

## Liquid Chromatographic Enantiomer Separation of $\alpha$ -Amino Acid Esters as Nitrobenzoxadiazole Derivatives Using Polysaccharide-Derived Chiral Stationary Phases

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### Abstract

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

**Keywords:**  $\alpha$ -amino Acid Esters, Chiral Stationary Phase, Enantiomer Separation, NBD Derivatives.

### 1. Introduction

In the fields of pharmaceutical industry,  $\alpha$ -amino acids and/or esters have been widely used as important chiral building blocks and their enantiomer separation for development of chiral drugs has been of great interest<sup>[1]</sup>. For the determination of enantiomeric purity of  $\alpha$ -amino acids and/or ester derivatives, several analytical methods have been developed<sup>[1,2]</sup>. Among them, the liquid chromatographic enantiomer separation on chiral stationary phase (CSP) has been known to be one of the most convenient and versatile methods. Particularly, polysaccharide-derived CSPs have been widely and successfully used for separating a variety of enantiomer compounds<sup>[2-4]</sup>. Related to this study, we have been reported enantiomer separation of  $\alpha$ -amino acids and/or esters as several aromatic moiety derivatives using these polysaccharide-derived CSPs<sup>[5-8]</sup>. In this study, we focused on nitrobenzoxadiazole (NBD) group which is a fluorescence active derivatizing group

of  $\alpha$ -amino acids esters, because NBD fluorescence detection may provide strong advantages of selectivity and sensitivity in enantiomer separation. In previous studies, some analytical results of amino acids as NBD derivatives have been reported<sup>[9-12]</sup>. In particular, for enantiomer resolution of amino acids as NBD derivatives, Pirkle type chiral columns of Sumochiral OA 2500, Chiralpak QN-AX, and Chiralpak QD-AX were used by Zaitso group<sup>[10-12]</sup>. Although they reported pretty good enantiomer separation results under aqueous HPLC conditions, but not high enantioselectivity. In general, for NBD derivatization of  $\alpha$ -amino acids, they have used aqueous sodium borate buffer system<sup>[9-12]</sup>. Here, we are to perform enantiomer separation of  $\alpha$ -amino acid esters as NBD derivatives by normal phase chromatography using polysaccharide-derived CSPs. Until now, no previous studies have been reported for enantiomer separation of amino acid esters as NBD derivatives using polysaccharide-derived CSPs by normal phase chromatography.

### 2. Experimental Section

Chromatographic analysis was carried out using an

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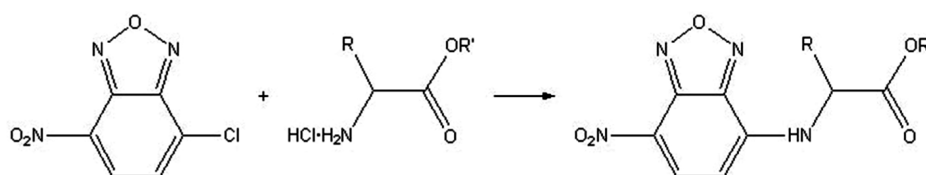


Fig. 1. Preparation of  $\alpha$ -amino acid esters as NBD derivatives.

HPLC system with HP series 1100 with G1310A Iso pump, an automatic sample injector and an HP 1046A programmable fluorescence detector. All covalently immobilized CSPs (Chiralpak IA, Chiralpak IB, Chiralpak IC, Chiralpak ID, Chiralpak IE and Chiralpak IF) derived from polysaccharides were purchased from the Daicel Chemical Company (Tokyo, Japan). HPLC grade hexane, 2-propanol and other solvents were obtained from J. T. Baker. All  $\alpha$ -amino acid methyl esters, 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD chloride) and several bases such as sodium bicarbonate were obtained from Aldrich (Milwaukee, WI), Sigma (St. Louis, MO), Advanced ChemTech (Louisville, KY) and Chem-Impex International (Wood Dale, IL). The NBD racemic or L-analytes used in this study were synthesized, as shown on Fig. 1. The NBD derivatives of  $\alpha$ -amino acid esters were prepared by stirring 0.25 mmol of  $\alpha$ -amino acid ester hydrochloride salts, 0.5 mmol of NBD chloride and 2.5 mmol of sodium bicarbonate in 5 mL ethanol at room temperature for 12 hours. After 12 hours, the reaction mixtures were sonicated at 50°C for 30 to 60 minutes and then filtered to remove sodium bicarbonate and diluted by adding ethanol. And then the resulting solutions were directly injected into the HPLC. Chromatography was performed at room temperature and a flow rate of 1 mL/min; 20-40% 2-propanol/hexane (V/V) were used as mobile phases. UV and fluorescence detectors were connected on-line with detection wavelength of UV 337 nm in HP 1100 series of HPLC system and fluorescence excitation 470 nm and emission 530 nm with HP 1046A fluorescence detector<sup>[9-12]</sup>.

### 3. Results and Discussion

For normal HPLC analytes, a new convenient, non-aqueous derivatization method of  $\alpha$ -amino acid esters as NBD derivatives was developed. The NBD derivatives were prepared by reacting NBD chloride and  $\alpha$ -amino

acid methyl ester HCl with sodium bicarbonate in ethanol at room temperature. For optimized non-aqueous derivatization, several solvents (methanol, ethanol, 2-propanol and acetonitrile) and bases (sodium bicarbonate, sodium carbonate, sodium borate, triethylamine and 1,8-diazabicycloundec-7-ene) were investigated and, finally, sodium bicarbonate in ethanol for reaction condition was used. Although the reaction in methanol was the fastest, it provided the by-product of methoxy NBD. Especially, the use of other bases gave the unexpected impurity during preparation process. After NBD derivatization with sodium bicarbonate in ethanol, the reaction mixture was filtered to remove the solid base and then the resulting solution was used.

Tables 1-6 show the chromatographic results for enantiomer separation of several  $\alpha$ -amino acid methyl esters as NBD derivatives on six covalently bonded CSPs derived from polysaccharides (Chiralpak IA, Chiralpak IB, Chiralpak IC, Chiralpak ID, Chiralpak IE and Chiralpak IF). Chiralpak IA in Table 1 showed the greatest enantioselectivity, while Chiralpak ID in Table 4 showed the smallest. In addition to Chiralpak IA,

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

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

Mobile phase: 25% 2-propanol/hexane (V/V);  $\alpha$ : Separation factor,  $k'_1$ : Capacity factor of first eluted enantiomer,  $R_s$ : resolution factor; \*indicates the absolute configuration of the second eluted enantiomer.

**Table 2.** Separation of enantiomers of  $\alpha$ -amino acid methyl esters as NBD derivatives on Chiralpak IB

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

Mobile phase: 25% 2-propanol/hexane (V/V);  $\alpha$ : Separation factor,  $k'_1$ : Capacity factor of first eluted enantiomer, Rs: resolution factor; \*indicates the absolute configuration of the second eluted enantiomer.

**Table 3.** Separation of enantiomers of  $\alpha$ -amino acid methyl esters as NBD derivatives on Chiralpak IC

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

Mobile phase: 40% 2-propanol/hexane (V/V);  $\alpha$ : Separation factor,  $k'_1$ : Capacity factor of first eluted enantiomer, Rs: resolution factor; \*indicates the absolute configuration of the second eluted enantiomer.

most analytes were separated in Chiralpak IB or Chiralpak IE but the resolution factor and separation factor is not as good as that on Chiralpak IA. The degree of enantioselectivity for six CSPs is as follows; Chiralpak IA > Chiralpak IE > Chiralpak IB > Chiralpak IF ~ Chiralpak IC > Chiralpak ID. Especially, all investigated analytes were base-line separated on Chiralpak IA ( $\alpha$  = 1.11-2.21, Rs = 1.54-9.10) and on Chiralpak IE ( $\alpha$  = 1.08-1.95, Rs = 1.16-10.43). Consistently, the L-isomers of all investigated analytes are secondly eluted on Chiralpak IA in Table 1 and Chiralpak IB in Table 2, except for phenylalanine methyl ester on Chiralpak IB. On the contrary, the D-isomers of all resolved analytes are sec-

**Table 4.** Separation of enantiomers of  $\alpha$ -amino acid methyl esters as NBD derivatives on Chiralpak ID

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

Mobile phase: 20% 2-propanol/hexane (V/V);  $\alpha$ : Separation factor,  $k'_1$ : Capacity factor of first eluted enantiomer, Rs: resolution factor; \*indicates the absolute configuration of the second eluted enantiomer.

**Table 5.** Separation of enantiomers of  $\alpha$ -amino acid methyl esters as NBD derivatives on Chiralpak IE

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

Mobile phase: 40% 2-propanol/hexane (V/V);  $\alpha$ : Separation factor,  $k'_1$ : Capacity factor of first eluted enantiomer, Rs: resolution factor; \*indicates the absolute configuration of the second eluted enantiomer.

ondly eluted on Chiralpak IE in Table 5 and Chiralpak IF in Table 6, except for serine methyl ester on Chiralpak IE. Therefore, the use of Chiralpak IA and Chiralpak IE is complementary to check the trace amount of the enantiomer of the analyte<sup>[11]</sup>, because Chiralpak IA in Table 1 and Chiralpak IE in Table 5 provide good base-line resolution for all analytes with the opposite elution order, except for serine methyl ester.

The stability test of optical purity for analytes after NBD derivatization of  $\alpha$ -amino acid methyl esters was performed. Table 7 shows stability test results of optical purity for NBD derivatives of L-methionine methyl ester (Sigma-Aldrich) after NBD derivatization in

**Table 6.** Separation of enantiomers of  $\alpha$ -amino acid methyl esters as NBD derivatives on Chiralpak IF

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

Mobile phase: 20% 2-propanol/hexane (V/V);  $\alpha$ : Separation factor,  $k'_1$ : Capacity factor of first eluted enantiomer, Rs: resolution factor; \*indicates the absolute configuration of the second eluted enantiomer.

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

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

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

<sup>a</sup>Average value of three times determined.

<sup>b</sup>Relative standard deviation.

ethanol on Chiralpak IA. During 20 days of storage at 4°C, its optical purity was almost unaffected, showing

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

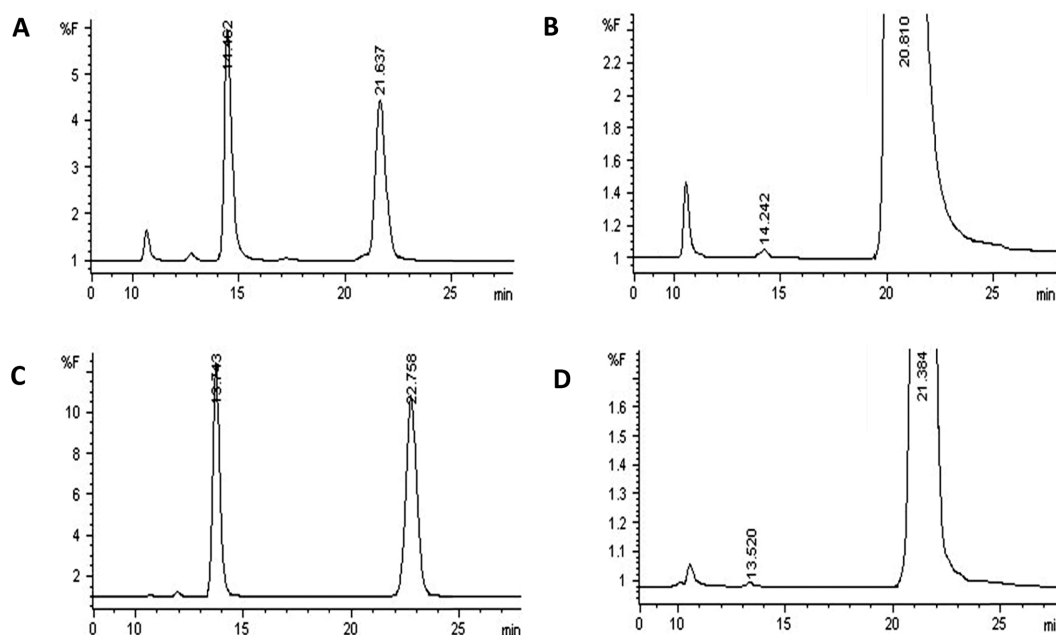
Sample	Company	D : L ratio <sup>a</sup>
L- Alanine methyl ester	Sigma-Aldrich	0.02 : 99.98
L- Leucine methyl ester	Sigma-Aldrich	0.09 : 99.91
L-Methionine methyl ester	Sigma Aldrich	0.05 : 99.95
L- Norleucine methyl ester	Chem-Impex International	0.66 : 99.34
L- Phenylalanine methyl ester	Advanced ChemTech	0.04 : 99.96
L-Phenylglycine methyl ester	Sigma-Aldrich	1.73 : 98.27

<sup>a</sup>Average value of three times determined.

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

#### 4. Conclusion

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



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

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