In part 2, the enantiomer separation of α-amino acid methyl esters as nitrobenzoxadiazole (NBD) derivatives was performed by normal HPLC using covalently immobilized chiral columns based on polysaccharide derivatives. The NBD derivatives were prepared by stirring α-amino acid methyl ester HCl and NBD-Cl with sodium bicarbonate in ethanol. The results of enantiomer separation of NBD derivatives, The performance of Chiralpak IA was superior to the other CSPs. In addition, analysis of NBD derivative of α-amino acid methyl ester was confirmed that the fluorescence detection is more selective and sensitive as compared with UV detection.

Keywords: Chiral stationary phase, Enantiomer separation, Chiral column, α-Amino acid ester, Benzophenone imine derivative, NBD derivative.

THANKS TO

석사학위를 큰 무리 없이 마칠 수 있게 하늘에서 돌봐주신 하느님께 감사드립니다. 항상 뒤에서 묵묵히 지원해 주신 아버지와 어머님, 감사드리고 사랑합니다. 하늘나라에 계시지만 그곳에서도 보살펴 주셨을 엄마, 새 어머니 감사드립니다. 가까이에 살진 않지만 멀리서나마 응원해주시고 만나게 되면 항상 덕담을 해 주시는 큰 고모님 내외, 작은 고모님, 막내 고모님 내외, 숙부님 내외, 막내 숙부님 내외, 형들, 형수님들, 그 외 친척들 감사하고 항상 건강하세요. 석사 학위를 하면서 모자란 저를 이끌어 주시고 챙겨주시고 새로운 길을 제시해 주셨던 이원재 교수님, 존경합니다. 그리고 여러 가지 조언과 관심을 가져 주셨던 백만정 교수님, 홍준희 교수님, 최홍석 교수님, 김은애 교수님, 기성환 교수님, 황호님, 김광준님, 김지연님 감사드리고 항상 하시는 일이 잘 되시기를 기원하겠습니다. 같은 석사과정 중이면서도 힘든 일이 있을 때마다 버틸 수 있도록 격려해 주신 소울메이트 준호형, 항상 감사드리고 형도 학위과정 하시느라 수고 많으셨어요. 자주 연락을 못 드렸지만 제 맘 아실 거라 생각합니다. 항상 건강하시고 좋은 일만 가득하시길 바라고 죽는 그날까지 끝까지 함께해요. 그리고 신경 쓰이는 일이 있거나 스트레스 받을 때면 날려버릴 수 있도록 같이 해준 진정한 내 친구들 철희, 재윤이, 기남이, 화, 대한이 고맙다. 너희들이 있어서 다른 생각 안하고 끝까지 올 수 있었던 것 같다. 특히 철희랑 재윤이, 너희들도 학위중이라 힘들 텐데 신경써줘서 고맙다. 학위 수여받고 마칠 때까지 너희들도 힘내고 항상 응원하고 있을게. 더불어 조대 생공과 07학번 우리 동기들도 항상 잘 되길!!! 밤샘 실험과 작업을 할 때면 향긋한 커피와 다양한 음료로 피로를 잊고 일에 집중할 수 있게 해준 스탠다드 커피, 특히 철오랑 영실이 누나, 땡큐. 음……. 누나가 내려주는 아메리카노가 제일 맛있었어요. 마지막으로 지금은 내 옆에 있진 않지만 지난 시간동안 만나고 싶을 때 못 만났더라도 기다리고 버텨줬던 한 사람, 네가 있어서 학위를 시작하게 되었고, 학위 중 극단적인 생각이 들 때면 너를 생각하면서 너를 위해 버티고 버텨서 이렇게 학위를 무사히 마친다. 많은 용기와 희망을 주고 격려해 줘서 고맙고 항상 행복하길 바란다. 앞서 언급한 사람들 외에 감사드릴 분들이 참 많습니다. 다 언급해 드리지 못해 죄송하고 모든 분들에게 감사인사 드립니다. 감사합니다.