2007 年 8 月

博士學位論文

Enantiomer Resolution of α-Amino Acids and *N*-Protected α-Amino Acids on Chiral Stationary Phases by HPLC

朝 鮮 大 學 校 大 學 院

藥 學 科

金 京 玉

Enantiomer Resolution of α-Amino Acids and *N*-Protected α-Amino Acids on Chiral Stationary Phases by HPLC

키랄고정상에서 HPLC를 이용한 α-아미노산 및 *N*-protected α-아미노산유도체들의 광학분할

2007 年 8 月 24 日

朝鮮大學校 大學院

藥 學 科

金 京 玉

Enantiomer Resolution of α-Amino Acids and *N*-Protected α-Amino Acids on Chiral Stationary Phases by HPLC

指導敎授　　李 元 宰

이 論文을 藥學 博士學位申請 論文으로 提出함.

2007 年　4 月

朝鮮大學校 大學院

藥學科

金　京　玉

金京玉의 博士學位論文을 認准함

委員長　朝鮮大學校 教授　白采善　印

委　員　朝鮮大學校 教授　高玉鉉　印

委　員　朝鮮大學校 教授　李範揆　印

委　員　朝鮮大學校 教授　洪俊憙　印

委　員　朝鮮大學校 教授　李元宰　印

2007年　6月

朝鮮大學校 大學院

Contents

List of Tables

List of Figures

ABSTRACT

Enantiomer Resolution of α-Amino Acids and *N*-Protected α-Amino Acids on Chiral Stationary Phases by HPLC

Jin, JingYu
Advisor: Prof. Wonjae Lee, Ph.D.
Department of Pharmacy
Graduate School of Chosun University

Liquid chromatographic comparisons for enantiomer resolution of α-amino acids and chiral primary amino compounds were made using chiral stationary phases (CSPs) prepared by covalently bonding (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (18-C-6-TA) of the same chiral selector. The resolution of all α-amino acids on CSP 1 developed in our group was found to be better than that on CSP 2 reported by Machida et al. All α-amino acids examined in this study were well enantioseparated on CSP 1 (α = 1.22–2.47), while four analytes were not resolved or all the other analytes were poorly resolved on CSP 2 than on CSP 1. However, in resolving the primary amino compounds without a carbonyl group, CSP 1 was comparable with CSP 2. Although (+)-18-C-6-TA of the same chiral selector was used to prepare CSP 1 and CSP 2, this study showed that different connecting methods for the CSPs might influence their ability to resolve the analytes depending on their structures related to the chiral recognition mechanism.

Chiral stationary phases (CSPs), CSP 1 and CSP 1', with a reverse stereochemical configuration, were prepared by covalently bonding (+)-and (−)-18-C-6-TA to silica gel, respectively. These CSPs were used to resolve the enantiomers of α-amino acids and primary amino compounds, affording reasonable and quite similar resolution behaviors except for the elution orders. The elution orders of the two enantiomers for the resolution of α-amino acids and other primary amino compounds were always opposite on the two CSPs. The reverse elution orders on the two CSPs with the antipode of the chiral selector were demonstrated to be very useful in the determination of the enantiomeric purity of optically enriched analytes.

The liquid chromatographic enantiomer separation of several *N*-hydrazide derivatives of 2-aryloxypropionic acids was performed on a crown ether type chiral stationary phase (CSP) derived from (18-C-6-TA). The behavior of chromatographic parameters by the change of mobile phases and additives for the resolution of these analytes was investigated. The enantiomers of all analytes were base-line resolved in the mobile phase of 100% methanol containing 20 mM H_2SO_4 as an acid additive. These results using the crown ether derived CSP are the first reported for enantiomer resolution of chiral acids of 2-aryloxypropionic acids as their *N*-hydrazide derivatives.

Liquid chromatographic comparisons for enantiomer resolution of *N*-fluorenylmethoxycarbonyl (FMOC) α-amino acids with fluorescence detection were made on covalently bonded type chiral stationary phases (CSPs) (Chiralpak IA and Chiralpak IB) and coated type CSPs (Chiralpak AD and Chiralcel OD) derived from polysaccharide derivatives of the same chiral selectors. This is the first study reported of enantiomer resolution with fluorescence detection on covalently bonded type CSPs, Chiralpak IA and Chiralpak IB. In general, covalently bonded type CSPs (Chiralpak IA and Chiralpak IB) showed lower enantioseparation than coated type CSPs (Chiralpak AD and Chiralcel OD) for enantiomer resolution of these analytes, respectively. Owing to higher sensitivity and broader solvent compatibility in fluorescence detection on Chiralpak IA and Chiralpak IB than in UV detection, however, this analytical method is expected to enlarge their application of enantiomer resolution, such as an online HPLC monitoring of asymmetric synthesis.

The liquid chromatographic enantiomer separation of *N*-FMOC protected α-amino acids ethyl ester derivatives was performed on polysaccharide-derived chiral stationary phases, covalently bonded type chiralpak IA and coated type Chiralpak AD. Although Chiralpak IA showed slightly lower enantioselectivity than Chiralpak AD, most of *N*-FMOC α-amino acids ethyl esters enantiomers were base-line separated on Chiralpak IA and Chiralpak AD. Owing to the compatibility with a broad range of solvents and column safety of Chiralpak IA, it is expected to enlarge its new application of enantiomer separation. Especially, it is expected to be useful for preparative separations, because the halogenated solvent like chloroform or methylene chloride shows often better solubility than the other solvents.

The liquid chromatographic enantiomer separation of *N*-phthaloyl (PHT) protected α-amino

acids on several coated and immobilized chiral stationary phases (CSPs) derived from polysaccharide derivatives was performed. The coated CSP of Chiralpak AD showed more or less enantioseparation than the covalently bonded CSP of Chiralpak IA with the same chiral selector of amylose tris(3,5-dimethylphenylcarbamate). However, the coated Chiralcel OD showed greater enantioseparation than the covalently bonded Chiralpak IB with the same chiral selector of cellulose tris(3,5-dimethylphenylcarbamate). Among all examined CSPs, Chiralcel OD afforded the greatest performance for enantiomer resolution of *N*-PHT α-amino acids and, therefore, all analytes enantiomers were base-line separated on Chiralcel OD. The chromatographic method developed in this study was usefully applied for determination of the enantiomeric purity of commercially available *N*-PHT α-amino acids analytes.

국문초록

키랄고정상에서 HPLC를 이용한 α-아미노산 및 *N*-protected α-아미노산유도체들의 광학분할

김경옥

지도교수: 이원재

조선대학교 대학원 약학과

액체크로마토그래피를 이용한 α-아미노산과 키랄 1차 아미노화합물의 광학이성질체의 분리를 위하여 키랄선택자 (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (18-C-6-TA)을 silica gel에 공유결합시킨 키랄고정상(CSPs)을 사용하였다. 연구결과 모든 α-아미노산은 키랄고정상(CSP 1)에서 Machida et al.등이 연구한 키랄고정상(CSP 2)에서보다 분리결과가 더 좋은 것으로 나타났다. α-아미노산의 광학이성질체는 α = 1.22–2.47로써 아주 이상적으로 분리되었다. 이와 반면에 Machida et al.등이 연구한 α-아미노산의 분석물질 중 4개 분석물질은 분리가 되지 않았고 기타 분석물질도 모두 CSP 1에서보다 분리결과가 낮음을 알 수 있다. 그러나 카르보닐기 그룹이 없는 1차 아미노화합물은 CSP 1와 CSP 2에서 비슷한 분리결과를 가져다 주었다. 두개의 키랄고정상은 동일한 (+)-18-C-6-TA 키랄선택자를 갖고 있지만 그들의 연결 방법의 다름에 따라 그들의 화학구조와 관련된 키랄 인식 메커니즘이 다르며 따라서 광학이성질체 분리결과에도 영향을 줌을 알 수 있다.

키랄고정상 CSP 1 와 CSP 1'는 서로 반대의 입체화학 원자배열을 갖고 있으며 각각 (+)-와 (–)-18-C-6-TA 키랄선택자를 silica gel에 공유결합시킨 것이다. α-아미노산과 1차 아미노화합물의 광학이성질체는 키랄고정상 CSP 1와 CSP 1'에서 분리 할 수 있으며 분리순서를 제외한 기타 광학이성질체 분리 효과는 유사하게 나타났다. α-아미노산과 1차 아미노 화합물의 광학이성질체 분리 순서는 정반대로

나타나며 이 결과는 정확한 광학이성질체 순도를 필요로 하는 키랄물질의 분석에 아주 유용하게 응용될 것이다.

액체크로마토그래피를 이용한 2-아릴록시프로피온산의 *N*-히드라진 유도체들의 광학이성질체 분리를 위하여 18-C-6-TA에서 유도된 크라운 에테르 타입의 키랄고정상을 사용하였다. 이동상 및 이동상에 첨가한 물질을 변경시키면서 크로마토그래피 변화를 관찰하였다. 모든 분석물질은 20 mM 황산을 포함한 100% 메탄올에서 base-line을 유지하면서 완전히 분리 되었다. 본 연구는 최초로 2-아릴록시프로피온산에 아미노기를 반응시켜 광학이성질체의 분리를 보고한 것이다. 이 결과는 아미노기를 가지고 있지 않는 키랄 카르복실산 분석물질에서 아미노기를 반응시켜 첨가함으로써 키랄고정상에서 이들의 광학이성질체 분리를 할 수 있음을 보여주고 있다.

액체크로마토그래피를 이용한 *N*-fluorenylmethoxycarbonyl (FMOC) α-아미노산의 광학이성질체의 분리를 위하여 키랄고정상으로는 다당유도체에서 유도된 공유결합타입의 키랄고정상(Chiralpak IA, Chiralpak IB)과 코팅타입의 키랄고정상 (Chiralpak AD, Chiralcel OD)을 사용하였으며 형광검출기를 사용하여 연구하였다. 본 연구는 최초로 공유결합의 키랄고정상에서 형광검출기를 사용하여 광학이성질체의 분리를 보고한 것이다. 연구결과 공유결합의 키랄고정상(Chiralpak IA, Chiralpak IB)에서는 대응되는 코팅타입의 키랄고정상(Chiralpak AD, Chiralcel OD)에 비해 광학이성질체 분리가 모두 낮아졌다. 형광검출기는 자외선검출기에 비해 감도가 높으며, 또한 Chiralpak IA와 Chiralpak IB는 이동상을 광범위하게 사용할 수 있는 장점을 갖고 있다. 본 연구의 분석방법은 비대칭 합성의 온라인 고속액체크로마토그래프 모니터링에 응용될 수 있는 바와 같이 광학이성질체의 분리를 위해 더 광범위하게 응용될 것으로 기대된다.

액체크로마토그래피를 이용한 *N*-FMOC α-아미노산 에틸에스터 유도체의 광학이성질체 분리를 위하여 다당유도체에서 유도된 공유결합타입의 키랄고정상(Chiralpak IA)과 코팅타입의 키랄고정상(Chiralpak AD)을 사용하여 비교하였다. 일반적으로 공유결합의 키랄고정상인 Chiralpak IA에서 코팅타입의

키랄고정상인 Chiralpak AD에서보다 광학이성질체 분리가 낮은 것으로 나타났다. 대부분의 *N*-FMOC α-아미노산 에틸에스터 유도체들은 Chiralpak IA와 Chiralpak AD에서 base-line을 유지하면서 분리되었다. Chiralpak IA는 공유결합의 키랄고정상이므로 이동상을 광범위하게 사용할 수 있고 컬럼의 안정성으로 인하여 광학이성질체의 분리에 더 광범위하게 응용될 것으로 기대된다. 특히 할로겐화 용매, 예를 들어 클로로포름, 염화메틸렌등을 이동상 용매로 사용하는 경우에도 광학분할을 할 수 있음을 보여주고 있다.

액체크로마토그래피를 이용한 *N*-프탈릴 α-아미노산의 광학이성질체의 분리를 위하여 다당유도체에서 유도된 키랄고정상을 사용하여 비교하였다. 일반적으로 동일한 아밀로오스 tris(3,5-dimethylphenylcarbamate) 키랄 선택자를 갖고 있는 코팅타입의 키랄고정상인 Chiralpak AD와 공유결합의 키랄고정상인 Chiralpak IA을 비교하면 Chiralpak AD에서는 Chiralpak IA에서보다 광학이성질체 분리가 낮은 것으로 나타났다. 그러나 동일한 셀루로오스 tris(3,5-dimethylphenylcarbamate) 키랄 선택자를 갖고 있는 코팅타입의 키랄고정상인 Chiralcel OD에서는 코팅타입의 키랄고정상인 Chiralpak IB에서보다 광학이성질체 분리가 크게 나타났다. 본 연구에서 사용된 키랄고정상중에서 *N*-프탈릴 α-아미노산은 Chiralcel OD에서 광학이성질체의 분리결과가 가장 이상적으로 나타났으며 또한 모든 분석물질은 base-line을 유지하면서 분리 되었다. 본 연구에서 개발된 크로마토그래피 방법은 상업적으로 쉽게 얻을 수 있는 *N*-프탈릴 α-아미노산의 광학이성질체의 순도 검출을 위하여 매우 유용하게 적용될 수 있다.

Chapter Ⅰ

Comparison of Enantiomer Separation on Two Chiral Stationary Phases Derived from (+)-18-Crown-6-2,3,11,12-Tetracarboxylic Acid of the Same Chiral Selector

ABSTRACT

Liquid chromatographic comparisons for enantiomer resolution of α-amino acids and chiral primary amino compounds were made using chiral stationary phases (CSPs) prepared by covalently bonding (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (18-C-6-TA) of the same chiral selector. The resolution of all α-amino acids on CSP 1 developed in our group was found to be better than that on CSP 2 reported by Machida et al. All α-amino acids examined in this study were well enantioseparated on CSP 1 (α = 1.22–2.47), while four analytes were not resolved or all the other analytes were poorly resolved on CSP 2 than on CSP 1. However, in resolving the primary amino compounds without a carbonyl group, CSP 1 was comparable with CSP 2. Although (+)-18-C-6-TA of the same chiral selector was used to prepare CSP 1 and CSP 2, this study showed that different connecting methods for the CSPs might influence their ability to resolve the analytes depending on their structures related to the chiral recognition mechanism.

Keywords: chiral stationary phases, enantiomer separation, 18-crown-6-tetracarboxylic acid

1.1. INTRODUCTION

Chiral crown ethers, a class of synthetic host molecules, have aroused considerable interest since Cram and his co-workers reported the chiral discrimination studies of host-guest complexation.[1,2] Many studies using crown ethers as chiral selectors have been performed to resolve racemic α-amino acids and primary amines by liquid-liquid extraction and high-performance liquid chromatography.[3,4] For the liquid chromatographic resolution of α-amino acids and the chiral primary amino compounds, a commercial Crownpak CR column based on the chiral crown ether has been developed and widely used.[5,6] Among the several chiral crown ether derivatives, (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (18-C-6-TA) has been utilized in capillary electrophoresis to resolve the enantiomers of the α-amino acids and chiral primary amines (Fig. 1.1).[7-10] Subsequent studies on the application of the same chiral selector of (+)-18-C-6-TA in HPLC have been performed. In our group a chiral stationary phase (CSP 1) prepared by covalently bonding (+)-18-C-6-TA to the aminopropyl silica gel was developed and applied to the chromatographic resolution of several racemic α-amino acids and primary amino compounds including the quinolone antibacterials by HPLC (Fig. 1.2).[11-16] Machida et al. also developed a covalently bonded CSP (CSP 2) derived from the same chiral selector of (+)-18-C-6-TA using different connecting methods (Fig. 1.1 and 1.2).[17,18] Recently, for the chiral recognition mechanism between (+)-18-C-6-TA as a chiral selector and an α-amino acid in the solution state detailed NMR studies were reported.[19] In this study we compare the chromatographic results of two CSPs prepared from (+)-18-C-6-TA of the same chiral selector using different connecting methods for resolution of the analytes depending on their structures related to the chiral recognition.

1.2. EXPERIMENTAL

Chromatography was performed at room temperature using an HPLC Breeze system consisting of a Waters model 1525 binary pump, a Rheodyne model 7125 injector with a 20 μL loop, a dual absorbance detector (Waters 2487 detector). CSP 1 was prepared by bonding (+)-18-C-6-TA to aminopropyl silica gel [Kromasil, EKA Chemicals, 100 , 5 μm; Micro analysis, found: C, 6.30%, N, 1.72%, calculated: 1.22 mmol/g (based on N)] via the previously reported method (Fig. 1.1).[12] The modified silica [Microanalysis, found: C, 9.30%, N, 1.32%, calculated: 0.30 mmol/g (based on C)] for CSP 1 was slurry packed into a 250 × 4.6 mm stainless steel column. HPLC-grade methanol was obtained from J.T. Baker. Water was purified using a milli-Q water purification system (Bedford, MA, USA). Sulfuric acid was obtained from Fluka Company (Switzerland). All chromatography samples were commercially available from Aldrich and Sigma.

1.3. RESULTS AND DISCUSSION

Table 1.1 shows the comparative results of the enantiomer resolution of the α-amino acids on CSP 1 and CSP 2 derived from the (+)-18-C-6-TA of the same chiral selector. All the α-amino acids used in this study were base-line resolved with good separation factors (α = 1.22-2.47) on CSP 1. The observed enantioselectivities and resolution factors on CSP 1. were superior to those on CSP 2. In particular, the enantiomers of the four analytes (entry 3, 4, 7, 15) that failed to resolve on CSP 2 were separated on CSP 1 with reasonable separation factors (α = 1.22–1.64). [17] The elution orders were determined by injecting the pure enantiomer with a known configuration except for 1-naphthylglycine (entry 19). All the R-enantiomers of the α-amino acids used in this study were selectively retained on the CSP 1 derived from (+)-18-C-6-TA. Although the elution order of the 1-naphthylglycine enantiomers was not determined, it is expected that the R-enantiomer will be selectively retained on the CSP 1 using a similar chiral recognition mechanism between the chiral crown ether on CSP 1 and the 1-naphthylglycine analyte. It was reported that the R-enantiomers of α-amino acids eluted prior to the S-enantiomers on Crownpak CR(+), the chiral crown ether type CSP.[5,6] Since the elution orders of the enantiomers of the α-amino acids on the Crownpak CR(+) were the opposite on CSP 1, it might be said that the CSP 1 derived from (+)-18-C-6-TA is equivalent to Crownpak CR(−) in terms of the elution order. Therefore, the elution orders mentioned by Machida et al. that (+)-18-C-6-TA derived CSP 2 is similar to Crownpak CR(+) was not correctly described.[17]

Table 1.2 shows the comparative results of the enantiomeric resolution of the primary amino compounds on CSP 1 and CSP 2. In general, the enantioselectivities and resolution factors on CSP 1 are comparable to those on CSP 2 except for two analytes (entries 1 and 2) possessing a carbonyl group. The alanine-β-naphthylamide enantiomers were greater separated on CSP 1 than on CSP 2 (entry 1). Also, the baclofen enantiomers were base-line separated on CSP 1, while they were not resolved on CSP 2 (entry 2). Compared to the other analytes, primaquine, which has an amino group in the remote δ position from the chiral center, showed fairly lower enantioseparation (entry 8). In general, the enantioselectivities and resolution factors for primary amino compounds lacking a carbonyl group were observed to be lower than those for α-amino acids on CSP 1. The typical chromatograms are shown in Fig. 1.3

As shown in Table 1.1 and Table 1.2, although (+)-18-C-6-TA of the same chiral selector was used to prepare CSP 1 and CSP 2, different connecting methods for these CSPs might influence their ability to resolve the analytes depending on their structures related to the chiral recognition mechanism. The structure of CSP 2 developed by Machida et al. was not clearly defined (Fig.1.2).[17] They reported that the bonding form of the chiral selector of (+)-18-C-6-TA to the silica gel appears to be a monoamide and diamide linking. Complexation of the primary ammonium moiety formed by protonating the α-amino acids under acidic conditions inside the chiral cavity of the 18-crown-6 ring of the CSP is believed to be essential for the chiral recognition.[7] According to our previous study of α-amino acid in the presence of (+)-18-C-6-TA as a chiral selector by NMR spectroscopy and molecular dynamics calculations, these tripod hydrogen bonding interactions were observed in not only the R-enantiomer/18-C-6-TA complex but also in the S-enantiomer/18-C-6-TA complex during chiral recognition.[19] In addition to these hydrogen bonding interactions, we reported that another hydrogen bonding interaction between one carboxylic acid of 18-C-6-TA and the carbonyl oxygen of the R-amino acid was observed to be crucial for effective chiral discrimination.[19] Based on the NMR and molecular dynamics results, Fig. 1.4 shows a schematic representation of the chiral recognition between the CSP 1 derived from (+)-18-C-6-TA and the strongly eluted enantiomer of the R-amino acid. However, the CSP 2 possessing a diamide tether may have a limitation for this type of hydrogen bonding, while CSP 1 is an axially chiral phase with a C2 symmetry structure, favorable for the chiral discrimination interaction of the hydrogen bonding.[20] Consequently, it is considered that the structural feature of CSP 2 might result in the inferior performance of CSP 2 in resolving α-amino acids, compared to CSP 1. On the other hand, such a hydrogen bonding interaction cannot be formed in primary amino compounds without a carbonyl group even on CSP 1. Therefore, it might be explained that the lower enantioselectivities for primary amino compounds without a carbonyl moiety than those for α-amino acids on CSP 1 are attributed to the absence of this hydrogen bonding. However, since alanine-β-naphthylamide and baclofen have a carbonyl group (entry 1 and 2) unlike the other primary amino compounds in Table1.2, it is considered that the greater enantioselectivities for these two analytes on CSP 1 than on CSP 2 are due to the carbonyl group, an essential interaction site for hydrogen bonding with the carboxylic acid of CSP 1.

1.4. CONCLUSION

Two covalently bonded CSP 1 and CSP 2 derived from (+)-18-C-6-TA using the same chiral selector, and prepared by different connecting methods were compared for their ability to resolve the α-amino acids and chiral primary amino compounds. CSP 1 showed a higher enantioselectivity for the resolution of all α-amino acids than CSP 2, while CSP 1 was comparable to CSP 2 in resolving primary amino compounds, lacking a carbonyl group. It is considered that the greater enantioselectivities of the amino acids on CSP 1 than on CSP 2 are due to the hydrogen bonding between one carboxylic acid of 18-C-6-TA and a carbonyl oxygen of amino acid, because CSP 2 with a diamide tether may limit this hydrogen bonding. In general, it is also considered that the lower enantioselectivities for the primary amino compounds without a carbonyl group on CSP 1 than those for α-amino acids are responsible for the absence of this hydrogen bonding.

REFERENCES

1. Kyba, E. B.; Koga, K.; Sousa, L. R.; Siegel, M. G.; Cram, D. J. Chiral recognition in molecular complexing. J. Am. Chem. Soc. **1973**, *95*, 2692–2693.

2. Sousa, L. R.; Hoffman, D. H.; Kaplan, L.; Cram, D. J. Total optical resolution of amino esters by designed host-guest relationships in molecular complexation. J. Am. Chem. Soc. **1974,** *96*, 7100–7101.

3. Zhang, X. X.; Bradshaw, J. S.; Izatt, R. M. Enantiomeric recognition of amine compounds by chiral macrocyclic receptors. Chem. Rev. **1997,** *97*, 3313–3362.

4. Shibukawa, A.; Nakagawa, T. The use of chiral crown ethers in liquid chromatography. In Chiral Separations by HPLC: Applications to Pharmaceutical Compounds, Krstulovic, A. M., Ed.; John Wiley and Sons, New York, 1989.

5. Shinbo, T.; Yamaguchi, T.; Nishimura, K.; Sugiura, M. Chromatographic separation of racemic amino acids by use of chiral crown ether-coated reversed-phase packings. J. Chromatogr. **1987**, *405*, 145–153.

6. Refer to “Application guide for chiral HPLC selection”, 2nd Ed., Daicel Chemical Industries, Ltd.

7. Kuhn, R.; Erni, F.; Bereuter, T.; Hausler, J. Chiral recognition and enantiomeric resolution based on host-guest complexation with crown ethers in capillary zone electrophoresis. Anal. Chem. **1992**, *64*, 2815–2820.

8. Kuhn, R.; Wagner, J.; Walbroehl, Y.; Bereuter, T. Potential and limitation of an optically active crown ether for chiral separation in capillary zone electrophoresis. Electrophoresis **1994,** *15*, 828–834.

9. Kuhn, R.; Riester, D.; Fleckenstein, B.; Wiesmuller, K.-H. Evaluation of an optically active crown ether for the chiral separation of di- and tripeptides, J. Chromatogr. A **1995**, *716* , 371–379.

10. Kuhn, R. Enantiomeric separation by capillary electrophoresis using a crown ether as chiral selector. Electrophoresis **1999**, *20*, 2605–2613.

11. Hyun, M. H.; Jin, J. S.; Lee, W. A new HPLC chiral stationary phase for the direct resolution of racemic quinolone antibacterials containing a primary amino group. Bull. Kor.

Chem. Soc. **1998**, *19*, 819–821.

12. Hyun, M. H.; Jin, J. S.; Lee, W. Liquid chromatographic resolution of racemic amino acids and their derivatives on a new chiral stationary phase based on crown ether. J. Chromatogr. A **1998**, *822*, 155–161.

13. Hyun, M. H.; Jin, J. S.; Koo, H. J.; Lee, W. Liquid chromatographic resolution of racemic amines and amino alcohols on a chiral stationary phase derived from crown ether. J. Chromatogr. A **1999**, *837*, 75–82.

14. Hyun, M. H.; Han, S. C.; Jin, J. S.; Lee, W. Separation of the stereoisomers of racemic fluoroquinolone antibacterial agents on a crown-ether-based chiral HPLC stationary phase. Chromatographia **2000**, *52*, 473–476.

15. Hyun, M. H.; Han, S. C.; Cho, Y. J.; Jin, J. S.; Lee, W. Liquid chromatographic resolution of gemifloxacin mesylate on a chiral stationary phase derived from crown ether. Biomed Chromatogr. **2002**, *16*, 356–360.

16. Lee, W.; Bang, E.; Lee, W. Chiral resolution of diphenylalanine by high-performance liquid chromatography on a crown-ether-based chiral stationary phase and by NMR spectroscopy. Chromatographia **2003,** *57*, 457–461.

17. Machida,Y.; Nishi, H.; Nakamura, K.; Nakai, H.; Sato, T. Enantiomer separation of amino compounds by a novel chiral stationary phase derived from crown ether. J. Chromatogr. A **1998,** *805,* 85–95.

18. Machida, Y.; Nishi, H.; Nakamura, K. Separation of the enantiomers of amino and amide compounds on novel chiral stationary phases derived from a crown ether. Chromatographia **1999,** *49*, 621–627.

19. Bang, E.; Jung, J.-W.; Lee, W.; Lee, D.-W.; Lee, W. Chiral recognition of (18-crown-6)-tetracarboxylic acid as a chiral selector determined by NMR spectrocropy. J. C. S. Perkin Trans. **2001**, *2*, 1685–1692.

20. Tichy, M.; Holanova, J.; Zavada, J. 6,6′-Dinitrobiphenyl-2,2′-dicarboxylic acid ionically bonded to aminopropyl silica: New axially chiral phase of C_2 symmetry for high-performance liquid chromatographic separation of enantiomeric amino alcohol derivatives, J. Chromatogr. A **1994**, *667*, 11–17.

Table1.1 Enantiomer resolution of the α-amino acids on CSP 1 and CSP 2 derived from (+)-18-C-6-TA of the same chiral selector

Entry	Analyte	CSP 1				CSP 2[a]		
		α	k'_1	Rs	Conf.[b]	α	k'_1	Rs
1	Alaning(Ala)	1.35	1.32	1.61	R	1.12	0.60	0.41
2	Arginine(Arg)	1.73	0.70	1.77	R	1.48	0.58	1.30
3	Asparagine(Asn)	1.22	1.04	1.26	R	1.00	0.34	–
4	Aspartic acid(Asp)	1.31	1.04	1.14	R	1.00	0.44	–
5	Histidine(His)	1.49	1.30	1.48	R	1.41	0.39	0.82
6	Leucine(Leu)	1.67	0.41	1.88	R	1.32	0.63	0.89
7	Isoleucine(Ile)	1.46	0.35	1.21	R	1.00	0.39	-
8	Norleucine(Norleu)	1.50	0.60	1.72	R	1.34	0.74	0.92
9	Norvaline(Norval)	1.48	0.65	1.69	R	–	–	–
10	Methionine(Met)	1.65	0.73	2.28	R	1.23	0.90	0.73
11	Phenylglycine(PG)	2.47	1.43	6.98	R	1.91	1.87	2.68
12	Phenylalanine(Phe)	1.75	0.51	2.20	R	1.53	1.22	1.61
13	Tryptophan(Trp)	1.61	0.59	1.78	R	1.41	2.76	1.77
14	Tyrosine(Tyr)	1.58	0.81	2.84	R	1.40	0.94	1.30
15	Valine(Val)	1.64	0.20	1.59	R	1.00	0.39	–
16	α-Amino-n-butyric acid	1.49	0.67	1.75	R	1.16	0.44	0.47
17	DOPA	1.63	0.95	2.76	R	1.44	0.80	1.33
18	5-Hydroxytryptophan	1.59	0.66	1.66	R	–	–	–
19	1-Naphthylglycine	1.49	2.24	2.94		–	–	–

Mobile phase = 80% methanol in water (v/v) containing 10 mM H_2SO_4; Flow rate = 1 mL/min; UV detection at 210 nm.

[a] Data taken from reference 17.

[b] The configuration of the second eluted isomer.

Table1.2 Enantiomer resolution of the primary amino compounds on CSP 1 and CSP 2 derived from the (+)-18-C-6-TA of the same chiral selector

Entry	Analyte	CSP 1				CSP 2[a]		
		α	k'_1	Rs	Conf.[b]	α	k'_1	Rs
1	Alanine-β-naphthylamide	1.71	4.01	4.11	R	1.49	2.80	2.23
2	Baclofen	1.29	8.00	2.05	S	1.00	8.63	–
3	α-Methylbenzylamine	1.30	2.90	2.37	S	–	–	–
4	α-Methyltryptamine	1.12	3.69[c]	0.63		1.07	3.00	0.48
5	Mexiletine	1.20	0.82	1.43	–	–	–	
6	1-(1-Naphthyl)ethylamine	1.27	2.68	2.10	R	1.48	2.69	2.13
7	Norephedrine	1.35	0.39[d]	1.56	1S,2R	1.38	1.54	1.39
8	Primaquine	1.10	1.37[e]	0.28		1.10	8.38	0.62

Mobile phase = 80% methanol in water (v/v) containing 10 mM H_2SO_4; Flow rate = 1 mL/min; UV detection at 210 nm.

[a] Data taken from reference 17.

[b] The configuration of the second eluted isomer.

[c,d] 100% and 30% methanol in water (v/v) containing 10 mM H_2SO_4 were used as a mobile phase, respectively.

[e] 10 mM H_2SO_4 aqueous solution as a mobile phase.

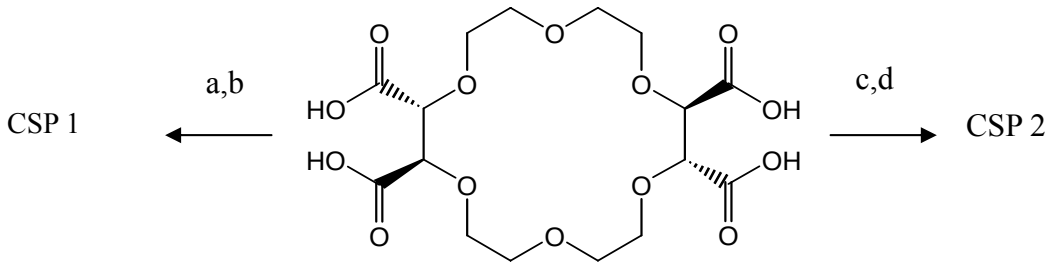


Figure 1.1 Two chiral stationary phases derived from the same chiral selector of (+)-18-C-6-TA: (a) acetyl chloride (b) aminopropyl silica gel, triethylamine (c) EEDQ, aminopropyl silica gel (d) acetic anhydride.

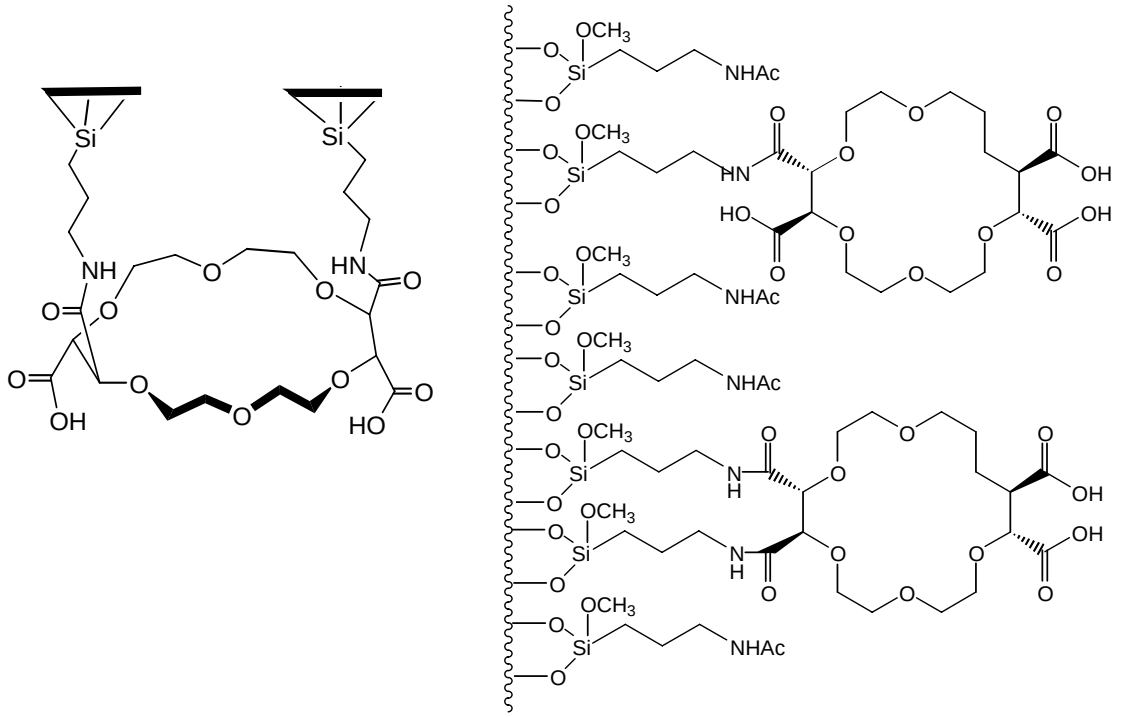


Figure 1.2 The structures of CSP 1 (left) and CSP 2 (right).

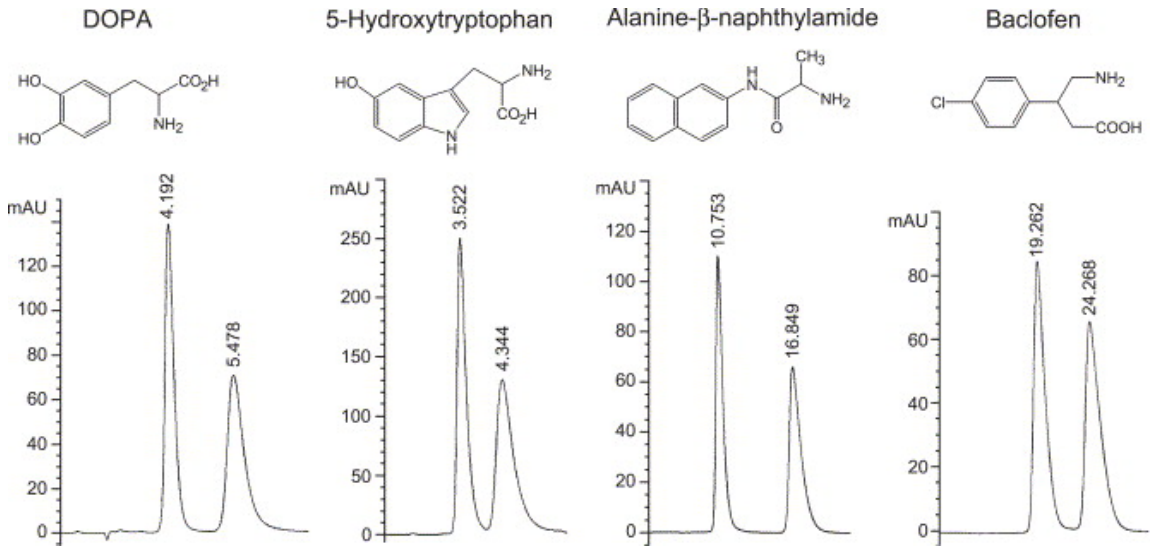


Figure 1.3 Chromatograms of the enantiomer separation on the CSP 1 derived from (+)-18-C-6-TA; See Tables for chromatographic conditions.; Injected amount 0.5−5 μg.

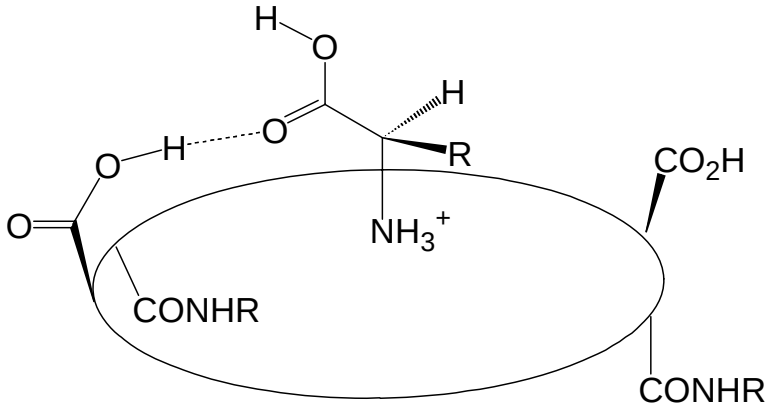


Figure 1.4 Schematic representation of the chiral recognition between the CSP 1 derived from (+)-18-C-6-TA and the strongly eluted enantiomer of the R-amino acid, showing intermolecular hydrogen bonding (dotted line) between the chiral selector and the carbonyl oxygen of the R-amino acid.

Chapter Ⅱ

Development of the Antipode of the Covalently-Bonded Crown Ether Type Chiral Stationary Phase for the Advantage of the Reversal of Elution Order

ABSTRACT

Chiral stationary phases (CSPs), CSP 1 and CSP 1', with a reverse stereochemical configuration, were prepared by covalently bonding (+)-and (−)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (18-C-6-TA) to silica gel, respectively. These CSPs were used to resolve the enantiomers of α-amino acids and primary amino compounds, affording reasonable and quite similar resolution behaviors except for the elution orders. The elution orders of the two enantiomers for the resolution of α-amino acids and other primary amino compounds were always opposite on the two CSPs. The reverse elution orders on the two CSPs with the antipode of the chiral selector were demonstrated to be very useful in the determination of the enantiomeric purity of optically enriched analytes.

Keywords: Chiral stationary phases, Enantiomer separation, 18-Crown-6-tetracarboxylic acid, Reversal of elution order

2.1. INTRODUCTION

Chiral stationary phases (CSPs) derived from chiral crown ethers have attracted considerable interest since Cram and his co-workers first introduced chiral crown ether type CSPs immobilized on a silica gel or on polystyrene in the 1970's.[1,2] Many studies using crown ethers as chiral selectors have been used to resolve racemic α-amino acids and primary amines by liquid-liquid extraction and high-performance liquid chromatography.[3,4] Among the several chiral crown ethers developed by Cram, (3,3'-diphenyl-1,1'-binaphthyl)-20-crown-6 as a chiral selector was dynamically coated on an octadecylsilica gel to afford the Crownpak CR CSP.[5] The Crownpak CR column of the chiral crown ether type has proven to be useful for the chromatographic resolution of not only amino acids but also primary amino compounds.[5-8] However, since the Crownpak CR is prepared by a dynamic coating of a chiral crown ether on a reversed-phase packing, it has an intrinsic drawback in the use of mobile phase.[5,6] The use of a mobile phase containing more than 15% methanol deteriorates the CSP performance due to leaching of the chiral crown ether selector. In particular, hydrophobic analytes have significantly long retention times.[7,8] Moreover, the use of other organic solvents except methanol is not permitted because they can compromise the safety of the column, which cause the column to over-pressurize and lose capacity. Recently, CSPs derived from (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (18-C-6-TA), which was first prepared by Behr and co-workers,[9] have been developed.[10-15] The covalently-bonded type column, CSP 1, shown in Fig. 2.1, has been successfully used to resolve the enantiomers of primary amines as well as α-amino acids.[11-14] In particular, since CSP 1 is prepared by covalently bonding (+)-18-C-6-TA to an aminopropyl silica gel, this crown ether type chiral column has the advantage of the use of a variety of mobile phases along with the wide applicability of the hydrophobic analytes.[13,14] More recently, detailed NMR studies on the chiral recognition mechanism between (+)-18-C-6-TA as a chiral selector and α-amino acid in the solution state were reported.[16] However, in the determination of enantiomeric composition of optically active primary amino compounds containing very small amount of second eluting enantiomer on CSP 1, some difficulties have been sometimes encountered because the small second HPLC peaks are sometimes embedded in the first big peaks. To solve these difficulties, the reversal of the elution orders of the two enantiomers is absolutely required.[17,18] In an affort to solve these

difficuties, in this study, we report the preparation of a new CSP 1' by covalently bonding (−)-18-C-6-TA and the comparison of the enantiomer resolutions on CSP 1 and CSP 1'.

2.2. EXPERIMENTAL

CSP 1 and CSP 1' were prepared by bonding (+)- and (−)-18-C-6-TA to aminopropyl silica gel, [Kromasil, EKA Chemicals, 100 , 5 μm; Micro analysis, found: C, 6.30%, N, 1.72%, calculated: 1.22 mmol/g (based on N)] respectively, as described previously.[11,12] The modified silica gels [Microanalysis, found: C, 8.70%; N 1.03% calculated: 0.28 mmol/g (based on C)] for CSP 1 and [Microanalysis, found: C, 9.02%; N 1.20% calculated: 0.29 mmol/g (based on C)] for CSP 1' were slurry packed into 250 × 4.6 mm stainless steel column, respectively. Chromatography was performed at room temperature using an HPLC Breeze system consisting of a Waters model 1525 binary pump, a Rheodyne model 7125 injector with a 20 μL loop, and a dual absorbance detector (Waters 2487 detector). The HPLC-grade methanol was obtained from J.T. Baker. Water was purified using a milli-Q water purification system (Bedford, MA, USA). Sulfuric acid was obtained from the Fluka Company (Switzerland). All the analytes were obatined from either Aldrich or Sigma.

2.3. RESULTS AND DISCUSSION

For the purpose of comparison, CSP 1 and CSP 1' were prepared under an identical condition by covalently bonding (+)-and (−)-18-C-6-TA to an aminopropyl silica gel, respectively, as reported previously (Fig. 2.1).[11,12] Each 18-C-6-TA was converted into its dianhydride by treating it with acetyl chloride. In order to prepare the modified silica gel, the dianhydride compound was treated in dry methylene chloride at 0℃ under nitrogen with triethylamine and 3-aminopropyl silica gel. The CSP 1 and CSP 1' columns packed with the covalently bonded silica gels prepared from (+)-and (−)-18-C-6-TA, respectively, were used to resolve several α-amino acids and primary amino compounds.

Tables 2.1 shows the chromatographic results of the enantiomeric resolution of α-amino acids on CSP 1 and CSP 1'. All α-amino acids used in this study were base-line resolved with good separation factors (α = 1.27–2.74) on CSP 1 and CSP 1'. The separation factors for the α-amino acids on CSP 1 were similar to those on CSP 1'. Probably, the longer retention times on CSP 1' might be due to the slightly higher loading of the chiral selector to the aminopropyl silica gel in CSP 1'. The elution orders were determined by injecting configurationally known enantiomers. The elution orders of the α-amino acids shown in Table 2.1 were consistent, the (D)-enantiomers of all the analytes investigated in this study being selectively retained on CSP 1 derived from (+)-18-C-6-TA while the (L)-enantiomers being selectively retained on CSP 1' derived from (−)-18-C-6-TA, with the exception of serine, threonine (entry 10 and 11 in Table 2.1) and serine and threonine methyl esters.

Since both threonine and serine analytes have a hydroxy group on the β-carbon, it is considered that the β-hydroxy moiety might directly influence the chiral recognition interaction with the crown ether as a chiral selector. Based on our previous NMR results and molecular dynamic calculations, it is considered that much stronger hydrogen bonding between the β-hydroxy moiety of threonine (or serine) and the COOH of 18-C-6-TA instead of the hydrogen bonding between the carbonyl oxygen of threonine (or serine) and the COOH of 18-C-6-TA might be formed.[16] This hydrogen bonding interaction between the β-hydroxy moiety of threonine (or serine) and the COOH of 18-C-6-TA, which is meant to be a favorable interaction in the spatial disposition upon forming the diastereomeric complex could reverse

the elution orders of these analytes. The separation factors (α = 1.30, 1.30) of the enantiomers of threonine and its methyl ester were observed to be lower than those (α = 2.14, 3.10) of serine and its methyl ester on CSP 1'. It is possible that the interference of the methyl group on the β-carbon in the threonine structure with the chiral interaction between threonine and 18-C-6-TA might be responsible for these reduced enantioselectivities.

Table 2.2 shows the enantiomer resolution of some primary amino compounds on CSP 1 and CSP 1'. All the primary amino compounds investigated in this study were well resolved except for the kynurenine and α-methyltryptamine analytes (entry 3 and 5). Generally, CSP 1' for all the analytes shows a slightly higher enantioselectivity with increased retention times than CSP 1. Again the elution orders of the examined analytes are exactly opposite on the two CSPs and the typical examples of the reversal of elution order on CSP 1 and CSP 1' are demonstrated in Fig. 2.2 and Fig. 2.3 The chromatograms for the determination of the enantiomeric purity of enantiomerically enriched thyroxine (L:D = 90:1) and DOPA samples (L:D = 100:1) on CSP 1 and CSP 1' are shown in Fig. 2.2 and Fig.2.3, respectively. The (D)-enantiomers are strongly retained on the CSP 1 derived from (+)-18-C-6-TA, while the (L)-enantiomers are strongly retained on the CSP 1' derived from (−)-18-C-6-TA. Especially, as shown in Fig.2.3, the second small peak corresponding to (D)-DOPA is slightly embed in the first big peak on CSP 1 and consequently the exact determination of the enantiomeric purity has some difficulty.[17,18] In contrast, the two peaks on CSP 1' are well separated because of the reversal of the elution order and consequently, it is much easier to determine the enantiomeric purity on CSP 1'. These results demonstrate that the change in the elution order is often desirable when attempting to detect the minor enantiomers in the front of the major isomer of the analyte.[17]

2.4. CONCLUSION

CSP 1 and CSP 1' derived from (+)- and (−)-18-C-6-TA with a reverse configuration, respectively, were prepared and successfully used to resolve several primary amino compounds as well as α-amino acids. Since the chiral selectors of CSP 1 and CSP 1' are covalently bonded to the silica gel, various mobile phases can be used without any deterioration. Therefore, it is expected that they will be quite useful for resolving the enantiomers of hydrophobic compounds and preparative separations.[14] In addition, it is expected that CSP 1 and CSP 1', with the antipode of the chiral selector, will be effective for determining the enantiomeric purity due to the advantage of a reversal of the elution order.

REFERENCES

1. Newcomb, M.; Cram, D. J. Chromatographic optical resolution through chiral complexation of amino esters salts by a host covalently bound to silica gel. J. Am. Chem. Soc. **1975**, *97*, 1259−1261.

2. Sogah, G. D. Y.; Cram, D. J. Host-guest complexation. 14. host covalently bound to polystyrene resin for chromatographic resolution of enantiomers of amino acid and esters salts. J. Am. Chem. Soc. **1979**, *101*, 3035−3042.

3. Zhang, X. X.; Bradshaw, J. S.; Izatt, R. M. Enantiomeric recognition of amine compounds by chiral macrocyclic receptors. Chem. Rev. **1997**, *97*, 3313−3361.

4. Shibukawa, A.; Nakagawa, T. The use of chiral crown ethers in liquid chromatography. In Chiral Separations by HPLC: Applications to Pharmaceutical Compounds, Krstulovic. A. M., Ed.; John Wiley and Sons, New York, 1989.

5. Shinbo, T.; Yamaguchi, T.; Nishimura, K.; Sugiura, M. Chromatographic separation of racemic amino acids by use of chiral crown ether-coated reversed-phase packings. J. Chromatogr. **1987**, *405*, 145−153.

6. Refer to "Application guide for chiral HPLC selection", 4th ed., Daicel Chemical Industries, Ltd.

7. Machida, Y.; Nishi, H.; Nakamura, K. Enantiomer separation of hydrophobic amino compounds by high-performance liquid chromatography using crown ether dynamically coated chiral stationary phase. J. Chromatogr. A **1999**, *830*, 311−320.

8. Lee, W.; Hong, C. Y. Direct liquid chromatographic enantiomer separation of new fluoroquinolones including gemifloxacin. J. Chromatogr. A **2000**, *879*, 113−120.

9. Behr, J.-P.; Girodeau, J.-M.; Heyward R. C.; Lehn, J.-M.; Sauvage, J.-P. Molecular receptors. Functionalized and chiral macrocyclic polyethers derived from tartaric acid. Helv. Chim. Acta. **1980**, *63*, 2096−2111.

10. Machida, Y.; Nishi, H.; Nakamura, K.; Nakai, H.; Sato, T. Enantiomer separation of amino compounds by a novel chiral stationary phase derived from crown ether. J. Chromatogr. A **1998**, *805*, 85−92.

11. Hyun, M. H.; Jin, J. S.; Lee, W. A new HPLC chiral stationary phase for the direct resolution of racemic quinolone antibacterials containing a primary amino group. Bull. Kor.

Chem. Soc. **1998**, *19*, 819–821.

12. Hyun, M. H.; Jin, J. S.; Lee, W. Liquid chromatographic resolution of racemic amino acids and their derivatives on a new chiral stationary phase based on crown ether. J. Chromatogr. A **1998**, *822*, 155–161.

13. Hyun, M. H.; Jin, J. S.; Koo, H. J.; Lee, W. Liquid chromatographic resolution of racemic amines and amino alcohols on a chiral stationary phase derived from crown ether. J. Chromatogr. A **1999**, *837*, 75–82.

14. Hyun, M. H.; Han, S. C.; Jin, J. S.; Lee, W. Separation of the stereoisomers of racemic fluoroquinolone antibacterial agents on a crown-ether-based chiral HPLC stationary phase. Chromatographia **2000**, *52*, 473–476.

15. Hyun, M. H.; Koo, H. J.; Jin, J. S.; Lee, W. Liquid chromatographic resolution of racemic compounds containing a primary amino group on a dynamic chiral stationary phase derived from chiral crown ether. J. Liq. Chrom. & Rel. Technol. **2000**, *23*, 2669–2682.

16. Bang, E.; Jung, J-W.; Lee, W.; Lee, D.-W.; Lee, W. Chiral recognition of (18-crown-6)-tetracarboxylic acid as a chiral selector determined by NMR spectrocropy. J. C. S. Perkin Trans. 2 **2001**, 1685–1692.

17. Okamoto, M. Reversal of elution order during the chiral separation in high performance liquid chromatography. J. Pharm. Biomed. Anal. **2002**, *27*, 401–407.

18. Meyer, V. R. Accuracy in the chromatographic determination of extreme enantiomeric ratios: a critical reflection. Chirality **1995**, *7*, 567–571.

Table 2.1 Enantiomer resolution of the α-amino acids on CSP 1 and CSP 1'

Entry	Analyte	CSP 1				CSP 1'			
		α	k'_1	Rs	Conf.[a]	α	k'_1	Rs	Conf.[a]
1	Ala	1.34	0.77	1.58	D	1.32	1.15	1.51	L
2	Asp	1.31	1.02	1.11	D	1.31	1.49	1.10	L
3	Ile	1.44	0.29	1.23	D	1.44	0.44	1.25	L
4	Leu	1.62	0.42	1.78	D	1.65	0.63	1.80	L
5	Met	1.66	0.70	2.30	D	1.64	1.02	2.25	L
6	Norleu	1.53	0.36	1.75	D	1.47	0.50	1.60	L
7	Norval	1.53	0.33	1.74	D	1.50	0.52	1.71	L
8	PG	2.74	0.82	7.18	D	2.57	1.31	7.05	L
9	Phe	1.60	0.44	2.01	D	1.54	0.67	1.92	L
10	Serine(Ser)	2.10	0.71	4.02	L	2.14	1.01	4.30	D
11	Thr	1.27	0.19	1.01	L	1.30	0.25	1.08	D
12	Trp	1.49	0.51	1.62	D	1.45	0.71	1.59	L
13	Tyr	1.55	0.45	2.80	D	1.52	0.65	2.65	L
14	Val	1.56	0.16	1.49	D	1.51	0.26	1.41	L
15	Diphenylalanine	2.00	0.11	2.56	D	2.04	0.16	2.67	L
16	DOPA	1.62	0.55	2.74	D	1.55	0.75	2.40	L
17	5-Hydroxytryptophan	1.49	0.61	1.46	D	1.55	0.63	1.56	L
18	Thy	1.88	1.50	2.65	D	1.90	1.72	2.90	L
19	m-Tyr	1.61	0.55	2.07	D	1.56	0.80	2.01	L

Mobile phase = 80% methanol in water (V/V) containing 10 mM H_2SO_4; Flow rate = 1 mL/min; UV detection at 210 nm; [a] the configuration of the second eluted isomer.

Table 2.2 Enantiomer resolution of the primary amino compounds on CSP 1 and CSP 1'

Entry	Analyte	CSP 1				CSP 1'			
		α	k'_1	Rs	Conf.[a]	α	k'_1	Rs	Conf.[a]
1	Alanine-β-naphthylamide	1.48	1.72	3.49	R	1.55	3.09	3.70	S
2	Baclofen	1.25	3.08	1.98	S	1.26	5.92	2.00	R
3	Kynurenine	1.10	1.43[b]	0.47	–	1.12	2.33[b]	0.59	–
4	α-Methylbenzylamine	1.37	0.95	2.47	S	1.30	2.16	2.36	R
5	α-Methyltryptamine	1.10	3.76[b]	0.70	–	1.13	5.09[b]	0.66	–
6	Mexiletine	1.17	0.28	1.39	–	1.20	0.69	1.42	–
7	1-(1-Naphthyl) ethylamine	1.22	0.85	1.98	R	1.27	2.05	2.09	S
8	Norepinephrine	1.20	1.07	1.60	R	1.21	1.52	1.63	S
9	Octopamine	1.29	0.61	1.30	–	1.35	0.81	1.60	–

Mobile phase = 80% methanol in water (v/v) containing 10 mM H_2SO_4; Flow rate = 1 mL/min; UV detection at 210 nm.

[a] the configuration of the second eluted isomer.

[b] 100% methanol containing 10 mM H_2SO_4 was used as a mobile phase.

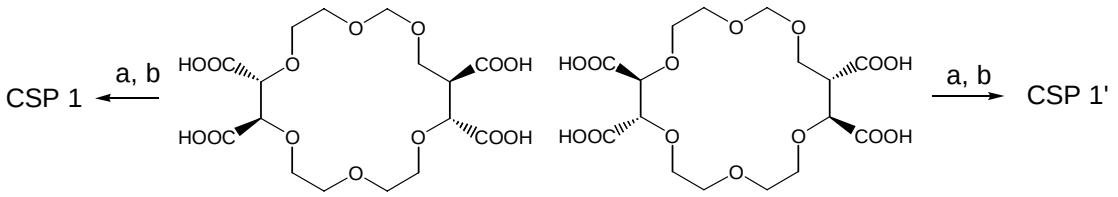


Figure 2.1 Covalently-bonded CSP 1 and CSP 1' derived from (+)-and (−)-18-C-6-TA, respectively; (a) acetyl chloride (b) aminopropyl silica gel, triethylamine.

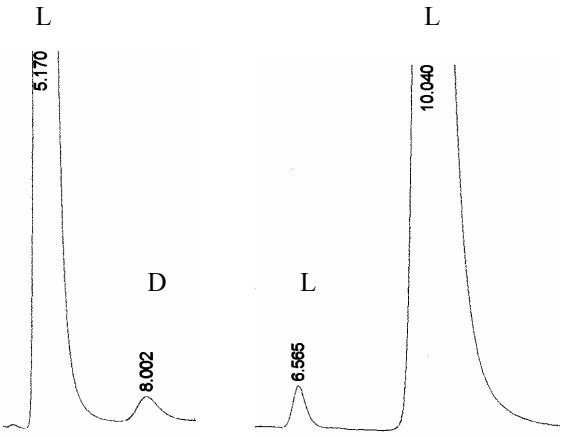


Figure 2.2 Chromatograms showing the resolution of an enantiomerically enriched Thyroxine (L:D = 90:1) on CSP 1 (the left) and CSP 1' (the right); Injection amount 1 μg.; The chromatographic conditions are given in Table 2.1.

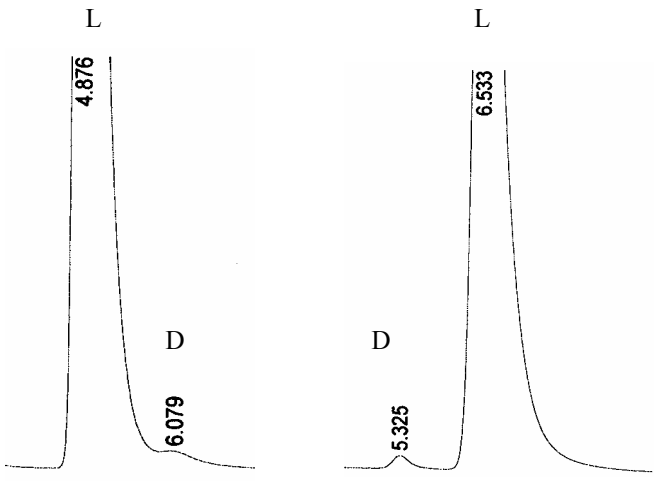


Figure 2.3 Chromatograms showing the resolution of an enantiomerically enriched DOPA (L: D = 100:1) on CSP 1 (the left) and CSP 1' (the right); Injection amount 1 μg.; The chromatographic conditions are given in Table 2.1.

Chapter Ⅲ

Liquid Chromatographic Enantiomer Resolution of *N*-Hydrazide Derivatives of 2-Aryloxypropionic Acids on a Crown Ether Derived Chiral Stationary Phase

ABSTRACT

The liquid chromatographic enantiomer separation of several *N*-hydrazide derivatives of 2-aryloxypropionic acids was performed on a crown ether type chiral stationary phase (CSP) derived from (18-crown-6)-2,3,11,12-tetracarboxylic acid. The behavior of chromatographic parameters by the change of mobile phases and additives for the resolution of these analytes was investigated. The enantiomers of all analytes were base-line resolved in the mobile phase of 100% methanol containing 20 mM H_2SO_4 as an acid additive. These results using the crown ether derived CSP are the first reported for enantiomer resolution of chiral acids of 2-aryloxypropionic acids as their *N*-hydrazide derivatives.

Keyword: chiral stationary phase; enantiomer separation, 2-aryloxypropionic acids, *N*-hydrazide derivative

3.1. INTRODUCTION

Chiral crown ethers have been considered to be important as chiral selectors for resolution of primary amine compounds and a number of their related studies using chiral crown ethers have been accomplished by liquid-liquid extraction and high-performance liquid chromatography.[1,2] Among several chiral crown ether derivatives, (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (18-C-6-TA) has been of great interest and chiral stationary phases prepared by covalently bonding (+)-18-C-6-TA to the aminopropyl silica gel have been developed.[3] And they have been successfully utilized in resolving the enantiomers of various racemic α-amino acids and primary amino compounds including amino alcohols and quinolone antibacterials by HPLC.[3-7] In addition to primary amino compounds, interesting results on enantiomer resolution of non-primary amino compounds like β-blockers and *N*-(3,5-dinitrobenzoyl)-α-amino acids on the crown ether derived CSP have been reported.[8,9] In terms of chiral mechanistic consideration between primary amino compounds and (+)-18-C-6-TA of chiral selector, complexation of the primary ammonium moiety formed under acidic conditions inside the chiral cavity of the 18-crown-6 ring of the CSP is considered to be important for the chiral recognition.[3] In order to investigate the chiral recognition mechanism of the diastereomeric complexes in solution state, detailed NMR studies for (+)-18-C-6-TA of a chiral selector and α-amino acid enantiomer have been performed and hydrogen bonding interactions during their complexation were observed in NMR experiments as well as their related molecular calculation studies.[10,11] We have attempted to extend the application of enantiomer separation using the crown ether derived CSP to chiral acids like 2-aryloxypropionic acids used as herbicides,[12] although several chromatographic methods for direct resolution of 2-aryloxypropionic acids and/or their esters on various CSPs have been reported.[13-18] Since the direct enantiomer resolution of 2-aryloxypropionic acids lacking primary amino moiety could not be applied on this type CSP, *N*-hydrazide derivative as an appending group for amino moiety was introduced to 2-aryloxypropionic acids for its complexation with crown ether in this study. Here, we report the chromatographic enantiomer separation of *N*-hydrazide derivatives of 2-aryloxypropionic acids on CSP 1 derived from (+)-18-C-6-TA (Fig. 3.1).

3.2. EXPERIMENTAL

All chemicals of ethyl 2-bromopropionate, 4-butoxyphenol, 2-chloro-4-methylphenol, 2-chloro-5-methylphenol, 4-chloro-2-methylphenol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, *p*-cresol, 2,4-dichlorophenol, 1-naphthol, 2-naphthol, phenol and 2,4,5-trichlorophenol were purchased from Aldrich. Anhydrous potassium carbonate and hydrazide monohydrate were purchased from Osaka Hayashi pure chemical and ACROS, respectively. According to the reported procedure, all 2-aryloxypropionic acids ethyl esters were prepared and converted to their corresponding *N*-hydrazide derivatives with reaction of hydrazine in ethanol.[18,19] Chromatography was performed at room temperature using an HPLC Breeze system consisting of a Waters model 1525 binary pump, a Rheodyne model 7125 injector with a 20 μL loop, and a dual absorbance detector (Waters 2487 detector). HPLC-grade methanol (J. T. Baker) was used. Water was purified using a milli-Q water purification system (Bedford, MA, USA). CSP 1 derived from (+)-18-C-6-TA (250 mm L × 4.6 mm I.D.) was obtained from RS Technologies (Daejon, Korea).

3.3. RESULTS AND DISCUSSION

Tables 3.1 and 3.2 show the effect of acid additives and mobile phases for the resolution of *N*-hydrazide derivatives of 2-aryloxypropionic acids on CSP 1, respectively. These data show that chromatographic parameters such as separation factors, retention times and resolution factors are considerably influenced by the nature of acid additive and mobile phases in the mobile phase.[4,20] An increase in the concentration of acid additive or methanol in the mobile phase has a tendency of the increase of the retention times with better resolution factors. When sulfuric acid as an acid additive among several acid additives is used in Table 3.1, the greatest separation is obtained. The use of acetic acid as an acid additive afforded no resolution or the lowest separation factor. Compared to sulfuric acid, the use of the other acid additives except acetic acid provided almost the same separation factors with lower resolution factors. Although good resolution was often obtained using perchloric acid as an lipophilic acid additive, significantly enhanced retention times for enantiomer separation *N*-hydrazide derivatives of 2-aryloxypropionic acids were observed.[20]

Tables 3.2 and 3.3 show chromatographic results for the separation of the enantiomers of several *N*-hydrazide derivatives of 2-aryloxypropionic acids on CSP 1. As shown in Table 3.2, when better enantioselectivities with greater retention times using the mobile phase of 100% methanol with 20 mM H_2SO_4 in Table 3.3 are observed than those using the mobile phase of 80% methanol/water (V/V) with 20 mM H_2SO_4 in Table 3.4. Using the former mobile phase the enantiomers of all analytes were base-line resolved in Table 3.3. Typical chromatograms of enantiomer resolution of 2-(2,4-dichlorophenoxy)- and 2-(1-naphthoxy)propionic acid as *N*-hydrazide derivatives are presented in Figure 3.2, respectively.

Unlike the enantioseparation of 2-aryloxypropionic acids and their derivatives on Pirkle type CSP,[18] it seems that the structural difference of aryloxy groups of analytes does not greatly affect the enantioselectivities on CSP 1. The separation factors for all analytes range from 1.13–1.25 in Table 3.3. However, the degree of enantioseparation on Pirkle type CSP is directly affected by aryloxy groups of the analytes, because π-π chiral interaction between aryloxy group and the *N*-dinitrobenzoyl substituted chiral selector of Pirkle CSP is very important.[18] In terms of mechanistic view, however, it is considered that the aryloxy group is not greatly involved for chiral recognition interaction on CSP 1, because it lies in the remote

position from the complexation center between the ammonium moiety of the analyte and the chiral cavity of the 18-crown-6 ring of the CSP. Elution orders were determined for three configurationally known analytes of 4-chlorophenoxy-, 2,4-dichlorophenoxy- and 1-naphthoxy substituted 2-propionic acid as *N*-hydrazide derivatives (entries 7, 9 and 10), the (S)-enantiomers being selectively retained on CSP 1.

3.4. CONCLUSION

In conclusion, liquid chromatographic separation of the enantiomers of several 2-aryloxypropionic acids as *N*-hydrazide derivatives was achieved on 18-crown–6 derived CSP 1. For all analytes, CSP 1 afforded good resolving ability with base-line separation using 100% methanol containing H_2SO_4 as a mobile phase. Although 2-aryloxypropionic acids do not possess primary amino group, it was shown that enantiomer resolution of 2-aryloxypropionic acids as their *N*-hydrazide derivatives could be possible on crown ether type CSP 1 after prederivatization of these chiral acids. The further application to other chiral acids on crown ether type CSP is currently in progress in our laboratory.

REFERENCES

1. Shibukawa, A.; Nakagawa, T. The use of chiral crown ethers in liquid chromatography, Krstulovic AM (Ed.), Chiral separations by HPLC: applications to pharmaceutical compounds. New York, John Wiley and Sons, **1989**.

2. Zhang, X. X.; Bradshaw, J. S.; Izatt, R. M. Enantiomeric recognition of amine compounds by chiral macrocyclic receptors. Chem. Rev. **1997**, *97*, 3313–3361.

3. Hyun, M. H. Preparation and application of HPLC chiral stationary phases based on (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid. J. Sep. Sci. **2006,** *29*, 750–761.

4. Hyun, M. H.; Jin, J. S.; Lee, W. Liquid chromatographic resolution of racemic amino acids and their derivatives on a new chiral stationary phase based on crown ether. J. Chromatogr. A **1998,** *822*, 155–161.

5. Hyun, M. H.; Jin, J. S.; Koo, H. J.; Lee, W. Liquid chromatographic resolution of racemic amines and amino alcohols on a chiral stationary phase derived from crown ether. J. Chromatogr. A **1999**, *837*, 75–82.

6. Hyun, M. H.; Han, S. C.; Jin, J. S.; Lee, W. Separation of the stereoisomers of racemic fluoroquinolone antibacterial agents on a crown-ether-based chiral HPLC stationary phase. Chromatographia **2000**, *52*, 473–476.

7. Jin, J. Y.; Lee, W.; Hyun, M. H. Development of the antipode of the covalently-bonded crown ether type chiral stationary phase for the advantage of the reversal of elution order. J. Liq. Chrom. & Rel. Tech. **2006**, *29*, 841–848.

8. Steffeck, R. J.; Zelechonok, Y.; Gahm, K. H. Enantioselective separation of racemic secondary amines on a chiral crown ether-based liquid chromatography stationary phase. J. Chromatogr. A **2002**, *947*, 301–305.

9. Hyun, M. H.; Tan, G.; Xue, J. Y. Unusual resolution of *N*-(3,5-dinitrobenzoyl)-α-amino acids on a chiral stationary phase based on (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid. J. Chromatogr. A **2005**, *1097*, 188–191.

10. Bang, E.; Jung, J. W.; Lee, W.; Lee, D. W.; Lee, W. Chiral recognition of (18-crown-6)-tetracarboxylic acid as a chiral selector determined by NMR spectrocropy. JCS. Perkin Transactions 2 **2001**, 1685–1692.

11. Park, H. J.; Choi, Y.; Lee, W.; Kim, K. R. Enantioseparation of aromatic amino acids and amino acid esters by capillary electrophoresis with crown ether and prediction of enantiomer migration orders by a three-dimensional quantitative structure-property relationship/ comparative field analysis model. Electrophoresis **2004**, *25*, 2755–2760.

12. Hopkins, W. L. Global herbicide directory. Indianapolis, Ag Chem Information Services, **1997**.

13. Dernoncour, R.; Azerad, R. High-performance liquid chromatographic separation of the enantiomers of substituted 2-aryloxypropionic acid methyl esters. J. Chromatogr. **1987**, *410*, 355–361.

14. Blessington, B.; Crabb, N. Proposed primary reference methods for the determination of some commercially important chiral aryloxypropionate herbicides in both free acid and ester forms. J. Chromatogr. **1989**, *483*, 349–358.

15. Tambute, A.; Siret, L.; Caude, M.; Rosset, R. Enantiomeric separation of substituted 2-aryloxy propionic esters: Application to the determination of the enantiomeric excess in herbicide formulations. J. Chromatogr. **1991**, *541*, 349–357.

16. Lin, S. H.; Chiou, A. J.; Wu, S. H.; Wang, K. T. Enantiomeric separation of 2-(phenoxy)propionate derivatives by chiral high-performance liquid chromatography. Chirality **1991**, *3*, 476–479.

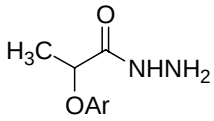
17. Bettoni, G.; Ferorelli, S.; Loiodice, F.; Tangari, N.; Tortorella, V.; Gasparrini, F.; Misiti, D.; Villani, C. Chiral α-substituted α-aryloxy acetic acids: Synthesis, absolute configuration, chemical resolution, and direct separation by hplc. Chirality **1992**, *4*, 193–203.

18. Pirkle, W. H.; Welch, C. J.; Lee, W. Chromatographic separation of the enantiomers of 2-aryloxypropionc acids, esters and amides. Enantiomer **1997**, *2*, 423–431.

19. Organic Syntheses. Collective volume 4, New York, John Wiley and Sons. **1963**, 819–823.

20. Lee, W.; Hong, C. Y. Direct liquid chromatographic enantiomer separation of new fluoroquinolones including gemifloxacin. J. Chromatogr. A **2000**, *879*, 113–120.

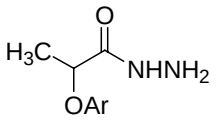
Table 3.1 The effect of acid additives on enantiomer separation of *N*-hydrazide derivatives of 2-aryloxypropionic acids on CSP 1



Ar	α	k'_1	Rs	Acid additive
4-Chlorophenyl	1.24	8.20	1.62	5 mM H_2SO_4
	1.24	10.13	1.72	10 mM H_2SO_4
	1.24	12.63	1.86	20 mM H_2SO_4
	1.23	3.17	1.02	20 mM CF_3COOH
	1.00	2.56	–	20 mM CH_3COOH
	1.23	12.84	1.19	20 mM HCl
	1.25	32.27	1.12	20 mM $HClO_4$
2,4-Dichlorophenyl	1.25	15.06	1.54	5 mM H_2SO_4
	1.25	20.32	2.01	10 mM H_2SO_4
	1.25	20.88	2.17	20 mM H_2SO_4
	1.23	3.43	1.10	20 mM CF_3COOH
	1.07	2.91	0.24	20 mM CH_3COOH
	1.24	20.18	1.86	20 mM HCl
	1.23	45.49	2.27	20 mM $HClO_4$

Mobile phase: 100% MeOH with acid additive; Flow rate: 1.0 ml/min; UV detection at 210 nm; Temperature ambient (about 25℃). α: Separation factor; k'_1: Retention factor of the first eluted enantiomer; R_S: Resolution factor.

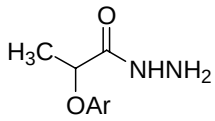
Table 3.2 The effect of methanol content on enantiomer separation of *N*-hydrazide derivatives of 2-aryloxypropionic acids on CSP 1



Ar	α	k'_1	Rs	Acid additive
4-Chlorophenyl	1.17	4.03	1.27	80% MeOH/H_2O with 20 mM H_2SO_4
	1.19	5.42	1.33	85% MeOH/ H_2O with 20 mM H_2SO_4
	1.20	6.78	1.64	90% MeOH/ H_2O with 20 mM H_2SO_4
	1.22	9.20	1.67	95% MeOH/ H_2O with 20 mM H_2SO_4
	1.24	12.63	1.86	100% MeOH with 20 mM H_2SO_4
2,4-Dichlorophenyl	1.15	5.23	1.28	80% MeOH/ H_2O with 20 mM H_2SO_4
	1.18	7.54	1.50	85% MeOH/ H_2O with 20 mM H_2SO_4
	1.19	8.76	1.79	90% MeOH/ H_2O with 20 mM H_2SO_4
	1.21	12.60	2.06	95% MeOH/ H_2O with 20 mM H_2SO_4
	1.25	20.88	2.17	100% MeOH with 20 mM H_2SO_4

Flow rate: 1.0 ml/min; UV detection at 210 nm; Temperature ambient (about 25℃). α: Separation factor; k'_1: Retention factor of the first eluted enantiomer; R_S: Resolution factor.

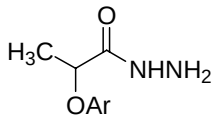
Table 3.3 Enantiomer separation of *N*-hydrazide derivatives of 2-aryloxypropionic acids on CSP 1



Analyte	Ar	α	k'_1	Rs
1	4-Butoxyphenyl	1.18	10.60	1.39
2	2-Chloro-4-methylphenyl	1.13	14.94	1.13
3	2-Chloro-5-methylphenyl	1.14	17.39	1.43
4	4-Chloro-2-methylphenyl	1.19	17.59	1.27
5	2-Chlorophenyl	1.13	17.04	1.13
6	3-Chlorophenyl	1.16	14.78	1.04
7	4-Chlorophenyl	1.24	12.63	1.86
8	4-Methylphenyl	1.16	11.24	1.14
9	2,4-Dichlorophenyl	1.25	20.88	2.17
10	1-Naphthyl	1.20	21.93	1.54
11	2-Naphthyl	1.13	16.16	0.86
12	Phenyl	1.14	12.48	1.05
13	2,4,5-Trichlorophenyl	1.14	21.37	1.01

Mobile phase: 100% MeOH with 20 mM H_2SO_4; Flow rate: 1.0ml/min; UV detection at 210 nm; Temperature ambient (about 25℃). α: Separation factor; k'_1: Retention factor of the first eluted enantiomer; R_S: Resolution factor.

Table 3.4 Enantiomer separation of *N*-hydrazide derivatives of 2-aryloxypropionic acids on CSP 1



Analyte	Ar	α	k'_1	Rs
1	4-Butoxyphenyl	1.14	3.21	1.08
2	2-Chloro-4-Methylphenyl	1.09	4.89	0.66
3	2-Chloro-5-Methylphenyl	1.08	5.40	0.60
4	4-Chloro-2-Methylphenyl	1.09	4.82	0.64
5	2-Chlorophenyl	1.08	5.37	0.57
6	3-Chlorophenyl	1.11	4.96	0.67
7	4-Chlorophenyl	1.17	4.03	1.27
8	4-Methylphenyl	1.11	3.76	0.74
9	2,4-Dichlorophenyl	1.15	5.23	1.28
10	1-Naphthyl	1.13	8.00	0.90
11	2-Naphthyl	1.06	6.05	0.33
12	Phenyl	1.10	4.03	0.65
13	2,4,5-Trichlorophenyl	1.11	6.70	0.73

Mobile phase: 80% MeOH/H_2O (V/V) with 20 mM H_2SO_4. Flow rate: 1.0 ml/min. Detection UV 210 nm. Temperature ambient (about 25℃). α: Separation factor. k'_1: Retention factor of the first eluted enantiomer. R_S: Resolution factor.

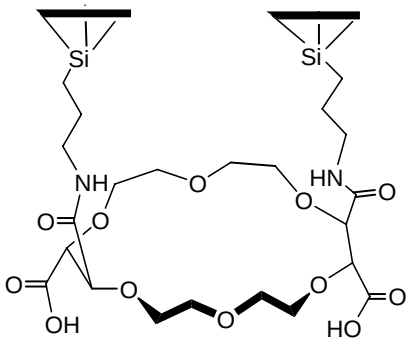


Figure 3.1 Chemical structure of CSP 1.

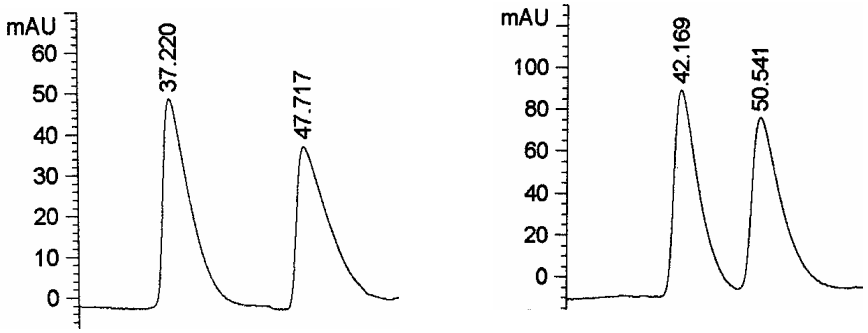


Figure 3.2 Chromatograms of enantiomer resolution of *N*-hydrazide derivatives of 2-(2,4-dichlorophenoxy)propionic acid (the left) and 2-(1-naphthoxy)propionic acid (the right). See Table 3.3 for the chromatographic condition. Injection amount 2.5 μg.

Chapter Ⅳ

Covalently Bonded and Coated Chiral Stationary Phases Derived from Polysaccharide Derivatives for Enantiomer Separation of *N*-Fluorenylmethoxycarbonyl α-Amino Acids with Fluorescence Detection

ABSTRACT

Liquid chromatographic comparisons for enantiomer resolution of *N*-fluorenylmethoxycarbonyl (FMOC) α-amino acids with fluorescence detection were made on covalently bonded type chiral stationary phases (CSPs) (Chiralpak IA and Chiralpak IB) and coated type CSPs (Chiralpak AD and Chiralcel OD) derived from polysaccharide derivatives of the same chiral selectors. This is the first reported for enantiomer resolution with fluorescence detection on covalently bonded type CSPs, Chiralpak IA and Chiralpak IB. In general, covalently bonded type CSPs (Chiralpak IA and Chiralpak IB) showed lower enantioseparation than coated type CSPs (Chiralpak AD and Chiralcel OD) for enantiomer resolution of these analytes, respectively. Owing to higher sensitivity and broader solvent compatibility in fluorescence detection on Chiralpak IA and Chiralpak IB than in UV detection, however, this analytical method is expected to enlarge their application of enantiomer resolution, such as an online HPLC monitoring of asymmetric synthesis.

Keywords: Enantiomer separation, Chiralpak IA, Chiralpak IB, Fluorescence detection, Chiral stationary phase

4.1. INTRODUCTION

Chiral stationary phases (CSPs) derived from polysaccharide derivatives have been extensively used for enantiomer separation of a number of racemic compounds.[1,2] Since chiral selectors of polysaccharide derivatives are coated on a silica matrix, these type CSPs have intrinsic drawbacks of column stability and a limitation of mobile phases. Therefore, the solvents such as halogenated solvents, tetrahydrofuran, ethyl acetate and acetone which partially or totally dissolve the chiral selectors of the polysaccharide derivatives must be excluded for mobile phases and analytes solvents.[1,2] These disadvantages are directly related to limitation of their applicability including preparative separation due to solubility of analytes. Many studies to solve these problems by covalently bonding the chiral selectors of polysaccharide derivatives to a silica matrix have been reported.[3-9] Recently, covalently bonded CSPs on silica matrix, Chiralpak IA[10-15] and Chiralpak IB[16] which have the same chiral selectors, amylose and cellulose tris(3,5-dimethylphenylcarbamate) of coated type Chiralpak AD and Chiralcel OD, respectively, have been developed. Therefore, it is considered that Chiralpak IA and Chiralpak IB are the covalently immobilized CSP of Chiralpak AD and Chiralcel OD, respectively. In this study, we present the comparative liquid chromatographic enantiomer resolution of *N*-fluorenylmethoxycarbonyl (FMOC) protected α-amino acids on polysaccharide-derived CSPs, covalently bonded type Chiralpak IA and Chiralpak IB, and coated type Chiralpak AD and Chiralcel OD with fluorescence detection.[17]

4.2. EXPERIMENTAL

Chromatographic analysis was carried out using an HPLC consisting of a Waters model 510 pump, a Rheodyne model 7125 injector with a 20 μL loop, a spectrofluorometric detector (Jasco FP-920) and an HP 3396 series II recorder. The excitation and emission wavelengths were 280 and 310 nm, respectively. Chiralpak IA, Chiralpak IB, Chiralpak AD and Chiralcel OD column (250 mm L × 4.6 mm I.D.) were purchased from Daicel Chemical Company (Tokyo, Japan). HPLC grade hexane (Hxn), 2-propanol, tetrahydrofuran (THF), ethyl acetate and dichloromethane were obtained from J. T. Baker (Phillipsburg, NJ). Trifluoroacetic acid (TFA) was obtained from Aldrich (Milwaukee, WI). The racemic and L-*N*-FMOC α-amino acids were prepared according to a reported procedure.[18]

4.3. RESULTS AND DISCUSSION

Tables 4.1 and 4.2 show the effect of mobile phase on the enantiomer separation of four *N*-FMOC α-amino acids on Chiralpak IA and Chiralpak IB with fluorescence detection. Due to their covalently bonded nature, these two CSPs are compatible with a large range of organic miscible solvents, including the halogenated solvent of dichloromethane or chloroform.[10,16] The separation factors and retention times on Chiralpak IA and Chiralpak IB are considerably influenced by the nature of mobile phase.[10,12,15] As shown in Tables 4.1 and 4.2, the highest enantioselectivities for all analytes were obtained using 5% 2-propanol in hexane with 0.1% TFA as a mobile phase, except for *N*-FMOC valine using 25% dichloromethane in hexane with 0.1% TFA. Interestingly, the elution orders of these analytes on Chiralpak IA are variable depending upon the used mobile phases in Table 4.1, while their elution orders on Chiralpak IB are unchanged regardless of the mobile phases in Table 4.2.

Especially, this fluorescence detection method for enantiomer separation on Chiralpak IA and Chiralpak IB has great advantages over UV detection method. It provides higher sensitivity and much wider solvent versatility irrelevant to the cut-offs of the used mobile phases, compared to UV detection method.[17] This is the first reported for enantiomer resolution with fluorescence detection on covalently bonded type CSPs, Chiralpak IA and Chiralpak IB.

Tables 4.3 and 4.4 show the comparative results of enantiomer separation of *N*-FMOC α-amino acids on covalently bonded type CSPs (Chiralpak IA and Chiralpak IB) and coated type CSPs (Chiralpak AD and Chiralcel OD) using 2-propanol in hexane with 0.1% TFA as a mobile phase with fluorescence detection. Chiralpak IB and Chiralcel OD derived from cellulose tris(3,5-dimethylphenylcarbamate) showed, in general, higher enantioselectivity than Chiralpak IA and Chiralpak AD derived from amylose tris(3,5-dimethylphenylcarbamate), respectively. Most of *N*-FMOC α-amino acids enantiomers were well separable on Chiralcel OD. Also, in general, Chiralpak IA and Chiralpak IB of covalently bonded type CSPs showed lower enantioseparation than Chiralpak AD and Chiralcel OD of coated type CSPs, respectively. It was reported that the reduction in enantioselectivity on covalently bonded CSPs is due to the lack of ordered arrangement of polysaccharide-derived chiral selectors bonded to the silica matrix.[4,6] It is interesting that the elution orders of the resolved *N*-FMOC

α-amino acids on covalently bonded type Chiralpak IB derived from cellulose tris(3,5-dimethylphenylcarbamate) are observed to be identical with those on coated type Chiralcel OD of the same chiral selector using 2-propanol in hexane with 0.1% TFA as a mobile phase in Table 4.4, as the similar results using different mobile phases are shown in Table 4.2. However, Chiralpak IA and Chiralpak AD derived from amylose tris(3,5-dimethylphenylcarbamate) showed three exceptions of the elution orders (entries 8, 11 and 18) in Table 4.3.

4.4. CONCLUSION

HPLC-fluorescence analysis of enantiomer separation of *N*-FMOC α-amino acids on covalently bonded type CSPs (Chiralpak IA and Chiralpak IB) and coated type CSPs (Chiralpak AD and Chiralcel OD) was performed. In general, cellulose tris(3,5-dimethylphenylcarbamate) derived CSPs, Chiralpak IB and Chiralcel OD showed higher enantioselectivity than amylose tris(3,5-dimethylphenylcarbamate) derived CSPs, Chiralpak IA and Chiralpak AD, respectively. Also, Chiralpak IA and Chiralpak IB showed, in general, lower enantioselectivity than Chiralpak AD and Chiralcel OD, respectively. However, this analytical method using fluorescence detection affords high sensitivity and compatibility with a much wider range of solvents on covalently bonded type CSPs (Chiralpak IA and Chiralpak IB). And it permits to create new applications of enantiomer separation for fluorescent analytes including a direct online HPLC monitoring for determination of enantiomeric purity during asymmetric synthesis procedures on these covalently bonded CSPs.[13]

REFERENCES

1. Yashima, E. Polysaccharide-based chiral stationary phases for high-performance liquid chromatographic enantioseparation. J. Chromatogr. A **2001,** *906*, 105–125.

2. Okamoto, Y.; Yashima, E. Polysaccharide derivatives for chromatographic separation of enantiomers. Angew. Chem. Int. Ed. **1998**, *37*, 1021–1043.

3. Okamoto, Y.; Aburatani, R.; Miura, S.; Hatada. K. Chiral stationary phases for HPLC: cellulose tris(3,5-dimethylphenylcarbamate) and tris(3,5-dichlorophenylcarbamate) chemically bonded to silica gel. J. Liq. Chromatogr. **1987**, *10*, 1613–1628.

4. Yashima, E.; Fukaya, H.; Okamoto, Y. 3,5-Dimethylphenylcarbamates of cellulose and amylose regioselectively bonded to silica gel as chiral stationary phases for high-performance liquid chromatography. J. Chromatogr. A **1994**, *677***,** 11–19.

5. Oliveros, L.; López, P.; Minguillón, C.; Franco, P. Chiral chromatographic discrimination ability of a cellulose 3,5-dimethylphenylcarbamate/ 10-undecenoate mixed derivative fixed on chromatographic matrices. J. Liq. Chromatogr. **1995**, *18*, 1521–1532.

6. Enomoto, N.; Furukawa, S.; Ogasawara, Y.; Akano, H.; Kawamura, Y.; Yashima, E.; Okamoto, Y. Preparation of silica gel-bonded amylose through enzyme-catalyzed polymerization and chiral recognition ability of its phenylcarbamate derivative in HPLC. Anal. Chem. **1996**, *68*, 2798–2804.

7. Franco, P.; Minguillón, C.; Oliveros, L. Solvent versatility of bonded cellulose-derived chiral stationary phases for high-performance liquid chromatography and its consequences in column loadability. J. Chromatogr. A **1998**, *793*, 239–247.

8. Franco, P.; Senso, A.; Oliveros, L.; Minguillión, C. Covalently bonded polysaccharide derivatives as chiral stationary phases in high-performance liquid chromatography. J. Chromatogr. A **2001**, *906*, 155–170.

9. Chen, X.; Liu, Y.; Qin, F.; Kong, L.; Zou, H. Synthesis of covalently bonded cellulose derivative chiral stationary phases with a bifunctional reagent of 3-(triethoxysilyl)propyl isocyanate. J. Chromatogr. A **2003**, *1010*, 185–194.

10. Zhang, T.; Kientzy, C.; Franco, P.; Ohnishi, A.; Kagamihara, Y.; Kurosawa, H. Solvent versatility of immobilized 3,5-dimethylphenylcarbamate of amylose in enantiomeric

separations by HPLC. J. Chromatogr. A **2005**, *1075*, 65–75.

11. Zhang, T.; Schaeffer, M.; Franco, P. Optimization of the chiral separation of a Ca-sensitizing drug on an immobilized polysaccharide-based chiral stationary phase: Case study with a preparative perspective. J. Chromatogr. A **2005**, *1083,* 96–101.

12. Li, Y. H.; Jin, J. Y.; Lee, W. Comparative liquid chromatographic enantiomer resolution on two chiral stationary phases derived from amylose tris(3,5-dimethylphenylcarbamate). Bull. Kor. Chem. Soc. **2005**, *26*, 1132–1134.

13. Ghanem, A.; Hoenen, H.; Y. Aboul-Enein, Y. H. Application and comparison of immobilized and coated amylose tris(3,5-dimethylphenylcarbamate) chiral stationary phases for the enantioselective separation of β-blockers enantiomers by liquid chromatography. Talanta **2006**, *68*, 602–609.

14. Ghanem, A.; Naim, L. Immobilized versus coated amylose tris(3,5-dimethylphenylcarbamate) chiral stationary phases for the enantioselective separation of cyclopropane derivatives by liquid chromatography. J. Chromatogr. A **2006**, *1101,* 171–178.

15. Cirilli, R.; Simonelli, A.; Ferretti, R.; Bolasco, A.; Chimenti, P.; Secci, D.; Maccioni, E.; Torre, L. F. Analytical and semipreparative high performance liquid chromatography enantioseparation of new substituted 1-thiocarbamoyl-3,5-diaryl-4,5-dihydro-(1*H*)-pyrazoles on polysaccharide-based chiral stationary phases in normal-phase, polar organic and reversed-phase conditions. J. Chromatogr. A **2006**, *1101*, 198–203.

16. Zhang, T.; Nguyen, D.; Franco, P.; Murakami, T.; Ohnishi, A.; Kurosawa, H. Cellulose 3,5-dimethylphenylcarbamate immobilized on silica: A new chiral stationary phase for the analysis of enantiomers. Anal. Chim. Acta **2006**, *557*, 221–228.

17. Brückner, H.; Lüpke, M. Use of chromogenic and fluorescent oxycarbonyl chlorides as reagents for amino acid analysis by high-performance liquid chromatography. J. Chromatogr. A **1995**, *697*, 295–307.

18. Bodansky, M.; Bodansky, A. The Practice of Peptide Synthesis, Springer: New York, 1984.

Table 4.1 Effect of mobile phase on the enantiomer separation of some *N*-FMOC α-amino acids on Chiralpak IA

Mobile[a] phase	5% 2-Propanol/Hxn with 0.1% TFA			15% THF/Hxn with 0.1% TFA			20% Ethyl acetate/Hxn with 0.1% TFA			25% Dichloromethane/ Hxn with 0.1% TFA		
Analyte	α[b]	k'_1[c]	Conf.[d]	α[b]	k'_1[c]	Conf.[d]	α[b]	k'_1[c]	Conf.[d]	α[b]	k'_1[c]	Conf.[d]
Ala	1.09	5.28	D	1.07	6.15	D	1.00	5.76	–	1.00	4.37	–
Leu	1.26	4.82	L	1.00	5.21	–	1.07	4.08	L	1.16	3.04	L
PG	1.31	5.02[e]	D	1.09	9.92	D	1.11	6.35	L	1.18	8.40	L
Val	1.08	5.92	L	1.08	4.02	D	1.00	3.59	–	1.21	2.81	L

[a] Mobile phase: Hexane (Hxn), Tetrahydrofuran (THF); Flow rate = 1 mL/min.

[b] Separation factor.

[c] Capacity factor for the first eluted enantiomer.

[d] Indicates the absolute configuration of the second retained enantiomer.

[e] 10% 2-Propanol/Hxn (V/V) with 0.1% TFA.

Table 4.2 Effect of mobile phase on the enantiomer separation of some *N*-FMOC α-amino acids on Chiralpak IB

Mobile[a] phase	5% 2-Propanol/Hxn with 0.1% TFA			15% THF/Hxn with 0.1% TFA			20% Ethyl acetate/Hxn with 0.1% TFA			20% Dichloromethane/ Hxn with 0.1% TFA		
Analyte	α[b]	k'_1[c]	Conf.[d]	α[b]	k'_1[c]	Conf.[d]	α[b]	k'_1[c]	Conf.[d]	α[b]	k'_1[c]	Conf.[d]
Ala	1.40	7.15	L	1.07	6.01	L	1.09	10.25	L	1.00	11.93	–
Leu	1.42	4.65	D	1.13	3.72	D	1.18	5.53	D	1.24	7.33	D
PG	1.34	3.65[e]	D	1.18	6.65	D	1.19	9.61	D	1.00	13.45	D
Val	1.21	4.61	D	1.10	3.12	D	1.20	5.04	D	1.17	6.21	D

[a] Mobile phase: Hexane (Hxn), Tetrahydrofuran (THF); Flow rate = 1 mL/min.

[b] Separation factor.

[c] Capacity factor for the first eluted enantiomer.

[d] Indicates the absolute configuration of the second retained enantiomer.

[e] 10% 2-Propanol/Hxn (V/V) with 0.1% TFA.

Table 4.3 Enantiomer separation of *N*-FMOC α-amino acids on Chiralpak IA and Chiralpak AD

Entry	Analyte	Chiralpak IA				Chiralpak AD			
		α[a]	k'$_1$[b]	Rs[c]	Conf.[d]	α[a]	k'$_1$[b]	Rs[c]	Conf.[d]
1	ABA[e]	1.07	6.90	0.96	D	1.13	3.46	1.40	D
2	ACA[f]	1.06	5.98	0.85	–	1.13	2.95	1.36	–
3	Ala	1.09	5.28	1.25	D	1.12	3.05	1.46	D
4	Asn	1.28	8.19[g]	1.96	L	1.20	6.97	2.03	L
5	Asp	1.07	3.70[g]	0.72	D	1.17	4.90	0.94	D
6	Glutamine(Gln)	1.10	6.67[g]	0.54	L	1.42	5.64	3.79	L
7	Glutamic acid(Glu)	1.11	4.01[g]	1.14	L	1.27	5.30	2.69	L
8	Ile	1.08	7.26	1.07	L	1.17	3.99	1.86	D
9	Leu	1.26	4.82	3.34	L	1.19	3.22	1.85	L
10	Met	1.05	12.11	0.71	L	1.00	5.57	–	–
11	Norleu	1.04	6.75	0.45	L	1.07	9.69[h]	0.92	D
12	Norval	1.00	7.12	–	–	1.12	10.63[h]	1.63	D
13	PG	1.31	5.02[g]	3.86	D	1.40	7.62	4.56	D
14	Phe	1.08	3.31[g]	0.91	L	1.13	4.89	1.50	L
15	Ser	1.07	3.65[g]	0.60	L	1.09	4.78	1.05	L
16	Thr	1.09	3.76[g]	0.92	D	1.14	5.05	1.55	D
17	Tyr	1.25	15.35[g]	2.76	L	1.31	26.55	3.79	L
18	Val	1.08	5.92	1.15	L	1.14	4.24	1.43	D

Mobile phase: 5% and 10% 2-propanol/hexane (V/V) containing 0.1% TFA on Chiralpak IA and Chiralpak AD, respectively; Flow rate = 1 mL/min. [a]Separation factor. [b]Capacity factor for the first eluted enantiomer. [c]Resolution factor. [d]Indicates the absolute configuration of the second eluted enantiomer. [e]2-Aminobutyric acid. [f]2-Aminocaprylic acid. [g,h]10% and 5% 2-propanol/hexane(V/V) containing 0.1% TFA, respectively.

Table4.4 Enantiomer separation of *N*-FMOC α-amino acids on Chiralpak IB and Chiralcel OD

Entry	Analyte	Chiralpak IB				Chiralcel OD			
		α[a]	k'$_1$[b]	Rs[c]	Conf.[d]	α[a]	k'$_1$[b]	Rs[c]	Conf.[d]
1	ABA[e]	1.23	6.28	2.02	L	1.38	5.23	2.89	L
2	ACA[f]	1.12	4.78	1.55	-	1.41	4.48	2.78	-
3	Ala	1.40	7.15	3.95	L	1.80	5.86	5.25	L
4	Asn	1.10	4.37[g]	0.48	L	1.60	5.05[g]	1.81	L
5	Asp	1.22	5.02[h]	1.50	L	1.58	6.77[g]	2.80	L
6	Gln	1.00	4.81[g]	–		1.17	14.01	0.86	D
7	Glu	1.16	6.00[h]	1.15	L	1.33	9.69	1.80	L
8	Ile	1.51	4.59	5.42	D	1.51	4.30	3.62	D
9	Leu	1.42	4.65	3.97	D	1.26	5.19	2.01	D
10	Met	1.07	4.35[h]	0.76	L	1.14	7.80	1.11	L
11	Norleu	1.07	5.84	0.80	L	1.15	4.69	1.19	L
12	Norval	1.05	6.06	0.68	L	1.07	4.98	0.61	L
13	PG	1.34	3.65[h]	3.14	D	1.71	7.88	3.79	D
14	Phe	1.08	3.69[h]	0.70	L	1.10	8.49	0.70	L
15	Ser	1.65	6.25[h]	3.73	L	2.56	2.70[g]	5.01	L
16	Thr	1.11	4.66[h]	0.74	L	1.48	7.29	2.98	L
17	Tyr	1.08	7.76[g]	0.56	L	1.10	20.50[g]	0.55	L
18	Val	1.21	4.61	2.25	D	1.13	4.55	1.10	D

Mobile phase; 5% and 10% 2-propanol/hexane (V/V) containing 0.1% TFA on Chiralpak IB and Chiralcel OD, respectively; Flow rate = 1 mL/min. [a]Separation factor. [b]Capacity factor for the first eluted enantiomer. [c]Resolution factor. [d]Indicates the absolute configuration of the second eluted enantiomer. [e]2-Aminobutyric acid. [f]2-Aminocaprylic acid. [g,h]10% 2-propanol/hexane(V/V) containing 0.1% TFA, respectively.

Chapter Ⅴ

Comparative Liquid Chromatographic Enantiomer Resolution on Two Chiral Stationary Phases Derived from Amylose Tris(3,5-dimethylphenylcarbamate)

ABSTRACT

The liquid chromatographic enantiomer separation of *N*-fluorenylmethoxycarbonyl (FMOC) protected α-amino acids ethyl ester derivatives was performed on polysaccharide-derived chiral stationary phases, covalently bonded type chiralpak IA and coated type Chiralpak AD. Although Chiralpak IA showed slightly lower enantioselectivity than Chiralpak AD, most of *N*-FMOC α-amino acids ethyl esters enantiomers were base-line separated on Chiralpak IA and Chiralpak AD. Owing to the compatibility with a broad range of solvents and column safety of Chiralpak IA, it is expected to enlarge its new application of enantiomer separation. Especially, it is expected to be useful for preparative separations, because the halogenated solvent like chloroform or methylene chloride shows often better solubility than the other solvents.

Keywords: Enantiomer resolution, Chiral stationary phase, Amylose tris(3,5-dimethylphenylcarbamate)

5.1. INTRODUCTION

Polysaccharide-derived chiral stationary phases (CSPs) are known to show high chiral recognition ability in HPLC and have been extensively used to separate a broad range of racemic compounds.[1-3] These type CSPs are usually prepared coating or adsorbing the polysaccharide derivatives on silica gel. Therefore, the solvents such as chloroform, methylene chloride, and tetrahydrofuran which dissolve or swell the chiral selectors of the polysaccharide derivatives cannot be used as mobile phases.[4] For example, in case of Chiralpak AD prepared by coating amylose tris(3,5-dimethylphenylcarbamate) derivative, one of the widely used polysaccharide type CSPs, suitable mobile phases like hexane, 2-propanol and ethanol should be used for the column safety.[3,4] It may be damaged in case of even the use of only a little amount of inappropriate solvents used as mobile phases and/or sample solvents. These limitations represent a disadvantage for new applications using these CSPs and, especially, preparative separation due to solubilization of analytes. To overcome these problems, therefore, the development of polysaccharide-derived covalently bonded CSPs has been of great interest and various results of different attempts have been reported.[4-12] Recently, Chiralpak IA prepared by chemically bonding amylose tris(3,5-dimethylphenylcarbamate) derivative on silica gel, which is used as the same chiral selector of coated type Chiralpak AD has been introduced. In this study, we present the comparative liquid chromatographic enantiomer resolution of *N*-fluorenylmethoxycarbonyl (FMOC) protected α-amino acids ethyl ester derivatives on two polysaccharide-derived CSPs, covalently bonded type Chiralpak IA and coated type Chiralpak AD.[13] This is the first report concerning the comparison of the chiral separations on Chiralpak IA and Chiralpak AD using normal mobile phases.

For enantiomer separation of *N*-FMOC α-amino acids ester derivatives, very a few results on CSPs have been reported.[14-17] Rizzi et al. have separated three FMOC α-amino acids methyl esters enantiomers on cellulose triacetate type column.[14] Miyazawa et al. have reported on the resolution of only one analyte of FMOC 2-aminobutanoic acid methyl ester enantiomers among many different *N*-protected amino acid derivatives.[15,16] Küsters et al. have reported on the resolution of fifteen *N*-FMOC α-amino acids methyl and isopropyl esters enantiomers on a polysaccharide-derived CSP.[17] To our knowledge, our results are the first reported for the enantiomer resolution of *N*-FMOC α-amino acids ethyl ester derivatives.

5.2. EXPERIMENTAL

Chromatography was performed at room temperature using an HPLC consisting of a Waters model 510 pump, a Rheodyne model 7125 injector with a 20 μL loop, a variable wavelength detector (Waters 490) and an HP 3396 series II recorder. Chiralpak IA and Chiralpak AD column (250 mm L × 4.6mm I.D.) were purchased from Daicel Chemical Company (Tokyo, Japan). HPLC-grade hexane (Hxn), 2-propanol, tetrahydrofuran (THF), ethyl acetate and chloroform were obtained from J. T. Baker (Phillipsburg,NJ). Trifluoroacetic acid (TFA) was obtained from Aldrich (Milwaukee, WI). The racemic (or enantiomerically pure) *N*-FMOC protected α-amino acids and their esters were prepared according to the conventional methods.[18]

5.3. RESULTS AND DISCUSSION

Table5.1 shows the effect of mobile phase on the enantiomer separation of some *N*-FMOC α-amino acids ethyl esters on Chiralpak IA. The enantioselectivities and retention times are greatly influenced by the nature of mobile phase. As shown in Table 5.1, in general, 5% 2-propanol in hexane as a mobile phase afforded the greatest enantioselectivity with the highest resolution factor, whereas 20% chloroform in hexane afforded the lowest enantioselectivity. It is notable that the elution orders of three analytes using 10% tetrahydrofuran or 10% ethyl acetate or 20% chloroform in hexane are different from those using 5% 2-propanol in hexane.

Table 5.2 shows the comparative data of enantiomer separation of *N*-FMOC α-amino acids ethyl esters on Chiralpak IA and Chiralpak AD using 2-propanol in hexane as a mobile phase. In general, Chiralpak IA showed slightly lower enantioseparation than Chiralpak AD for enantioresolution of *N*-FMOC α-amino acids ethyl esters. Several results have reported that a certain decrease in the enantioseparation was observed on polysaccharide-derived covalently bonded CSPs, compared to the results on the corresponding coated type CSPs.[5,7,10] The lowered enantioselectivity on bonded CSPs might be responsible for the lack of ordered arrangement of the chiral selector bonded to the matrix.[7,10]

Most of *N*-FMOC α-amino acids ethyl esters enantiomers were well separable on Chiralpak IA and Chiralpak AD. Especially, Chiralpak IA afforded base-line separation (α = 1.14–2.63, Rs = 1.63–9.34) for all investigated *N*-FMOC α-amino acids ethyl esters enantiomers except for asparagine analyte (entry 4). It is noted that the consistent elution order of the resolved *N*-FMOC α-amino acids ethyl esters is observed on Chiralpak IA and Chiralpak AD using 2-propanol in hexane as a mobile phase, the L-enantiomers being preferentially retained.

5.4. CONCLUSION

we demonstrated the comparative liquid chromatographic separation of enantiomers of *N*-FMOC protected α-amino acids ethyl esters on covalently bonded type Chiralpak IA and coated type Chiralpak AD derived from amylose tris(3,5-dimethylphenylcarbanmate) of the same chiral selector. Although Chiralpak IA showed slightly lower enantioselectivity than Chiralpak AD, most of *N*-FMOC α-amino acids ethyl esters enantiomers were base-line separated on Chiralpak IA and Chiralpak AD. Owing to the compatibility with a broad range of solvents and column safety of Chiralpak IA, it is expected to enlarge its new application of enantiomer separation. Especially, it is expected to be useful for preparative separations, because the halogenated solvent like chloroform or methylene chloride shows often better solubility than the other solvents.

REFERENCES

1. Yashima, E. Polysaccharide-based chiral stationary phases for high-performance liquid chromatographic enantioseparation. J. Chromatogr. A **2001**, *906*, 105–125.

2. Okamoto, Y.; Yashima, E. Polysaccharide derivatives for chromatographic separation of enantiomers. Angew. Chem. Int. Ed. **1998**, *37*, 1021–1043.

3. Refer to "Application guide for chiral HPLC selection", 3rd Edition, Daicel Chemical Industries, Ltd.

4. Franco, P.; Senso. A.; Oliveros, L.; Minguillón, C. J. Chromatogr. A **2001**, *906*, 155–170.

5. Okamoto, Y.; Aburatani, R.; Miura, S.; Hatada. K. Chiral stationary phases for HPLC: cellulose tris(3,5-dimethylphenylcarbamate) and tris(3,5-dichlorophenylcarbamate) chemically bonded to silica gel. J. Liq. Chromatogr. **1987**, *10*, 1613–1628.

6. Kimata, K.; Tsuboi, R.; Hosoya, K.; Tanaka, N. Chemically bonded chiral stationary phase prepared by the polymerization of cellulose p-vinylbenzoate. Anal. Methods Instrum. **1993**, *1*, 23–29.

7. Yashima, E.; Fukaya, H.; Okamoto, Y. 3,5-Dimethylphenylcarbamates of cellulose and amylose regioselectively bonded to silica gel as chiral stationary phases for high-performance liquid chromatography. J. Chromatogr. A **1994**, *677*, 11–19.

8. Oliveros, L.; López, P.; Minguillón, C.; Franco, P. Chiral chromatographic discrimination ability of a cellulose 3,5-dimethylphenylcarbamate/ 10-undecenoate mixed derivative fixed on chromatographic matrices. J. Liq. Chromatogr. **1995**, *18*, 1521–1532.

9. Minguillón, C.; Franco, P.; Oliveros, L.; López, P. Bonded cellulose-derived high-performance liquid chromatography chiral stationary phases I. Influence of the degree of fixation on selectivity. J. Chromatogr. A **1996**, *728*, 407–414.

10. Enomoto, N.; Furukawa, S.; Ogasawara, Y.; Akano, H.; Kawamura, Y.; Yashima, E.; Okamoto, Y. Preparation of silica gel-bonded amylose through enzyme-catalyzed polymerization and chiral recognition ability of its phenylcarbamate derivative in HPLC. Anal. Chem. **1996**, *68*, 2798–2804.

11. Franco, P.; Minguillón, C.; Oliveros, L. Solvent versatility of bonded cellulose-derived chiral stationary phases for high-performance liquid chromatography and its consequences in

column loadability. J. Chromatogr. A **1998**, *793,* 239–247.

12. Franco, P.; Senso, A.; Minguillón, C.; Oliveros, L. 3,5-Dimethylphenylcarbamates of amylose, chitosan and cellulose bonded on silica gel: Comparison of their chiral recognition abilities as high-performance liquid chromatography chiral stationary phases. J. Chromatogr. A **1998**, *796,* 265–272.

13. Kim, B.-H.; Lee, W. Direct liquid chromatographic enantiomer separation of *N*-tert-butoxycarbonyl and *N*-benzyl-oxycarbonyl α-amino acids using polysaccharide derived chiral stationary phases. J. Liq. Chromatogr. & Rel. Tech. **1999**, *22*, 523–530.

14. Rizzi, A.M. Band broadening in high-performance liquid chromatographic separations of enantiomers with swollen microcrystalline cellulose triacetate packings : I. Influence of capacity factor, analyte structure, flow velocity and column loading. J. Chromatogr. **1989**, *478*, 71–86.

15. Miyazawa, T.; Shindo, Y.; Yamada, T.; Kuwata, S. Enantiomeric separation of *N*-protected non-protein amino acid esters by chiral high-performance liquid chromatography**.** Anal. Lett. **1993**, *26*, 457–473.

16. Spöndlin, C.; Küsters, E. Direct determination of enantiomeric purity of FMOC amino acids with high performance liquid chromatography. Chromatographia **1994**, *38,* 325–329.

17. Miyazawa, T.; Shindo, Y.; Yamada, T. Separation of enantiomers of *N*-protected non-protein amino acid esters by chiral high-performance liquid chromatography. chromatographia **2004**, *60*, 419–424.

18. Bodansky, M.; Bodansky, A. The Practice of Peptide Synthesis, Springer: New York, 1984.

Table 5.1 Effect of mobile phase on the enantiomer separation of some *N*-FMOC α-amino acids ethyl esters on Chiralpak IA

Mobile phase[a]	5% 2-Propanol/ Hxn			10% THF/Hxn			10% Ethyl acetate/Hxn			20% Chloroform/ Hxn		
Analyte	α^b	$k'_1{}^c$	Conf.[d]	α^b	$k'_1{}^c$	Conf.[d]	α^b	$k'_1{}^c$	Conf.[d]	α^b	$k'_1{}^c$	Conf.[d]
Ala	1.60	4.29	L	1.30	5.81	L	1.13	7.46	L	1.00	6.61	–
Leu	2.63	3.69	L	1.69	4.51	L	1.14	5.18	L	1.00	5.76	–
Phe	1.30	7.05	L	1.10	9.75	D	2.41	9.68	D	1.20	7.15	D
PG	1.14	8.18	L	1.09	8.73	D	1.21	8.95	D	1.07	7.37	D
Val	1.42	3.15	L	1.22	4.07	D	1.12	4.23	D	1.40	2.75	D

[a] Mobile phase: Hexane (Hxn), Tetrahydrofuran (THF); Flow rate = 1 mL/min; UV detection at 254 nm.

[b] Separation factor.

[c] Capacity factor for the first eluted enantiomer.

[d] Indicates the absolute configuration of the second retained enantiomer.

Table 5.2 Enantiomer separation of *N*-FMOC α-amino acids ethyl esters on Chiralpak IA and Chiralpak AD

Entry	Analyte	Chiralpak IA				Chiralpak AD			
		α[a]	k'_1[b]	Rs[c]	Conf.[d]	α[a]	k'_1[b]	Rs[c]	Conf.[d]
1	Ala	1.60	4.29	5.84	L	1.61	2.56	5.42	L
2	ABA[e]	1.86	3.97	6.90	L	1.84	2.45	6.73	L
3	ACA[f]	1.77	3.54	6.21		1.77	1.79	5.49	
4	Asn	1.06	8.82[g]	0.75	L	1.00	8.93	–	–
5	Asp	1.25	9.70	3.18	L	1.04	5.04	0.76	L
6	Glu	1.48	10.59	5.22	L	1.57	4.91	5.39	L
7	Gln	1.27	8.57[g]	2.81	L	1.33	7.21	3.90	L
8	Ileu	1.63	2.92	5.91	L	1.72	1.79	5.40	L
9	Leu	2.63	3.69	9.34	L	3.03	2.00	8.59	L
10	Met	1.68	7.96	7.83	L	1.89	4.26	8.08	L
11	Norleu	2.06	3.76	7.61	L	2.30	2.05	7.14	L
12	Norval	2.22	3.89	8.58	L	2.48	2.24	8.91	L
13	Phe	1.30	7.05	3.77	L	1.38	4.15	4.64	L
14	PG	1.14	8.18	1.63	L	1.15	4.93	1.84	L
15	Ser	1.50	4.78[g]	5.66	L	1.71	4.27	7.17	L
16	Thr	1.91	4.57[g]	8.42	L	1.89	4.81	8.36	L
17	Tyr	1.22	15.14[g]	2.89	L	1.27	11.85[h]	3.15	L
18	Val	1.42	3.15	4.30	L	1.52	1.98	4.72	L

Mobile phase: 5% and 10% 2-propanol/hexane(V/V) on Chiralpak IA and Chiralpak AD, respectively; Flow rate = 1 mL/min; UV detection at 254 nm. [a] Separation factor. [b] Capacity factor for the first eluted enantiomer. [c] Resolution factor. [d] Indicates the absolute configuration of the second retained enantiomer. [e] 2-Aminobutyric acid. [f] 2-Aminocaprylic acid. [g,h] 10% and 15% 2-propanol/hexane(V/V), respectively.

Chapter Ⅵ

Liquid Chromatographic Enantiomer Separation of *N*-Phthaloyl Protected α-Amino Acids on Coated and Immobilized Chiral Stationary Phases Derived from Polysaccharide Derivatives

ABSTRACT

The liquid chromatographic enantiomer separation of *N*-phthaloyl (PHT) protected α-amino acids on several coated and immobilized chiral stationary phases (CSPs) derived from polysaccharide derivatives was performed. The coated CSP of Chiralpak AD showed more or less enantioseparation than the covalently bonded CSP of Chiralpak IA with the same chiral selector of amylose tris(3,5-dimethylphenylcarbamate). However, the coated Chiralcel OD showed greater enantioseparation than the covalently bonded Chiralpak IB with the same chiral selector of cellulose tris(3,5-dimethylphenylcarbamate). Among all examined CSPs, Chiralcel OD afforded the greatest performance for enantiomer resolution of *N*-PHT α-amino acids and, therefore, all analytes enantiomers were base-line separated on Chiralcel OD. The chromatographic method developed in this study was usefully applied for determination of the enantiomeric purity of commercially available *N*-PHT α-amino acids analytes.

Keywords: Enantiomer separation, *N*-Phthaloyl α-amino acids, Chiral stationary phase

6.1. INTRODUCTION

N-Protected α-amino acids in the fields of pharmaceutical chemistry and biochemistry have been extensively used as important chiral building blocks of peptides and proteins.[1,2] Consequently, the studies of the determination of enantiomeric purity of *N*-protected α-amino acids have been of great interest and many techniques for these determinations have been developed and employed.[3] Among several *N*-protecting groups for α-amino acids, the *N*-phthaloyl (PHT) group is attractive in certain instances,[4] because it has often been used for protecting moiety of not only α-amino acids but also primary amine compounds and it can be readily removed under mild reaction conditions of hydrazine reagents.[5,6] Also the *N*-PHT α-amino acid derivatives have been usefully employed as chiral auxiliaries or chiral resolving agents for asymmetric synthesis.[7-9] And it has been reported that the *N*-PHT group as a chromophoric derivative of the α-amino acids derivatives can be applied for circular dichroism studies.[10] In spite of these chiral potential utility of the *N*-PHT α-amino acid acids, very a few of results for enantiomer separation of *N*-PHT α-amino acids have been reported.[11,12] For example, the enantioseparation of five *N*-PHT α-amino acids as well as their ester and amide derivatives has been performed on Pirkle-type CSP with reasonable separation factors.[11] And on macrocyclic antibiotic ristocetin A bonded CSP enantiomer resolution of only two analytes of *N*-PHT methionine and *N*-PHT α-amino-n-butyric acid has been reported.[12] In this study, we present the liquid chromatographic enantiomer resolution of several *N*-PHT protected α-amino acids on several coated and immobilized CSPs derived from polysaccharide derivatives.[13-15]

6.2. EXPERIMENTAL

Chromatography was performed at room temperature using an HPLC consisting of a Waters model 510 pump, a Rheodyne model 7125 injector with a 20 μL loop, a variable wavelength UV detector (Waters 484) and an HP 3396 series II recorder. All CSPs columns were purchased from Daicel Chemical Company (Tokyo, Japan). HPLC-grade hexane and 2-propanol were obtained from J. T. Baker (Phillipsburg, NJ). Trifluoroacetic acid (TFA) was obtained from Aldrich (Milwaukee, WI). The *N*-PHT racemic and L-α-amino acids were prepared according to the reported procedures.[2,16] And *N*-PHT L-glutamic acid and L-phenylalanine were obtained from Fluka company.

6.3. RESULTS AND DISCUSSION

Tables 6.1 and 6.2 show chromatographic results for the separation of the enantiomers of *N*-PHT α-amino acids not only on the most commonly used coated CSPs such as Chiralcel OD, Chiralpak AD, Chiralpak AS and Chiralcel OF[17,18] but also on the covalently CSPs, Chiralpak IA and Chiralpak IB.[13-15] Among all examined CSPs, in general, Chiralcel OD, the cellulose tris(3,5-dimethylphenylcarbamate) coated type CSP showed the greatest separation factor (α = 1.10-2.64) and, therefore, all investigated *N*-PHT α-amino acids enantiomers were base-line separated on Chiralcel OD, whereas Chiralpak AS showed the smallest separation factor. In Table 6.2 Chiralcel OF shows generally lower performance than Chiralcel OD and, however, it provided base-line separation except two analytes. Also Chiralcel OD, the cellulose tris(3,5-dimethylphenylcarbamate) coated CSP showed generally greater enantioselectivities than Chiralpak AD, the amylose tris(3,5-dimethylphenylcarbamate) coated CSP in Table 6.1 Similarly, Chiralpak IB, the immobilized CSP of the same chiral selector of Chiralcel OD showed generally better performance than Chiralpak IA, the immobilized CSP of the same chiral selector of Chiralcel AD, as shown in Table 6.3. Although the separation factors of all analytes on the covalently bonded Chiralpak IB showed lower enantioseparation than those on the coated Chiralcel OD with the same chiral selector, all analytes except for two cases (entries 5 and 10) provided quite good enantioselectivities (α = 1.13–1.73) on Chiralpak IB using 2-propanol in hexane with 0.1% TFA as a mobile phase.[14,15] However, Chiralpak IA of the covalently bonded CSP showed more or less enantioseparation than Chiralpak AD of the coated CSP with the same chiral selector.[13,15]

It is noted that the L-enantiomers of all examined *N*-PHT α-amino acids are selectively retained on Chiralcel OD, Chiralpak AS, Chiralcel OF and Chiralpak IB and, however, the elution orders of enantiomer separation of *N*-PHT α-amino acids are not always consistent on Chiralpak AD and Chiralpak IA derived from amylose tris(3,5-dimethylphenylcarbamate). It is considered that more than one chiral recognition process between *N*-PHT α-amino acid analyte and the chiral selector of amylose tris(3,5-dimethylphenylcarbamate) might be involved. Contrary to Chiralcel OD and Chiralpak IB, the D-enantiomers of all examined analytes except for *N*-PHT glutamic acid are selectively retained on Chiralpak IA and, therefore, these CSPs in Tables 6.1 and 6.3 might be complementarily used for the reversal of elution order of the

determination of enantiomeric purity.[19] The chromatographic method developed in this study was used for determination of the enantiomeric purity of two commercially available reagents of *N*-PHT L-glutamic acid and *N*-PHT L-phenylalanine. The enantiomeric impurities of 0.4% for these two samples were determined on Chiralcel OD. Chromatograms of determination of the enantiomeric purity of these analytes are presented in Figures 6.1 and 6.2.

6.4. CONCLUSION

we demonstrated the liquid chromatographic separation of enantiomers of *N*-PHT protected α-amino acids on several coated and immobilized CSPs derived from polysaccharide derivatives. This is the first reported for the enantiomer resolution of several *N*-PHT α-amino acids using polysaccharide-derived CSPs. Among all examined CSPs in this study, the cellulose tris(3,5-dimethylphenylcarbamate) derived CSPs, Chiralcel OD and Chiralpak IB showed quite good enantioselectivities. Especially, since Chiralcel OD showed excellent resolving ability for the enantiomer resolution of all *N*-PHT α-amino acids, it is expected to be quite useful for determination of the enantiomeric purity of these analytes and their related compounds.

REFERENCES

1. Sheldon, R. A. Chirotechnology: Industrial synthesis of optically active compounds, Marcel Dekker: New York, 1993.

2. Bodansky, M.; Bodansky, A. The Practice of Peptide Synthesis, Springer: New York, 1984.

3. Subramanian, G., Ed.; A Practical Approach to Chiral Separations by Liquid Chromatography, New York: VCH, 1994.

4. Greene, T. W.; Wuts, P. G. M. Protective Groups in Organic Synthesis, John Wiley & Sons. Inc, 3rd Ed., New York, 1999.

5. Birch, D.; Hill, R. R.; Jeffs, G. E.; North, M. Stereochemical preference for heterochiral coupling controls selectivity in competitive peptide synthesis. J.C.S. Chem. Commun. **1999**, 941–942.

6. Lei, A.; Wu, S.; He, M.; Zhang, X. Highly enantioselective asymmetric hydrogenation of α-phthalimide ketone: an efficient entry to enantiomerically pure amino alcohols. J. Am. Chem. Soc. **2004**, *126*, 1626–1627.

7. Müller, R.; Goesmann, H.; Waldmann, H. *N*,*N*-Phthaloylamino acids as chiral auxiliaries in asymmetric Mannich-type reactions. Angew. Chem. Int. Ed. **1999**, *38*, 184–187.

8. Müller, R.; Röttele, H.; Henke, H.; Waldmann, H. Asymmetric steering of the Mannich reaction with phthaloyl amino acids. Chem. Eur. J. **2000**, *6*, 2032–2043.

9. Krasnov, V. P.; Levit, G. L.; Kodess, M. I.; Charushin, V. N.; Chupakhin, O. N. *N*-Phthaloyl-(*S*)-alanyl chloride as a chiral resolving agent for the kinetic resolution of heterocyclic amines, Tet. Asymm. **2004**, *15*, 859–862.

10. Skowronek, P.; Katrusiak, A.; Gawronski, J. Phthalimide as a chromophoric tag in the circular dichroism determination of absolute configuration of α-amino acid amides and dipeptides. A case of a dipeptide isostructurality. Tetrahedron **2002**, *58*, 10463–10468.

11. Pirkle, W. H.; Lee, W. Separation of the enantiomers of *N*-benzyloxycarbonyl, 9-fluoromethoxycarbonyl and phthaloyl protected α-amino acids and their ester or amide derivatives. Bull. Kor. Chem. Soc. **1998**, *19*, 1277–1280.

12. Ekborg-Ott, K. H.; Liu, Y.; Armstrong, D. W. Highly enantioselective HPLC separations using the covalently bonded macrocyclic antibiotic, ristocetin A, chiral stationary phase. Chirality **1998**, *10*, 434–483.

13. Zhang, T.; Kientzy, C.; Franco, P.; Ohnishi, A.; Kagamihara, Y.; Kurosawa, H. Solvent versatility of immobilized 3,5-dimethylphenylcarbamate of amylose in enantiomeric separations by HPLC. J. Chromatogr. A **2005**, *1075*, 65–75.
14. Zhang, T.; Nguyen, D.; Franco, P.; Murakami, T.; Ohnishi, A.; Kurosawa, H. Cellulose 3,5-dimethylphenylcarbamate immobilized on silica: A new chiral stationary phase for the analysis of enantiomers. Anal. Chim. Acta **2006**, *557*, 221–228.
15. Jin, J. Y.; Lee, W.; Park, J. H.; 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. J. Liq. Chrom. & Rel. Tech. **2006**, *29*, 1793–1801.
16. McArthur, C. R.; Worster, P. M.; Okon, A. U. Amino group blocking: Improved method for *N*-phthaloylation using *N*-(ethoxycarbonyl)phthalimide. Syn. Commun. **1983**, *13*, 311–318.
17. Lee, W. Separation of the enantiomers of amino acid esters as benzophenone imine derivatives using polysaccharide derived chiral stationary phases. Chromatographia **1999**, *49*, 61–64.
18. Refer to Application guide for chiral HPLC selection 4th ed.; Daicel Chemical Industries, Ltd.
19. Jin, J. Y.; Lee, W.; Hyun, M. H. Development of the antipode of the covalently-bonded crown ether type chiral stationary phase for the advantage of the reversal of elution order. J. Liq. Chrom. & Rel. Tech. **2006**, *29*, 841–848.

Table 6.1 Enantiomer separation of *N*-PHT α-amino acids on Chiralpak AD and Chiralcel OD

Entry	Analyte	Chiralpak AD				Chiralcel OD			
		α^a	$k'_1{}^b$	Rs^c	Conf.[d]	α^a	$k'_1{}^b$	Rs^c	Conf.[d]
1	2-Aminobutyric acid	1.03	8.87	0.27	D	2.01	3.96	7.60	L
2	2-Aminocaprylic acid	1.00	6.93	–		1.91	2.70	9.70	
3	Ala	1.05	12.09	0.71	L	1.47	4.27	4.55	L
4	Glu	1.13	6.61[e]	1.66	L	2.64	6.78[e]	8.49	L
5	Ile	1.08	7.94	1.32	L	1.10	2.44	0.95	L
6	Leu	1.31	5.93	4.34	D	1.20	3.87	1.96	L
7	Met	1.07	14.65	1.22	D	1.90	6.68	7.02	L
8	Norleu	1.13	6.58	1.89	D	1.76	3.05	5.82	L
9	Norval	1.00	8.36	–		2.11	3.13	7.40	L
10	Phe	1.86	10.44	9.72	D	1.38	8.25	3.08	L
11	PG	1.28	16.32	4.03	D	1.76	7.52	5.52	L
12	Val	1.16	8.77	2.32	L	1.34	2.91	2.84	L

Mobile phase: 5% 2-propanol/hexane (V/V) containing 0.1% TFA; UV detection at 254 nm, Flow rate = 1 mL/min. [a] Separation factor. [b] Capacity factor for the first eluted enantiomer. [c] Resolution factor. [d] Indicates the absolute configuration of the second eluted enantiomer. [e] 10% 2-propanol/hexane (V/V) containing 0.1% TFA.

Table 6.2 Enantiomer separation of *N*-PHT α-amino acids on Chiralpak AS and Chiralcel OF

Entry	Analyte	Chiralpak AS				Chiralcel OF			
		α[a]	k'$_1$[b]	Rs[c]	Conf.[d]	α[a]	k'$_1$[b]	Rs[c]	Conf.[d]
1	2-Aminobutyric acid	1.16	4.44	1.29	L	1.00	9.22	-	
2	2-Aminocaprylic acid	1.00	3.71	–		1.74	3.67	2.67	
3	Ala	1.36	6.43	3.26	L	1.44	8.05	2.54	L
4	Glu	1.41	11.35[e]	2.17	L	1.37	9.68[e]	1.53	L
5	Ile	1.41	3.09	3.24	L	2.17	3.33	4.15	L
6	Leu	1.00	3.30	–		1.93	3.40	3.24	L
7	Met	1.00	11.21	–		1.56	5.16[e]	2.35	L
8	Norleu	1.07	4.14	0.40	L	1.77	4.57	3.18	L
9	Norval	1.15	4.34	1.25	L	1.82	4.92	3.42	L
10	Phe	1.00	8.57	–		1.60	4.16[e]	2.17	L
11	PG	1.35	9.25	2.35	L	1.07	7.96[e]	0.36	L
12	Val	1.66	3.97	3.72	L	2.05	3.87	3.88	L

Mobile phase: 5% 2-propanol/hexane (V/V) containing 0.1% TFA; UV detection at 254 nm, Flow rate=1 mL/min. [a] Separation factor. [b] Capacity factor for the first eluted enantiomer. [c] Resolution factor. [d] Indicates the absolute configuration of the second eluted enantiomer. [e] 10% 2-propanol/hexane (V/V) containing 0.1% TFA.

Table 6.3 Enantiomer separation of *N*-PHT α-amino acids on Chiralpak IA and Chiralpak IB

Entry	Analyte	Chiralpak IA				Chiralcel IB			
		α^a	$k'_1{}^b$	Rs^c	Conf.[d]	α^a	$k'_1{}^b$	Rs^c	Conf.[d]
1	2-Aminobutyric acid	1.08	6.47	1.23	D	1.39	2.91	4.29	L
2	2-Aminocaprylic acid	1.09	5.24	1.31		1.16	1.96	2.00	
3	Ala	1.00	8.06	–		1.15	3.44	1.84	L
4	Glu	1.07	5.86[e]	0.74	L	1.73	5.14[e]	5.63	L
5	Ile	1.10	6.03	1.46	D	1.07	1.99	0.81	L
6	Leu	1.33	4.56	4.95	D	1.12	2.25	1.52	L
7	Met	1.14	10.35	2.20	D	1.32	5.02	3.75	L
8	Norleu	1.19	4.94	2.79	D	1.27	2.28	2.57	L
9	Norval	1.12	5.83	1.85	D	1.41	2.52	5.11	L
10	Phe	1.63	7.66	8.70	D	1.00	5.32	–	
11	PG	1.35	11.65	4.73	D	1.25	5.39	2.55	L
12	Val	1.04	6.48	0.54	D	1.13	2.18	1.57	L

Mobile phase; 5% 2-propanol/hexane (V/V) containing 0.1% TFA; UV detection at 254 nm, Flow rate = 1 mL/min. [a]Separation factor. [b]Capacity factor for the first eluted enantiomer. [c]Resolution factor. [d]indicates the absolute configuration of the second eluted enantiomer. [e]10% 2-propanol/hexane (V/V) containing 0.1% TFA.

.

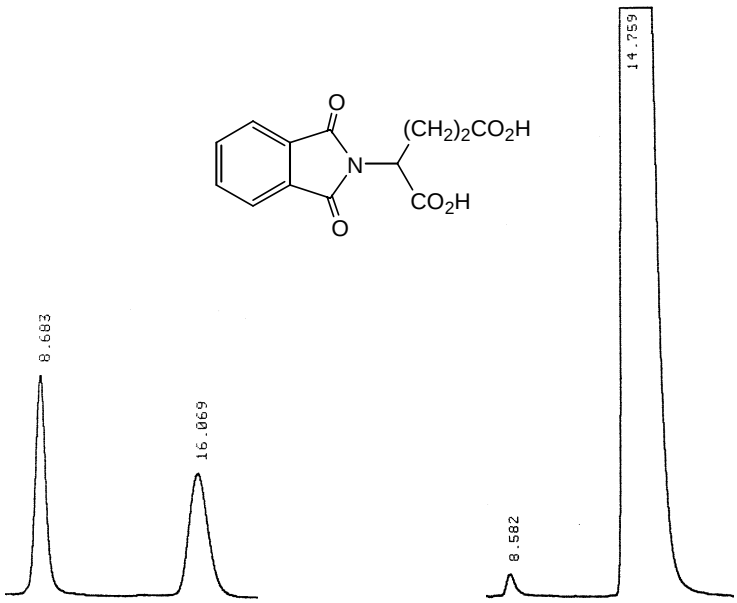


Figure 6.1 Chromatograms of enantiomer separation of racemic *N*-PHT glutamic acid (the left) and *N*-PHT L-glutamic acid (Fluka reagent) (the right, D:L = 0.4: 99.6) on Chiralcel OD; Mobile phase: 20% 2-propanol/hexane (V/V) containing 0.1% TFA; Flow rate = 1 mL/min; UV detection at 254 nm; Injected amount 20–100 μg.

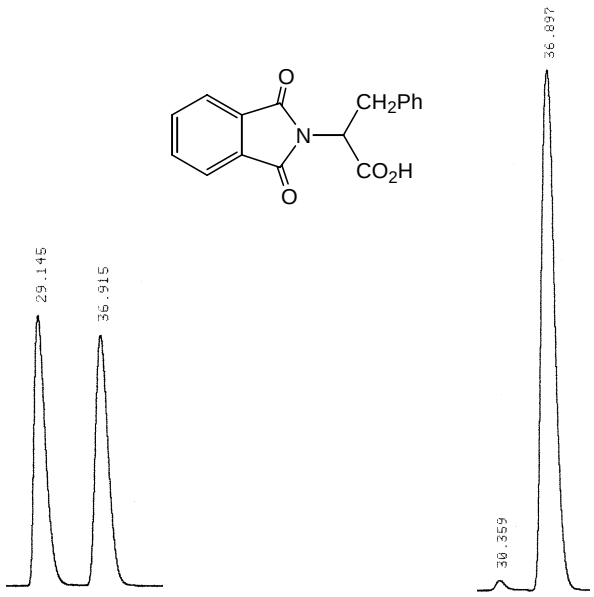


Figure 6.2 Chromatograms of enantiomer separation of racemic *N*-PHT phenylalanine (the left) and *N*-PHT L-phenylalanine (Fluka reagent) (the right, D:L = 0.4: 99.6) on Chiralcel OD; Mobile phase: 5% 2-propanol/hexane (V/V) containing 0.1% TFA; Flow rate = 1 mL/min; UV detection at 254 nm; Injected amount 40–60 μg.

Acknowledgements

I would like to express my gratitude to my advisor, 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 offered many helpful suggestions. The thesis committee members, professor Chae-Sun Baek, Ok-Hyun Ko, Joon-Hee Hong and Beom-Gyu Lee to whom I extend my sincere thanks, for giving me the excellent comments. I would like to remenber the help from professor Chae-Sun Baek who gave me many instructions and advices in grasping the experimental technology and pharmacy knowledge. My gratitude also extends to Chun-Shan An and Fu Kim, for their kind supports. Also, I would like to thank all the Chinese graduates in my Pharmacy College with the good relationship among us like a family which make me sweetly during these years. Here, I wish to say to my parents that there are no achievements I got today and I would not be brave enough to study abroad, without their warmful supports. At last, I wish to say “thank you” to my husband, Zhi-Song Li, and my lovely son, Zhong-Ze Li, for your help and encouragement both on my work and my life. I will miss my time in graduate school.