February 2022
Master's Degree Thesis

Enantiomer separation of α-amino acids and α-amino acid ester derivatives using polysaccharide-derived chiral stationary phases by high-performance liquid chromatography

Graduate School of Chosun University

Department of Pharmacy

Alisha Bhandari

Enantiomer separation of α-amino acids and α-amino acid ester derivatives using polysaccharide-derived chiral stationary phases by high-performance liquid chromatography

고성능 액체 크로마토그래피에서 다당 유도체의
키랄고정상을 이용한 아미노산과 에스터 유도체의 광학분리

2022 년 02 월 25 일

Graduate School of Chosun University

Department of Pharmacy

Alisha Bhandari

Enantiomer separation of α-amino acids and α-amino acid ester derivatives using polysaccharide-derived chiral stationary phases by high-performance liquid chromatography

지도교수 이 원 재

이 논문을 약학 석사학위신청 논문으로 제출함

2021 년 10 월

Graduate School of Chosun University

Department of Pharmacy

Alisha Bhandari

Alisha Bhandari's dissertation is certified

Chairperson Chosun Univ.	Prof. Hong, Joon Hee	(인)
Member Chosun Univ.	Prof. Jee, Jun Phil	(인)
Member Chosun Univ.	Prof. Lee, Wonjae	(인)

December 2021

Graduate School of Chosun University

Table of Contents

List of Tables

List of Figures

ABSTRACT (ENGLISH)

Enantiomer separation of α-amino acids and α-amino acid ester derivatives using polysaccharide-derived chiral stationary phases by high-performance liquid chromatography

Alisha Bhandari

Advisor: Prof. Wonjae Lee, Ph.D.

College of Pharmacy

Graduate School of Chosun University

In Chapter 1, the liquid chromatographic enantiomeric resolution of several α-amino acids as 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was performed using ten chiral stationary phases (CSPs) derived from amylose and cellulose tris phenylcarbamates by normal phase HPLC under simultaneous ultraviolet (UV) and fluorescence detection (FLD). Most of the analytes under consideration were baseline-separated with good separation and resolution factors. In general, the enantioselectivity observed on coated type columns were better than those on covalently

bonded type columns. Out of six covalently bonded CSPs, Chiralpak IA showed the highest enantiomer separation and resolution for most of the analytes. Chiralpak AD-H and Lux Amylose-1, both amylose-based coated type CSPs, demonstrated better enantiomer separation of NBD-F derivatized α-amino acids than cellulose-based Chiralcel OD-H and Lux Amylose-1.This developed analytical method is expected to be quite selective for the separation of other α-amino acids as NBD derivatives by normal high performance liquid chromatography (HPLC).

In Chapter 2, the normal phase liquid chromatographic enantiomer separation of α-amino acid esters and chiral amines as 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl) was performed on amylose and cellulose-based CSPs. The degree of enantioselectivity was affected by the type of analyte (α-amino acid esters or amines), backbone structure (cellulose or amylose) and the substituents of the phenyl moiety on the chiral selector of CSP. Amongst the examined CSPs, in particular, for the α-amino acid ethyl ester analytes, the covalently bonded Chiralpak IA with an amylose based chiral selector showed the best enantiomer separation and resolution, while among the coated type CSPs, Chiralpak AD-H (or Lux Amylose-1) showed the highest performance. Similarly, the degree of enantioselectivity for α-amino acid methyl esters was greater for Chiralpak AD-H (or Lux

Amylose-1). Further, for the enantiomeric resolution of chiral amines, Chiralpak IE and Chiralcel OD-H (or Lux Cellulose-1) columns showed the highest enantioselectivity. In general, the degree of enantioselectivity was greater on coated type α-amino acid esters as compared to that of chiral amines. This analytical method is expected to be quite useful for the enantiomeric resolution of other α-amino acid esters and chiral amines as NBD derivatives by normal high performance liquid chromatography (HPLC).

Index Terms: Amino acid, Amino acid ester, Chiral amine, Chiral high performance liquid chromatography, Enantiomer separation, Polysaccharide-derived chiral stationary phase

국문 초록

고성능 액체 크로마토그래피에서 다당 유도체의 키랄고정상을 이용한 아미노산과 에스터 유도체의 광학분리

알리샤 반다리

지도교수: 이 원 재

약학과

조선대학교 대학원

고성능 액체 크로마토그래피에서 키랄 선택자로 다당 유도체를 기초로 하는 키랄 컬럼을 사용하여 여러 종류의 α-amino acid 와 ester 그리고 키랄 아민의 NBD 유도체의 광학분리를 수행하였다.

Part 1에서는

NBD-F 유도체 시약을 이용하여 NBD α-amino acid 유도체를 만든 분석물질을 다당 유도체를 기초로 하는 키랄 컬럼 (6개의 키랄

선택자가 공유결합된 컬럼과 4개의 코팅된 컬럼)에서 자외선과 형광 검출의 동시분석을 진행하였다. 6개의 키랄 선택자가 공유결합된 컬럼에서는 Chiralpak IA가 가장 좋은 광학분할을 보여주었다. 4개의 키랄 선택자가 코팅된 컬럼에서는 아밀로오스 유도된 Chiralpak AD-H 과 Lux Amylose-1 가 셀룰로오스 유도된 Chiralcel OD-H and Lux Amylose-1 보다 더 나은 분석 결과를 보여주었다. 좋은 광학분할 결과와 함께, NBD 유도체의 성질로 형광분석 검출이 가능할 수 있기에 고성능 액체 크로마토그래피에서 키랄 선택자로 다당 유도체를 기초로 하는 키랄 컬럼에서 NBD α-amino acid 유도체의 광학분할은 매우 유용한 것으로 보인다.

Part 2에서는

NBD-Cl 유도체 시약을 이용하여 여러 NBD α-amino acid esters와 키랄 아민 유도체로 만든 분석물질을 다당 유도체를 기초로 하는 키랄 컬럼 (6개의 키랄 선택자가 공유결합된 컬럼과 4개의 코팅된 컬럼)에서 자외선과 형광 검출의 동시분석을 진행하였다. 광학분할 결과는 α-amino acid esters와 키랄 아민의 분석물질 종류에 따라, 키랄 선택자가 아밀로오스 또는 셀루로오스 종류에 따라, 키랄 선택자가 코팅 또는 공유결합 된 것에 따라 다르게 나왔다. NBD α-amino acid esters 의 광학분할은 NBD α-amino acid와 비슷하다. 그래서 6개의 키랄 선택자가

공유결합된 컬럼에서는 Chiralpak IA가 가장 좋은 광학분할을 보여주었다. 4개의 키랄 선택자가 코팅된 컬럼에서는 아밀로오스 유도된 Chiralpak AD-H 과 Lux Amylose-1 가 셀룰로오스 유도된 Chiralcel OD-H and Lux Amylose-1 보다 더 나은 분석 결과를 보여주었다. 그러나 NBD 키랄 아민의 경우, 공유 결합된 컬럼에서는 Chiralpak IE 그리고 코팅컬럼에서는 Chiralcel OD-H (또는 Lux Cellulose-1) 에서 가장 좋은 광학분할 결과를 보인다.

Index Terms: Amino acid, Amino acid ester, Chiral amine, Chiral high performance liquid chromatography, Enantiomer separation, Polysaccharide-derived chiral stationary phase

Abbreviation

CS	Chiral selector
CSP	Chiral Stationary Phase
UV	Ultraviolet
FL	Fluorescence
HPLC	High Performance Liquid Chromatography
NBD	Nitrobenzoxadiazole
NBD-Cl	4-chloro-7-nitro-2,1,3-benzoxadiazole
NBD-F	4-fluoro-7-nitro-2,1,3-benzoxadiazole

Chapter 1

4-Fluoro-7-nitro-2,1,3-benzoxadiazole as a Derivatizing Agent for Enantiomeric Separation of Amino Acids Using Polysaccharide-Derived Chiral Stationary Phases by Normal HPLC

Abstract

The liquid chromatographic enantiomer separation of several α-amino acids as 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was performed on six covalently bonded and four coated type amylose or cellulose derived chiral stationary phases (CSPs) under simultaneous ultraviolet (UV) and fluorescence (FL) detection. In general, among the six covalently bonded CSPs, the performance of Chiralpak IA was superior for seven α-amino acid analytes while Chiralpak IB showing the worst separation. On the other hand, Chiralpak AD-H and Lux Amylose-1 showed the greatest enantioselectivity while Chiralcel OD-H and Lux Cellulose-1 the least among the coated type CSPs. All the analytes were enantiomerically resolved at amylose-derived CSPs having chiral selector as amylose tris 3,5-dimethylphenylcarbamate with identical secondly eluted (S)-isomers. This analytical method is expected to be useful for the enantiomeric separation and resolution of other α-amino acids as NBD derivatives by normal high performance liquid chromatography (HPLC).

Keywords: α-Amino acids, Chiral stationary phase, Enantiomer separation, Nitrobenzoxadiazole derivative, HPLC.

1.1 Introduction

Before the 1980s, the majority of drugs were either achirals or racemates; an equimolar mixture of the two enantiomers [1]. The advancement with the new separation technologies has made it possible to separate and develop stereochemically pure chiral drugs [2]. Chiral drugs have been of great importance in the development of pharmacotherapy, in the fields of biochemistry and the pharmaceutical industry,from the earliest to the modern age [3]. The administered enantiomerically pure drugs should be able to fit into the target or receptor site for the optimal biological effects. Thus, the safety and quality of chiral drugs is a must and it requires a convenient and analytical method [4]. Enantiomer separation of the α-amino acids has been an essential area in the field of pharmaceutical research and biochemistry [5]. In the pharmaceutical field, several methods have been developed to determine the enantiomeric purities and/or configuration of α-amino acids [6]. Among those various methods, the liquid chromatographic enantiomer separation on chiral stationary phases (CSPs) especially derived from polysaccharide (cellulose and amylose) have been extensively used to separate a wide range of enantiomeric compounds [7]. The 4-chloro-7-nitro-2,1,3-benzoxadiazole

(NBD-Cl) has previously been used as a derivatizing reagent for enantiomer separation of amino acids and analysis by the Imai and Zaitsu research groups [8]. In this study, the potent derivatizing agent, 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) has been used to enhance selectivity and sensitivity for the ease in enantiomeric resolution of seven α-amino acid analytes using the normal phase HPLC. Here, we present the comparative liquid chromatographic enantiomer separation of α-amino acids on six covalently bonded CSPs, Chiralpak IA, Chiralpak IB, Chiralpak IC, Chiralpak ID, Chiralpak IE and Chiralpak IF and four coated type polysaccharide derived CSPs, Chiralcel OD-H, Lux Cellulose-1, Chiralpak AD-H and Lux Amylose-1 under simultaneous ultraviolet (UV) and fluorescence (FL) detection.

1.2 Experimental Section

1.2.1 Instrumentation and Experimental Conditions

An Agilent 1100 HPLC system with micro-vaccum degasser, G1310A isocratic pump, automatic sample injector, a thermostatic column compartment and a HP1046A programmed fluorescence detector was used for the chromatographic analysis. The six covalently bonded Chiralpak IA [amylose tris(3,5-dimethylphenylcarbamate)], Chiralpak IB [cellulose tris(3,5-dimethylphenylcarbamate)], Chiralpak IC [cellulose tris(3,5-dichlorophenlycarbamate)], Chiralpak ID [amylose tris(3-chlorophenylcarbamate)], Chiralpak IE [amylose tris(3,5-dichlorophenylcarbamate)], and Chiralpak IF [amylose tris(3-chloro-4-methylphenylcarbamate)] (250 mm × 4.6 mm, I.D., 5 μm) columns were obtained from Daicel company (Tokyo, Japan). The four coated type Chiralcel OD-H [cellulose tris(3,5-dimethylphenylcarbamate] and Chiralpak AD-H [amylose tris(3,5-dimethylphenylcarbamate)] (250 mm × 4.6 mm, I.D., 5 μm) columns were purchased from Daicel company (Tokyo, Japan); Lux Cellulose-1 [cellulose tris(3,5-dimethylphenylcarbamate)] and Lux Amylose-1 [amylose tris(3,5-dimethylphenylcarbamate)] (250 mm × 4.6 mm, I.D., 5 μm) columns were

procured from Phenomenex (Torrance, CA, USA). Separation of enantiomers using liquid chromatography was performed at room temperature with a flow rate of 1 mL/min. The isocratic mobile phase used for the separation procedure was 10-20 % 2-propanol/hexane (V/V) with 0.1% TFA. A simultaneous detection of UV 337 nm and FL (excitation 470 nm and emission 530 nm) were employed for the enantiomeric separation.

1.2.2 Reagents and Sample Preparation

The derivatizing agent along with the α-amino acid analytes were obtained from Tokyo Chemical Industry Co. Ltd. (Chuo-ku, Tokyo, Japan) or Junsei Chemical Co., Ltd. (Chuo-ku, Tokyo, Japan) or Katayama Chemical Industries Co., Ltd. (Chuo ward, Osaka, Japan) or Aldrich Chemical Co Inc. (Milwaukee, Wisconsin, USA) or Acros organics (Fair lawn, NJ, USA) or Sigma-Aldrich (St. Louis, MO, USA) or Janssen Chimica (Turnhoutseweg 30, Beerse, Belgium). HPLC grades of 2-propanol, ethanol and hexane were purchased from Avantor Performance Materials Korea Limited (Gangnam-Gu, Seoul, Korea) or Fischer Scientific Korea Ltd. (Kangnam-gu, Seoul, Korea) or Avantor Performance Materials Korea Limited (Suwon-si, Gyeonggi-do, Republic of Korea). The amino acids sample solution was prepared by dissolving amino acids with 50mM borate buffer (pH 8.0) with 20mM EDTA and then 300 µl of the sample solution and 100 µl of 100 mM NBD-F were mixed in a reaction vial. The vial was heated at 60°C for 1 min and then cooled it on an ice bath and then 40 µl of 50 mM HCL aqueous solution was added to the reaction mixture and this mixture was used for HPLC analysis. 0.1% TFA was used as a mobile phase additive to maintain a good peak shape and efficiency by minimizing peak tailing and/or broadening [9].

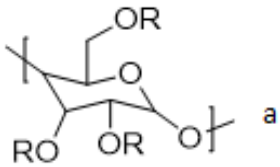
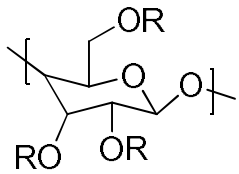
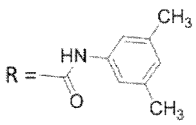
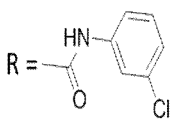
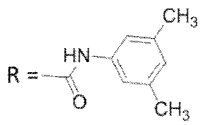
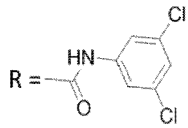
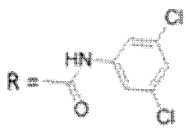
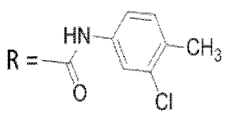
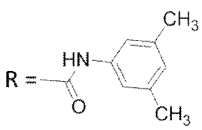
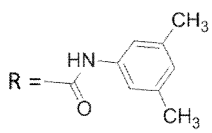
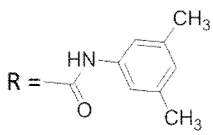
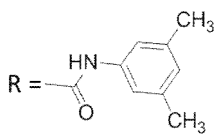
Amylose based CSPs		**Cellulose based CSPs**	
			
Chiralpak IA	**Chiralpak ID**	**Chiralpak IB**	**Chiralpak IC**
			
Chiralpak IE	**Chiralpak IF**		
			
Chiralpak AD-H	**Lux Amylose-1**	**Chiralcel OD-H**	**Lux Cellulose-1**
			

Figure 1. Chemical structures of tris phenylcarbamate derived amylose and cellulose based CSPs immobilized or coated on 5 μm silica gel.

1.3 Results and Discussion

The enantiomeric separation of α-amino acids as NBD derivatives was performed on polysaccharide based chiral stationary phases (CSPs) using normal phase HPLC under simultaneous UV and FL detection. Among the commercially available CSPs, the polysaccharide based CSPs derived from phenylcarbamates of cellulose and amylose were used for the analysis and separation of enantiomers [10]. The ten phenylcarbamates derived covalently bonded Chiralpak IA, Chiralpak IB, Chiralpak IC, Chiralpak ID, Chiralpak IE, Chiralpak IF and coated type Chiralcel OD-H, Lux Cellulose-1, Chiralpak AD-H and Lux Amylose-1 are being used for the enantio-discrimination of α-amino acids.

Table 1 and 2 represent the enantiomeric resolution of α-amino acids on six covalently bonded and four coated type CSPs having different nature and selector backbone. Among the six covalently bonded CSPs (Table 1), Chiralpak IA and Chiralpak IE showed the best separation for most of the analytes while Chiralpak IB showing the worst separation. Among 7 analytes, the analyte phenylalanine (Table 1, entry 5) showed the highest separation and resolution factor (α = 2.69, Rs = 13.54) at Chiralpak IA. The degree of enantioselectivity of six immobilized type CSPs was found

to be: Chiralpak IA > Chiralpak IE > Chiralpak IF > Chiralpak IC > Chiralpak ID > Chiralpak IB.

Different chiral recognism mechanism was observed in six immobilized type CSPs as secondly eluted order of enantiomers were not identical and difficult to rationalize. It might be the difference between backbone structures and different functional groups attached to the phenylcarbamate group. The analyte 2-aminobutyric acid (Table 1, entry 2) was baseline separated at mobile phase 10% 2-propanol/hexane with 0.1% TFA showed the highest retention factor in Chiralpak IC. The low interaction between the backbone structure and mobile phase might have attributed to the highest retention factor of this analyte.

On the other hand, among the four coated type CSPs (Table 2), the amylose-based Chiralpak AD-H and Lux Amylose-1 were superior and showed the greatest enantioselectivities as compared to that of the cellulose-based Chiralcel OD-H and Lux Cellulose-1. This might be due to the differences in the structure of the polysaccharide-based chiral selector (amylose or cellulose) derivative and type of the column used (coated or covalently bonded) [9,11]. Similar trend can be seen as that of immobilized CSPs as analyte phenylalanine (Table 2, entry 5) also showed the highest separation and resolution factor (α = 3.56, Rs = 15.07) at Chiralpak AD-H

and (α = 3.23, Rs = 15.74) at Lux Amylose-1. Consistently, (S)-isomers was secondly eluted and all the analytes of amylose derived Chiralpak AD-H and Lux Amylose-1 showing similar chiral recognition mechanism during separation process. Similarly, among the cellulose-based CSPs, the analyte phenylalanine showed comparatively good resolution as compared to that of other analytes. However, all the analytes were not baseline resolved and didn't showed good separation at all. The order of enantioselectivity of coated type CSPs was as follows: Lux Amylose-1 > Chiralpak AD-H > Lux Cellulose-1 > Chiralcel OD-H.

Morever, the covalently bonded and coated type CSPs is compared with the CSPs of same chiral selectors. The performance of the CSPs Chiralpak IA, Chiralpak AD-H and Lux Amylose-1 having the same chiral selector as amylose tris(3,5-dimethylphenyl carbamate) were compared. In general, all the analytes were enantiomerically resolved while Chiralpak AD-H and Lux Amylose-1 showed comparatively greater enantiomer resolution than that of the covalently bonded Chiralpak IA. Similarly for the cellulose tris(3,5-dimethylphenylcarbamate) based CSPs (Chiralpak IB, Chiralcel OD-H and Lux Cellulose-1) the analytes were not well enantiomerically resolved on the coated CSPs. Only few analytes at Chiralcel OD-H and Lux Cellulose-1 were enantiomerically resolved and the covalently bonded

Chiralpak IB showing the worst separation. The low enantioselectivity on the covalently bonded CSPs than that of coated CSPs might be due to the lack of ordered arrangement of chiral selectors bonded to the silica of the CSPs. Though the enantioselectivity of the covalently bonded CSPs is generally lower than that of coated CSPs having the same chiral selector, the former affords the significant advantages in terms of versatility and application having a much wider range of eluents because of their higher column stability [12]. The unreacted NBD-F peak appeared around 6 min on two chromatograms observed under 337 nm, but it did not appear at FLD as NBD-F is not fluorescence active. As shown in Figure 2, simple analytical chromatograms under high sensitive fluorescence detection showed an advantage of this present analytical method.

Table 1: Enantiomeric separation of α-amino acids on covalently bonded CSPs

Entry	Analytes	Chiralpak IA			Chiralpak IB			Chiralpak IC		
		α	k'_1	R_s	α	k'_1	R_s	α	k'_1	R_s
1	Alanine	1.50	3.86	3.98(S)	1.00	3.75	-	1.08	6.66	1.24(S)
2	2-Aminobutyric acid	1.66	3.06	6.68(S)	1.00	2.70	-	1.05	17.10*	1.05(R)
3	Leucine	1.43	3.71	5.30(S)	1.03	1.74	0.31(S)	1.00	4.49	-
4	Methionine	2.15	5.01	10.71(S)	1.00	3.97	-	1.12	8.06	1.72(R)
5	Phenylalanine	2.69	3.68	13.54(S)	1.04	3.73	0.42(R)	1.09	6.47	1.16(R)
6	Serine	1.13	10.15	0.78(S)	1.00	8.05	-	1.13	5.37	1.47(R)
7	Valine	1.33	3.30	4.00(S)	1.06	1.95	0.60(S)	1.00	5.58	-
Entry	**Analytes**	**Chiralpak ID**			**Chiralpak IE**			**Chiralpak IF**		
		α	k'_1	R_s	α	k'_1	R_s	α	k'_1	R_s
1	Alanine	1.09	3.70	1.24(R)	1.72	6.46	4.88(R)	1.18	4.39	1.55(R)
2	2-Aminobutyric acid	1.00	2.92	-	1.09	7.42	0.81(S)	1.13	3.18	1.40(R)
3	Leucine	1.03	1.94	0.51(S)	1.21	3.28	1.78(R)	1.00	2.14	-
4	Methionine	1.06	4.39	0.73(R)	1.36	6.82	2.78(R)	1.44	4.74	3.72(R)
5	Phenylalanine	1.00	3.55	-	1.45	6.01	3.09	1.14	3.38	1.42(S)
6	Serine	2.30	4.82	6.99(R)	1.74	8.49	3.29(R)	2.15	5.64	3.43(R)
7	Valine	1.05	2.31	0.70(S)	1.14	4.95	1.25(R)	1.04	2.51	0.41(R)

Mobile phase: 20% 2-propanol/hexane (V/V) with 0.1% TFA; Flow rate: 1mL/min; Detection: UV 310 nm, fluorescence 470 nm excitation, 530 nm emission; k'_1: Retention factor of the first eluted enantiomer; α: Separation factor; R_s: Resolution factor; [a]the absolute configuration of the second eluted enantiomer, *10% 2-propanol/hexane (V/V).

Table 2: Enantiomeric separation of α-amino acids on coated CSPs

Entry	Analytes	Chiralcel OD-H			Lux Cellulose-1		
		α	k'_1	R_s	α	k'_1	R_s
1	Alanine	1.04	5.41	0.36	1.00	4.92	-
2	2-Aminobutyric acid	1.08	3.31	0.59(R)	1.06	3.90	0.76(R)
3	Leucine	1.00	2.22	-	1.00	2.41	-
4	Methionine	1.13	4.80	0.94(S)	1.05	5.39	0.62(S)
5	Phenylalanine	1.28	5.43	1.96(R)	1.16	5.92	1.76(R)
6	Serine	1.12	7.15	0.47(S)	1.09	7.11	1.04(S)
7	Valine	1.00	2.81	-	1.03	5.50	0.63(S)
Entry	**Analytes**	**Chiralpak AD-H**			**Lux Amylose-1**		
		α	k'_1	R_s	α	k'_1	R_s
1	Alanine	1.99	3.41	8.73(S)	1.92	3.92	10.67(S)
2	2-Aminobutyric acid	2.10	3.34	9.87(S)	2.03	3.53	11.17(S)
3	Leucine	1.54	4.47	5.72(S)	1.51	4.78	5.98(S)
4	Methionine	2.47	5.65	11.48(S)	2.43	6.30	12.54(S)
5	Phenylalanine	3.56	4.05	15.07(S)	3.23	4.50	15.74(S)
6	Serine	1.90	6.90	7.59(S)	1.79	8.25	8.54(S)
7	Valine	1.60	3.73	6.56(S)	1.54	4.22	6.93(S)

Mobile phase: 20% 2-propanol/hexane (V/V) with 0.1% TFA; Flow rate: 1mL/min; Detection: UV 310 nm, fluorescence 470 nm excitation, 530 nm emission; k'_1: Retention factor of the first eluted enantiomer; α: Separation factor; R_s: Resolution factor; [a]the absolute configuration of the second eluted enantiomer.

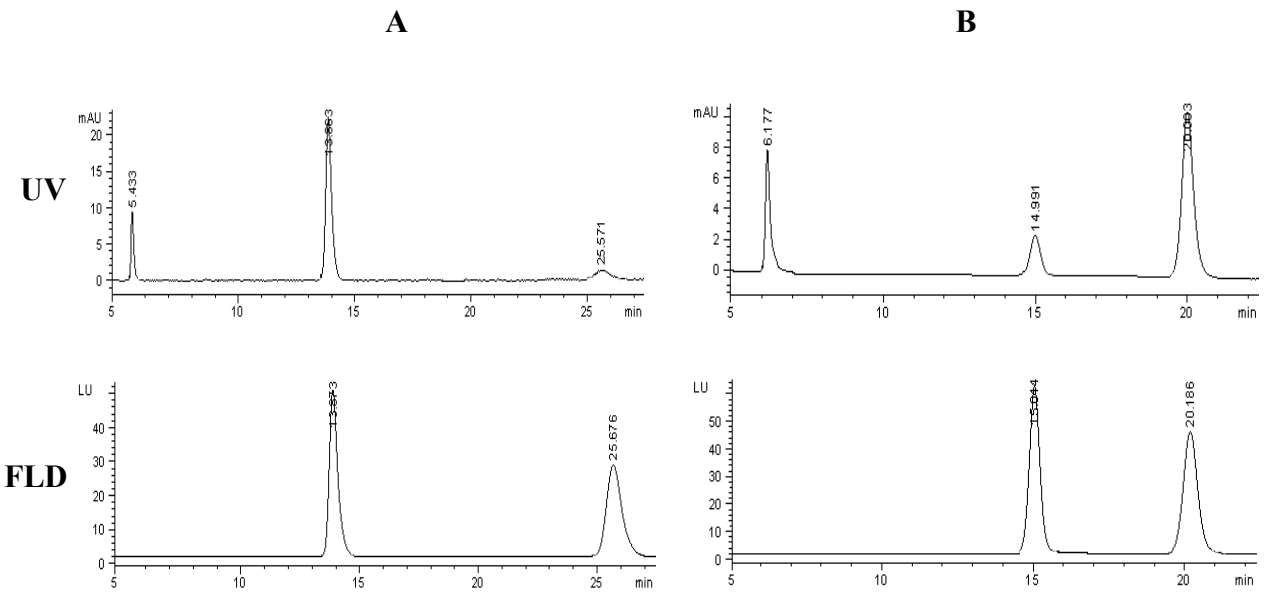


Figure 2. Typical chromatograms for enantiomer resolution of NBD 2-aminobutyric acid derivatives (**A**) on coated type Chiralpak AD-H and NBD leucine derivatives **(B)** on covalently bonded type Chiralpak IA under simultaneous UV and FL detection. Mobile phase: 20% 2-propanol/hexane (V/V) with 0.1% TFA, Flow Rate: 1 mL/min, Detection: UV 337 nm, FLD: excitation; 470 nm, emission; 530 nm. The unreacted NBD-F peak appeared around 6 min under UV 337 nm.

1.4 Conclusion

The potent derivatizing agent, 4-fluoro-7-nitro-2,1,3-benzoxadizole (NBD-F) was used for the enantiomeric separation of several α-amino acids using polysaccharide-based CSPs under simultaneous UV and FL detection. The degree of enantioselectivity was affected by the differences in structures of the used chiral selector (polysaccharide amylose or cellulose derivative) and the type of CSP (covalently bonded or coated type). Among the investigated six covalently bonded and four coated CSPs, the amylose-based showed the best separation for α-amino acids than that of the cellulose based CSPs. The covalently bonded, Chiralpak IA showed the superior performance while the coated type Chiralpak AD-H and Lux Amylose-1 showed enhanced enantioseparation and resolution of the investigated analytes. This described analytical method proved to be suitable and selective for the separation of α-amino acids as NBD derivatives and is expected to be quite useful for the enantiodiscrimination of other α-amino acids and related compounds which ultimately aids the pharmaceutical industry for developing and marketing enantiomerically pure chiral drugs.

1.5 References

1. Lin, Q.-G.; You, D.-Q.; Cheng, F.-J.; Eds.; Chiral drugs: chemistry and biological action, John Wiley and Sons, Inc., New York, 333, 2011.
2. Davies, M.-N.; Teng, W.-X.; Importance of chirality in drug therapy and pharmacy practice: Implications for psychiatry, Advances in Pharmacy, 1, 242, 2003.
3. Francotte E.; Lindner W.; Eds.; Isolation and production of optically pure drugs by enantioselective chromatography, Chirality in drug research, Wiley-VCH, Weinheim, Germany, 2006.
4. Subramanian G (ed) Chiral separation techniques: a practical approach, 3rd edn. Wiley, Weinheim, 2007.
5. Huang H.; Jin, Y.-J.; Hong, H.-J.; Lee W.; Han, K.-H.; Kang, S.-J.; Liquid Chromatographic Enantiomer Separation of Amino Acid Esters as 9-Anthraldimine Schiff Bases Using Polysaccharide-Derived Chiral Stationary Phase, Journal of Liquid Chromatography & Related Technologies, 34, 209, 2011.
6. Huang H.; Xu, J.-W.; Jin, Y.-J.; Hong, H.-J.; Shin, J.-H.; Lee W.; A Convenient Method for the Enantiomeric Separation of α-Amino

Acid Esters as Benzophenone Imine Schiff Base Derivative, 35, 1015, 2012.

7. Jin, Y.-J.; Lee W.; Park, H.-J.; Ryoo, J.-J.; Covalently Bonded and Coated Chiral Stationary Phases Derived from Polysaccharide Derivatives for Enantiomer Separation of N-Fluorenylmethoxycarbonyl α-amino acids with Fluorescence Detection, Journal of Liquid Chromatography & Related Technologies, 29, 1793, 2006.

8. Adhikari S.; Kang, J.-S.; Lee W.; A convenient and validated enantiomer separation of chiral aliphatic amines as nitrobenzoxadiazole derivatives on polysaccharide-derived chiral stationary phases under simultaneous ultraviolet and fluorescence detection, Chirality, 28, 789, 2016.

9. Chen X.; Yamamoto C.; Okamoto Y.; Polysaccharide derivatives as useful chiral stationary phases in high-performance liquid chromatography, 79, 1561, 2007.

10. Zhang T.; Franco, P.; Nguyen D.; Hamasaki, R.; Miyamoto S.; Ohnishi A.; Murakami T.; Complementary enantiorecognition patterns and specific method optimization aspects on immobilized

polysaccharide-derived chiral stationary phase, J. Chromatogr.A., 1269, 178, 2012.

11. Okamoto Y.; Yahima E.; Polysaccharide Derivatives for Chromatographic Separation of Enantiomers, Angew. Chem. Int. Ed., 37, 1020, 1998.

12. Chankvetadze B.; Recent developments on polysaccharide-based chiral stationary phases for liquid-phase separation of enantiomers, J. Chromatogr. A.; 1269, 26, 2012.

Chapter 2

Enantiodiscrimination of Amino Acid Esters and Chiral Amines as Nitrobenzoxadiazole Derivatives under Simultaneous Ultraviolet and Fluorescence Detection

Abstract

An efficient chiral HPLC method was developed for the enantiomeric resolution of α-amino acid esters and some chiral amines as nitrobenzoxadiazole (NBD) derivatives on amylose and cellulose phenylcarbamates derived chiral stationary phases (CSPs) under simultaneous ultraviolet and fluorescence detection. The degree of enantioselectivity was affected by the type of analyte (α-amino acid esters or amines), backbone structure (cellulose or amylose) and the substituents of phenyl moiety on the chiral selector of CSP. Among the α-amino acid ethyl esters, the covalently bonded Chiralpak IA showed the superior performance while among the coated type CSPs, Chiralpak AD-H (or Lux Amylose-1) showed the highest performance. Similarly, the degree of enantioselectivity for α-amino acid methyl esters was higher for Chiralpak AD-H (or Lux Amylose-1). Further, for the enantiomeric resolution of chiral amines, Chiralpak IE and Chiralcel OD-H (or Lux Cellulose-1) columns showed the highest enantioselectivities. In general, the degree of enantioselectivity was greater on coated type α-amino acid esters as compared to that of chiral amines. This analytical method is expected to be quite useful for the enantiomer separation of other α-amino acid esters and chiral amines as NBD derivatives by normal high performance liquid chromatography (HPLC).

Keywords: Amino acid esters, Chiral amine, Chiral stationary phase, Enantiomer separation, Nitrobenzoxadiazole derivative, HPLC

2.1 Introduction

Chiral drugs are ~~a~~ subgroup of drug substances which contain one or more chiral centers. More than one half of the marketed drugs are chiral. The opposite enantiomer of a chiral drug differs significantly in its pharmacological, toxicological, pharmacodynamics and pharmacokinetic properties [1]. The analysis of the chiral compounds and the preparation of optically active compounds have become important in the field of agrochemicals, foods, fragrances and functional materials [2,3]. The enantioselective separation of enantiomers by chromatography on chiral stationary phases (CSPs) has gained recognition over the last 15 years and the technique is now considered as a powerful approach for the preparation of optically pure compounds [4]. Among a large number of CSPs developed, the polysaccharide and its derivatives are highly used as chiral selectors for HPLC because of their high chiral recognition ability [5]. Chiral amines, α-amino acids and α-amino acid esters are being widely used as an chiral building blocks for the synthesis of many pharmaceuticals and biologically active molecules and as an scaffolds for the development of new drugs [6,7]. In this study, we present the comparative liquid chromatographic enantiomer separation of amino acid esters and chiral

amines on six covalently bonded Chiralpak IA, Chiralpak IB, Chiralpak IC, Chiralpak ID, Chiralpak IE and Chiralpak IF and four coated type polysaccharide derived CSPs Chiralcel OD-H, Lux Cellulose-1, Chiralpak AD-H and Lux Amylose-1. The derivatizing agent, 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl) has been used in order to increase selectivity and detection of α-amino acid ethyl and methyl ester analytes, and chiral amines using the normal phase HPLC [8].

2.2 Experimental section

2.2.1 Instrumentation and Experimental Conditions

The liquid chromatographic analysis was performed by using an Agilent Technologies (Palo Alto, CA, USA) 1100 HPLC system with a solvent degasser, an isocratic pump G1310A, an automatic sample injector, a thermostatic column compartment, a simultaneous multiwavelength G1315A ultraviolet, and a programmed fluorescence detector HP1046A [8]. Hewlett-Packard ChemStation chromatographic data software was used for instrument control, data acquisition and analysis. Ten amylose or cellulose phenylcarbamates derived chiral columns categorised as six covalently bonded type Chiralpak IA, Chiralpak IB, Chiralpak IC, Chiralpak ID, Chiralpak IE and Chiralpak IF were procured from Daicel company (Tokyo, Japan) while the four coated type columns, Chiralcel OD-H and Chiralpak AD-H were purchased from Daicel Company (Tokyo, Japan) and Lux Cellulose-1 and Lux Amylose-1 columns were supplied from Phenomenex (Torrance, CA, USA) [9]. All the columns used for the separation were of the size (250 mm × 4.6 mm, I.D., 5 μm). Separation of the enantiomers was performed at room temperature with a flow rate of 1 mL/min. The mobile phase used for the separation consists of 10~40 % 2-

propanol/hexane (V/V). Enantiomeric separation was simultaneously monitored by UV 310 nm and fluorescence (excitation: 470 nm; emission: 530 nm).

2.2.2 Reagents and Sample Preparation

For the preparation of the sample and mobile phase, HPLC grades of 2-propanol, ethanol, hexane, triethylamine (NEt3) and dimethylformamide (DMF) were obtained from Avantor Performance Materials Korea Limited (Gangnam-Gu, Seoul, Korea) or Fischer Scientific Korea Ltd. (Kangnam-gu, Seoul, Korea) or Daejung Chemical and Metals Co. Ltd. (Gyeonggi-do, South Korea). All α-amino acid esters, chiral amines along with the derivatizing agent, 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl) were procured from Sigma Aldrich (St. Louis, MO, USA) or Alfa Aesar (Haverhill, Massachusetts, USA) or Acros organics (Fair Lawn, NJ, USA) or Aldrich Chemical Co Inc. (Milwaukee, Wisconsin, USA) or Bachem AG (Bubendorf, Switzerland) and sodium bicarbonate was obtained from Katayama Chemical Industries Co. Ltd. (Chuo ward, Osaka, Japan) respectively [9]. The NBD derivatives of α-amino acid esters were prepared by stirring 0.25 mmol of α-amino acid esters hydrochloride salts, 0.5 mmol of NBD chloride and 2.5 mmol of sodium bicarbonate in 5 mL ethanol at room temperature for 6 hours [8]. After 6 hours, the reaction mixtures were sonicated at 50°C for 30 to 60 minutes and then filtered to remove sodium bicarbonate and diluted to a proper concentration by

adding ethanol. Then, the resulting solution were directly injected into the HPLC. Similarly, for chiral amines, a new preparation method was employed by stirring chiral amines and NBD-Cl (1 equivalent) with excess triethylamine (10 equivalents) in DMF (5 mL) at room temperature for 6 h. Mild reaction conditions were preferred and the resultant sample solution was then filtered and further diluted to a proper concentration for the injection in normal HPLC.

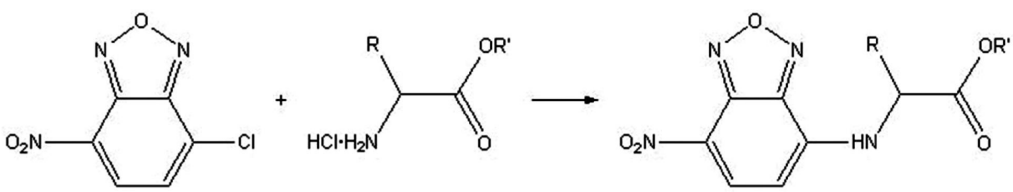


Figure 3. Preparation of α-amino acid esters as NBD derivatives.

2.3 Results and Discussion

The enantiomeric discrimination results of three α-amino acid ethyl ester analytes on six covalently bonded and four coated type polysaccharide derived CSPs are shown in Table 3 and 4 and five α-amino acid methyl esters on four coated type polysaccharide derived CSPs in Table 5. All the α-amino acid ethyl and methyl esters are well enantiomerically resolved and showed good baseline resolution. Identical (S)-isomers are secondly eluted in covalently bonded or coated type CSPs of similar backbone while (R)-isomers are secondly eluted in covalently bonded type Chiralpak ID, Chiralpak IE and Chiralpak IF of substituents attached at different position in tris phenylcarbamate moiety. This might be due to the nature of the chiral selector backbone (amylose or cellulose) and the nature of substituents of the phenylcarbamate moiety which might have influenced not only the separation performance but also the elution sequence [10,11]

As observed from Table 3 and Table 4, among the covalently bonded type CSPs, Chiralpak IA showed the best separation followed by Chiralpak IE while Chiralpak IC showing the worst separation. Among all, the analyte Leucine (Table 3, entry 1) at Chiralpak IA having amylose tris(3,5-dimethylphenylcarbamate) showed the highest resolution and separation factor (α = 2.43, Rs = 8.33) followed by the analytes phenylglycine and valine (Table 3, entries 2 and 3). However, the analyte leucine and phenylglycine at Chiralpak IC showed the worst separation. Consistently, (S)-isomer was secondly eluted at Chiralpak IA, Chiralpak IB and Chiralpak IC while (R)-isomer was secondly eluted at Chiralpak ID,

Chiralpak IE and Chiralpak IF. From Table 4, the amylose based CSPs, Chiralpak AD-H (or Lux Amylose-1) having the amylose-based chiral selector as amylose tris(3,5-dimethylphenylcarbamate) showed the greatest enantioselectivity with highest resolution and separation factor. The analyte leucine (Table 4, entry 1) and phenylglycine (Table 4, entry 2) showed the highest separation and resolution factor (α = 2.87, Rs = 12.90; α = 2.48, Rs = 13.50) at Lux Amylose-1. In contrast, the cellulose-derived CSPs, having chiral selector as cellulose tris(3,5-dimethylphenylcarbamate), Chiralcel OD-H (or Lux Cellulose-1) showed comparatively lowest enantioselectivity. This might be due to the differences in the polymer backbone structure (cellulose or amylose) as chiral selector and it showed that the investigated analytes fitted better to the amylose type CSPs than that of cellulose type CSPs [10,12]. Moreover, it can be observed that the coated CSPs seems more advantageous than that of the immobilized ones, but in general the employment of the covalent phases are preferred more due to their high robustness [13]. Identical (S)-isomers was secondly eluted in all the coated type CSPs depicting similar chiral discrimination mechanisms

Table 5 shows the enantiomeric separation of α-amino acid methyl esters as NBD derivatives on four coated type CSPs. Five analytes, alanine, leucine, phenylglycine, serine and valine were used for the enantiomeric discrimination. Interestingly, all the analytes were well-enantiomerically resolved and showed the good baseline resolution. Among the four coated type CSPs, chiral selector having amylose tris(3,5-dimethylphenylcarbamate), Chiralpak AD-H (or Lux amylose-1) showed the best separation and were comparable with each other as having the

same chiral selector. The analyte leucine (Table 5, entry 2) showed the greatest separation and resolution factor as (α = 2.31, Rs = 11.20) at Lux Amylose-1 and (α = 2.48, Rs = 10.62) at Chiralpak AD-H. Similar trend can be seen at α-amino acid ethyl and methyl esters as amylose based CSPs showing the highest resolution and separation factor (Table 4 and 5). The analyte phenylglycine showed the comparatively lowest separation for both amylose derived CSPs and was separated at mobile phase 10% 2-propanol/hexane. In contrast, the cellulose derived CSPs, Chiralcel OD-H and Lux Cellulose-1, having the chiral selector as cellulose tris(3,5-dimethyl phenylcarbamate) showed comparatively worst separation. The degree of enantioselectivity and resolution for four coated type CSPs can be shown as: Lux Amylose-1> Chiralpak AD-H> Lux Cellulose-1> Chiralcel OD-H. Consistently, (S)-isomers were preferentially retained as that of α-amino acid ethyl esters. Overall, Chiralpak AD-H and Lux Amylose-1 showed the superior performance among the four coated type CSPs and was more selective in enantiodiscrimination of α-amino acid methyl esters as compared to that of other CSPs.

Table 6 and 7 shows the enantio discrimination results of five chiral amines as NBD derivatives on six covalently bonded and four coated type CSPs under simultaneous ultraviolet and fluorescence detection. The three aliphatic amines 3,3-dimethyl-2-butylamine, 1,3-dimethylbutylamine, 1,2-dimethylpropylamine and two aromatic amines as α-methylbenzylamine and norephedrine were selected for the enantioseparation. The α-amino acid esters showed the enhanced performance in terms of enantio-selectivity and resolution as compared to that of chiral amines on ten phenylcarbamates derived CSPs. Among the six covalently bonded CSPs,

the performance of Chiralpak IE having the amylose tris(3,5-dichlorophenylcarbamate) and coated type Lux Cellulose-1 derived from the cellulose tris(3,5-dimethylphenylcarbamate) showed the excellent resolving ability and were able to resolve all five chiral amines to baseline separation. Among the three aliphatic amines (entries 1-3), the enantiomeric discrimination was highly successful on covalently bonded type Chiralpak ID, Chiralpak IE, Chiralpak IF and coated type Chiralcel OD-H (or Lux Cellulose-1) while that of the aromatic moiety (entry 5) on Chiralpak IE, Chiralpak IF and Lux Amylose-1. The aromatic moiety might have impact on different chiral interaction with chiral selector of CSPs and found to favor Chiralpak IE, Chiralpak IF and Lux Amylose-1. Amongst the six covalently bonded CSPs, the amylose based Chiralpak IA and the cellulose based Chiralpak IB were not satisfactory except for one analyte (Table 6, entry 5). Similarly, the coated type Chiralpak AD-H and Lux Amylose-1 were not satisfactory except for two analytes (Table 7, entries 2 and 5). From Tables 6 and 7 the employment of the covalently bonded CSPs seems more advantageous than that of coated type CSPs in enantiodiscrimination of chiral amines.

Typical simultaneous chromatograms of the enantiomer resolution of leucine methyl and ethyl esters as NBD derivatives on Lux Amylose-1 under simultaneous ultraviolet and fluorescence detection were shown in Figure 4 while the HPLC chromatograms showing the enantiomeric separation of 3,3-dimethyl-2-butylamine on Chiralpak IE under simultaneous UV and FL detection is shown in Figure 5. The unreacted NBD-Cl peak appeared around 18 min on Figure 4 on two chromatograms observed under 310 nm, but it did not appear at FLD as NBD-Cl is not

fluorescence active. As shown in Figure 4, simple analytical chromatograms under high sensitive fluorescence detection show an advantage of this present analytical method.

Table 3. Enantiomer separation of α-amino acid ethyl esters as NBD derivatives on covalently bonded CSPs

Entry	Analytes	Chiralpak IA			Chiralpak IB			Chiralpak IC		
		α	k'_1	R_s	α	k'_1	R_s	α	k'_1	R_s
1	Leucine	2.43	1.32	8.33(S)[a]	1.14	1.77	0.93(S)	1.03	11.34	0.53(S)
2	Phenylglycine	1.73	2.27	6.59(S)	1.13	3.96	1.06(S)	1.03	19.80	0.55(S)
3	Valine	1.48	1.75	4.74(S)	1.15	2.28	2.00(S)	1.12	14.25	2.05(S)
Entry	**Analytes**	**Chiralpak ID**			**Chiralpak IE**			**Chiralpak IF**		
		α	k'_1	R_s	α	k'_1	R_s	α	k'_1	R_s
1	Leucine	1.19	3.32	1.70(R)[a]	1.46	8.06	4.55(R)	1.34	3.11	1.51(R)
2	Phenylglycine	1.04	10.14	0.60(R)	1.18	20.89	2.07(R)	1.23	6.30	3.05(R)
3	Valine	1.51	3.16	5.76(R)	1.26	15.14	2.39(R)	1.16	3.69	2.09(R)

Mobile phase: 20% 2-propanol/hexane (V/V); Flow rate: 1mL/min; Detection: UV 310 nm, fluorescence 470 nm excitation, 530 nm emission; k'_1: Retention factor of the first eluted enantiomer; α: Separation factor; R_s: Resolution factor; [a]the absolute configuration of the second eluted enantiomer.

Table 4: Enantiomer separation of α-amino acid ethyl esters as NBD derivatives on coated CSPs

Entry	Analytes	Chiralcel OD-H			Lux Cellulose-1		
		α	k'_1	R_s	α	k'_1	R_s
1	Leucine	1.57	3.73	2.20(S)[a]	1.54	3.90	2.60(S)
2	Phenylglycine	1.18	9.46	1.79(S)	1.21	10.07	1.77(S)
3	Valine	1.37	4.36	2.81(S)	1.40	4.57	3.61(S)
Entry	**Analytes**	**Chiralpak AD-H**			**Lux Amylose-1**		
		α	k'_1	R_s	α	k'_1	R_s
1	Leucine	3.12	1.27	11.77(S)[a]	2.87	1.42	12.90(S)
2	Phenylglycine	2.41	1.68	11.20(S)	2.48	1.85	13.50(S)
3	Valine	1.70	1.73	5.97(S)	1.71	1.90	7.37(S)

Mobile phase: 20% 2-propanol/hexane (V/V); Flow rate: 1mL/min; Detection: UV 310 nm, fluorescence 470 nm excitation; 530 nm emission; k'_1: Retention factor of the first eluted enantiomer; α: Separation factor; R_s: Resolution factor; [a]the absolute configuration of the second eluted enantiomer.

Table 5: Enantiomer separation of α-amino acid methyl esters as NBD derivatives on coated CSPs

Entry	Analyte	Chiralcel OD-H			Lux Cellulose-1		
		α	k'_1	R_s	α	k'_1	R_s
1	Alanine	1.23	11.71	1.82(S)[a]	1.10	11.46	1.33(S)
2	Leucine	1.61	5.99	1.94(S)	1.52	5.81	2.75(S)
3	Phenylglycine	1.17	14.31	1.64(S)	1.16	13.86	2.10(S)
4	Serine	1.38	12.43	2.72(S)	1.26	12.56	3.11(S)
5	Valine	1.23	6.53	1.79(S)	1.26	6.38	3.00(S)
Entry	Analyte	Chiralpak AD-H			Lux Amylose-1		
		α	k'_1	R_s	α	k'_1	R_s
1	Alanine	1.62	2.49	6.10(S)[a]	1.68	2.92	8.22(S)
2	Leucine	2.48	1.52	10.62(S)	2.31	1.71	11.20(S)
3	Phenylglycine	1.15	10.08*	2.50(S)	1.16	10.80*	3.08(S)
4	Serine	1.82	5.30	7.11(S)	1.82	6.16	9.82(S)
5	Valine	1.84	1.78	6.74(S)	1.80	1.97	7.81(S)

Mobile phase: 20% 2-propanol/hexane (V/V); Flow rate: 1 mL/min; Detection: UV 310 nm, fluorescence 470 nm excitation; 530 nm emission; α: Separation factor; k'_1: Retention factor of the first eluted enantiomer, Rs: Resolution factor, [a]the absolute configuration of the second eluted enantiomer, *10% 2-propanol/hexane (V/V).

Table 6. Enantiomer separation of chiral amines as NBD derivatives on covalently bonded CSPs

Entry	Analytes	Chiralpak IA			Chiralpak IB			Chiralpak IC		
		α	k'_1	R_s	α	k'_1	R_s	α	k'_1	R_s
1	3,3-Dimethyl-2-butylamine	1.00	0.98	-	1.00	3.91	-	1.15	6.90**	1.88(S)[a]
2	1,3-Dimethylbutylamine	1.13	2.22	1.62	1.05	1.61	0.34	1.10	6.74**	0.94
3	1,2-Dimethylpropylamine	1.00	3.15*	-	1.00	4.70*	-	1.10	14.50	2.40
4	α-Methylbenzylamine	1.00	1.88	-	1.11	4.82	0.90(R)	1.24	7.40**	2.44(S)
5	Norephedrine	1.70	2.74	6.31(R)	1.30	4.66	2.62(R)	1.07	10.90	0.88(R)
Entry	**Analytes**	**Chiralpak ID**			**Chiralpak IE**			**Chiralpak IF**		
		α	k'_1	R_s	α	k'_1	R_s	α	k'_1	R_s
1	3,3-Dimethyl-2-butylamine	1.09	7.12	1.77(S)[a]	1.30	12.02	4.06(R)	1.17	2.80	1.76(S)
2	1,3-Dimethylbutylamine	1.20	3.29	1.19	1.67	6.29	2.24	1.12	2.80	1.21
3	1,2-Dimethylpropylamine	1.15	10.07*	2.51	1.42	11.90**	4.26	1.07	3.29	1.02
4	α-Methylbenzylamine	1.06	6.37	0.52(S)	1.17	13.10	1.52(S)	1.37	4.93	1.85(S)
5	Norephedrine	1.22	3.31	1.84(R)	2.93	2.46***	7.26(R)	1.30	3.72	2.75(R)

Mobile phase: 20% 2-propanol/hexane (V/V); Flow rate: 1mL/min; Detection: UV 310 nm, fluorescence 470 nm excitation, 530 nm emission; k'_1: Retention factor of the first eluted enantiomer; α: Separation factor; R_s: Resolution factor; [a]the absolute configuration of the second eluted enantiomer; *10% or **30% or ***40% 2-propanol/hexane (V/V)

Table 7: Enantiomer separation of chiral amines as NBD derivatives on coated CSPs

Entry	Analytes	Chiralcel OD-H			Lux Cellulose-1		
		α	k'_1	R_s	α	k'_1	R_s
1	3,3-Dimethyl-2-butylamine	1.23	7.35	3.35(S)[a]	1.09	3.95	1.10(S)
2	1,3-Dimethylbutylamine	2.45	4.33	2.60	2.11	5.19	3.47
3	1,2-Dimethylpropylamine	1.54	5.90	2.90	1.25	5.78	1.92
4	α-Methylbenzylamine	1.08	8.44	0.73(R)	1.09	9.77	1.20(R)
5	Norephedrine	2.20	2.26***	3.50(R)	1.79	2.14***	4.19(R)
Entry	**Analytes**	**Chiralpak AD-H**			**Lux Amylose-1**		
		α	k'_1	R_s	α	k'_1	R_s
1	3,3-Dimethyl-2-butylamine	1.00	0.88	-	1.04	1.94	0.58(R)[a]
2	1,3-Dimethylbutylamine	1.27	0.63	1.09	1.17	1.92	2.34
3	1,2-Dimethylpropylamine	1.00	2.63*	-	1.00	2.95*	-
4	α-Methylbenzylamine	1.00	1.68	-	1.00	1.78	-
5	Norephedrine	2.06	2.55	7.58(R)	1.96	2.93	8.40(R)

Mobile phase: 20% 2-propanol/hexane (V/V); Flow rate: 1mL/min; D etection: UV 310 nm, fluorescence 470 nm excitation, 530 nm emiss ion; k'_1: Retention factor of the first eluted enantiomer; α: Separation factor; R_s: Resolution factor; [a]the absolute configuration of the secon d eluted enantiomer; *10% or **30% or ***40% 2-propanol/hexane (V/V)

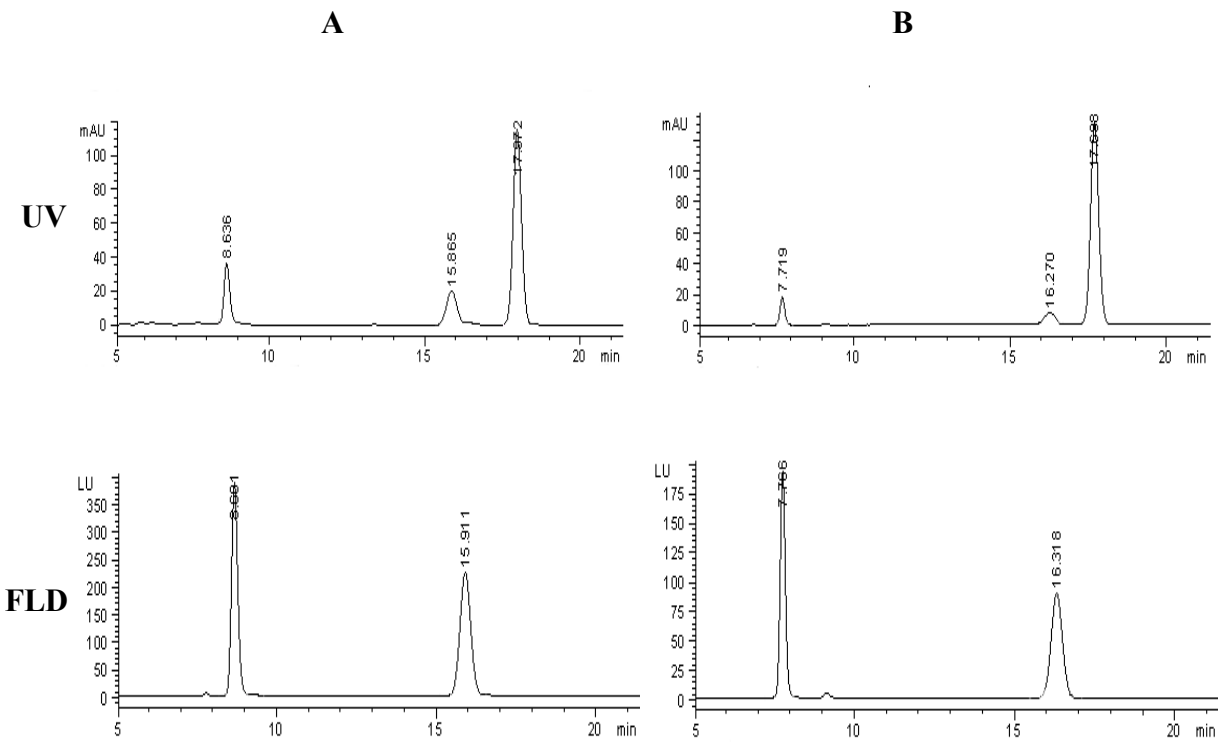


Figure 4. Typical simultaneous chromatograms of the enantiomeric separation of leucine methyl (**A**) and ethyl **(B)** esters as NBD derivatives on the coated type CSP, Lux Amylose-1. Mobile phase: 20% 2-propanol/hexane (V/V), Flow Rate: 1 mL/min, Detection: UV 310 nm, FLD: excitation; 470 nm, emission; 530 nm. The unreacted NBD-Cl peak appeared around 18 min under UV 310 nm.

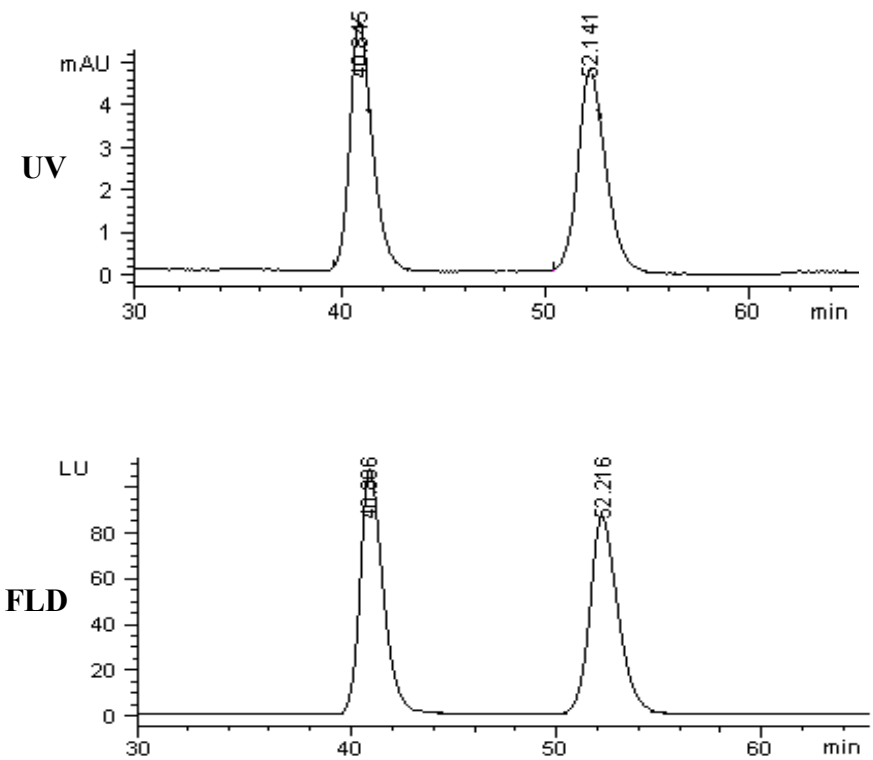


Figure 5. HPLC chromatograms showing the enantiomeric separation of 3,3-dimethyl-2-butylamine on Chiralpak IE under simultaneous UV and FL detection. Mobile phase: 20% 2-propanol/hexane (V/V), Flow Rate: 1 mL/min, Detection: UV 310 nm, FLD: excitation; 470 nm, emission; 530 nm.

2.4 Conclusion

A derivatized analytical method was employed for the enantiomer separation of α-amino acid esters and chiral amines (aliphatic or aromatic) as NBD derivatives using normal phase HPLC under simultaneous UV and FL detection. Covalently bonded and coated type CSPs based on different amylose and cellulose derivatives were used and their performances were evaluated on the basis of mentioned chiral selectors. Among the covalently bonded CSPs for α-amino acid ethyl esters, Chiralpak IA showed the best enantiomer separation and for the chiral amines, Chiralpak IE showed the best separation. Similarly, for the coated type CSPs, the amylose-based Chiralpak AD-H (or Lux Amylose-1) showed the higher separation for α-amino acid esters while the cellulose based Chiralcel OD-H (or Lux Cellulose-1) showed the higher enantiomer separation for the chiral amines.This developed analytical method proved to be suitable for the enantiomer separation of α-amino acid esters and chiral amines as NBD derivatives.

2.5 References

1. Reddy, K.-I.; Mehvae, R.; Chirality in drug design and development, Marcel Dekker, New York, 2004.
2. Subramanian G (ed) Chiral separation techniques: a practical approach, 3rd edn. Wiley, Weinheim, 2007.
3. Okamoto, Y.; Ikai, T.; Chiral HPLC for efficient resolution of enantiomers, Chem. Soc. Rev., 37, 2593, 2008.
4. Francotte, E.; Lindner, W.; Eds.; Isolation and production of optically pure drugs by enantioselective chromatography, Chirality in drug research, Wiley-VCH, Weinheim, Germany.
5. Padró, J. -M.; Keunchkarian, S.; State-of-the-art and recent developments of immobilized polysaccharide-based chiral stationary phases for enantioseparations by high performance liquid chromatography, Microchem. J., 140, 142, 2018.
6. Adhikari, S.; Paik, M.-J.; Lee, W.; Liquid chromatographic enantiomeric separation of chiral aliphatic amines using 2-hydroxynaphthaldehyde as a derivatizing agent on polysaccharide-derived chiral stationary phases, Chromatographia, 81, 1337, 2018.
7. Biancalana, L.; Bortoluzzi, M.; Ferretti, E.; Hayatifar, M.; Marchetti, F.; Pampaloni, G.; Zacchini, S.; The reactions of α-

amino acids and α-amino acid esters using high valent transition metal halides: synthesis of co-ordination complexes, activation processes and stabilization of α-ammonium acylchloride cations†, 7, 10158, 2017.

8. Adhikari, S.; Kang, J.-S.; Lee, W.; A convenient and validated enantiomer separation of chiral aliphatic amines as nitrobenzoxadiazole derivatives on polysaccharide-derived chiral stationary phases under simultaneous ultraviolet and fluorescence detection, Chirality, 28, 789, 2016.

9. Adhikari, S.; Kim, J.-K.; Chromatographic Enantiomer Separation of Chiral Amines as Fluorene-2-carboxaldimine Derivatives Using Polysaccharide-derived Chiral Stationary Phases, Yakhak Hoeji, 63, 328, 2019.

10. Ikai, T.; Okamoto, Y.; Structure Control of Polysaccharide Derivatives for Efficient Separation of Enantiomers by Chromatography, Chem. Rev., 109, 6077, 2009.

11. Tanács, D.; Orosz, T.; Szakonyi, Z.; Le, M. −T.; Fülöp, F.; Lindner, W.; Ilisz, I.; Péter, A.; High-performance liquid chromatographic enantioseparation of isopulegol-based β-amino lactone and β-amino

amide analogs on polysaccharide-based chiral stationary phases focusing on the change of the enantiomer elution order, J. Chromatogr. A. 1621, 461054, 2020.

12. Chankvetadze, B.; Recent developments on polysaccharide-based chiral stationary phases for liquid-phase separation of enantiomers, J. Chromatogr. A. 1269, 26, 2012.

13. Padró, J. -M.; Keunchkarian, S.; State-of-the-art and recent developments of immobilized polysaccharide-based chiral stationary phases for enantioseparations by high performance liquid chromatography, Microchem. J., 140, 142, 2018.

Publications

1. **Enantiomeric resolution of α-amino acid esters on polysaccharide-based chiral stationary phases under simultaneous ultraviolet and fluorescence detection**

   Bhandari A, Lee W

   J. Pharm. Res. 2021November; 42(1): 43-50

2. **Enantiomer Separation of Chiral Amines and Amino Acid Esters on Polysaccharide Phenylcarbamates Derived Chiral Stationary Phases**

   Adhikari S, Bhandari A, Lee W

   Yakhak Hoeji. 2021June; 65(3): 209-216

   DOI: 10.17480/psk.2021.65.3.209

Acknowledgement

Individual efforts alone can never contribute totally in a successful completion of any endeavor. The hard work, dedication, proper guidance, support, inspiration and motivation from the loved ones has made this Master's degree program a success.

My profound gratitude to National Institute For International Education (NIIED), a branch of the Ministry of Education in the Republic of Korea for selecting me as an Global Korea Scholarship (GKS) Scholar for my graduate studies and providing me an great opportunity to study at South Korea.

I would like to express my sincere gratitude to my advisor Prof. Dr. Wonjae Lee, for initiating and encouraging me in the research of pharmaceutical analysis area, for the time he devoted and for the precise way in which he has corrected and criticized the thesis and gave many helpful suggestions. Actually, I am indebted to him for accepting me in his laboratory, supervising my research works and encouraging me to do new things with great enthusiasms.

Besides my advisor, I would like to express my heartiest thankfulness to my senior Dr. Suraj Adhikari for the continuous guidance, support and help during my Master's degree journey. The insightful comments and encouragements as well as expertise feedback during the preparation of this dissertation was very helpful for me.

My sincere thanks also goes to my family and friends for their continuous motivation and support. I am very grateful and thankful to them for always being with me in my success and in failure and always inspiring and motivating me during my good and hard times.

Finally, I am obliged and hugely indebted to the Almighty God, for providing me strength and letting me through all the difficulties. The blessings from the god will definitely help me keep going in the coming future too.

Alisha Bhandari

February, 2022