Chiral Separation of *N*-Fluorenylmethoxycarbonyl α-Amino Acids on Quinine Carbamate-Bonded Silica by High Performance Liquid Chromatography

Munrak Lee, Myung Duk Jang,† Wonjae Lee,‡ and Jung Hag Park*

*Department of Chemistry, Yeungnam University, Gyeongsan 712-749, Korea. *E-mail: jhpark@ynu.ac.kr*
†Department of Gas Safety, Kundong University, Andong 760-833, Korea
‡College of Pharmacy, Chosun University, Gwangju 501-759, Korea
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Amino acids are essential chiral compounds in pharmaceutical and biochemical fields and *N*-protected α-amino acids have been widely used as important chiral building blocks.[1] The fluorenylmethoxycarbonyl (FMOC) group is one of the most useful *N*-protecting groups for α-amino acids and provides the advantage of high sensitivity in fluorescence detection.[2-4] Due to the importance of optical purity of *N*-FMOC α-amino acids, convenient and accurate methods to determine the enantiopurity of these compounds have been required. Several methods for the liquid chromatographic separation of the enantiomers of *N*-FMOC α-amino acids have been reported using chiral stationary phases (CSPs) derived from cyclodextrin, macrocyclic antibiotics and cellulose derivatives as well as small molecules derived brush-type CSPs.[5-14] Although the enantioseparation of some *N*-FMOC secondary amino acids has been performed on 1-naphthylethyl carbamoylated β-cyclodextrin bonded CSP with good resolution,[6] most *N*-FMOC α-amino acids enantiomers have not been resolved on β- and γ-cyclodextrin bonded CSPs.[7] It has been reported that nine *N*-FMOC α-amino acids enantiomers were separated on macrocyclic antibiotics derived CSP.[8,9] Brush type CSPs derived from α-amino acids including Whelk-O CSP have been employed to resolve some *N*-FMOC α-amino acids enantiomers.[10,11] There have been some reports on resolution of only a few of *N*-FMOC α-amino acids enantiomers on quinine derivatives-bonded stationary phases by LC.[15-19] Xiong *et al.* resolved five *N*-FMOC α-amino acids enantiomers on a *tert*-butyl carbamoylated quinine CSP in normal phase LC.[15] Five or six *N*-FMOC α-amino acids enantiomers resolved on quinine-calixarene hybrid type CSPs in polar organic mobile phases by Krawinkler and coworkers.[16,17] However, on quinine carbamate-bonded CSPs in reversed-phase LC (RPLC) enantiomers of only one *N*-FMOC α-amino acid were resolved and resolution of a series of these analytes has not been well investigated.[18,19] Quinine carbamate-bonded silica CSPs are simpler to prepare than other quinine derivative CSPs and yet provide good resolution for *N*-protected α-amino acids other than *N*-FMOC derivatives. In this work, we report the separation of sixteen *N*-FMOC α-amino acids on a carbamoylated quinine-bonded silica (QNS) in RPLC. We prepared a microbore (1 mm I.D.) column that leads to many advantages such as low consumption of both mobile and stationary phases *etc.*[20-22] Retention and enantioselectivity values on QNS were measured in mobile phases of varying pH, type and concentration of buffer and type and composition of organic modifier to find optimum separation conditions for the *N*-FMOC α-amino acids on QNS in RPLC.

Experimental Section

Reagents and materials. All reagents used for the preparation of the stationary phase were reagent grade or better. Quinine, anhydrous toluene, petroleum ether, *N,N*-dimethylformamide and anhydrous tetrahydrofuran were obtained from Aldrich (Milwaukee, USA). Methanol was HPLC grade (J.T. Baker, Phillipsburg, USA). *n*-Hexane was purchased from EM Sciences (Gibbstown, HPLC-grade methanol was obtained from J.T. Baker (Phillipsburg, USA). Water was processed with an Elgastat UHQ water purification system (Bucks, UK). All the chemicals were of the best quality available and used as received without any further purification. *N*-FMOC protected α-amino acids were prepared according to the literature.[23] Silica, having a mean pore size of 10 nm, specific surface area of 350 $m^2\ g^{-1}$ and a mean particle diameter of 5 μm, was obtained from Macherey-Nagel (Düren, Germany). Carbamoylated quinine-bonded silica (QNS) was prepared according to a procedure in the literature.[19] Bonding density based on the percent carbon from microanalysis was found to be 1.36 $\mu mol\ m^{-2}$.

Chromatography. QNS was suspended in a (1:1) hexane/2-propanol mixture and packed into 15 cm × 1 mm (ID) columns using the downward slurry method at *ca.* 5000 psi. 2-Propanol was employed as the displacing solvent. A chromatographic system consisting of a Model 7520 injector with a 0.5-μL internal loop (Rheodyne, CA, USA), a Model 530 column oven (Alltech, IL, USA) set at 25 °C and a Linear Model 200 UV/VIS detector (Alltech, IL, USA) with a 0.25-μL flowcell set at 254 nm was used. A Younglin Autochro-2000 chromatographic data acquisition system was used to record chromatogram. The mobile phases were mixtures of methanol and ammonium acetate or sodium hydrogen phosphate buffer. They were filtered through a

membrane filter of 0.5-μm pore size and degassed prior to use. The flow rate was 70 μL min^{-1}. Deuterated water was used as the dead time marker by noting the baseline disturbance due to the refractive index difference. Peak identification was carried out by injecting solutions of each enantiomer of amino acids. The plate numbers for an unretained marker on the column was found to be around 8000 per meter in the methanol-aqueous buffer mobile phases while varying somewhat with the composition of the eluent.

Results and Discussion

Retention of acidic analytes on quinine-bonded CSPs is controlled by a mixed mode of ion-pairing and reversed phase mechanism.[19] A number of mobile phase variables such as type and concentration of buffer, pH, type and content of organic modifier can be used to control the analyte retention and enantioselectivity on QNS. Methanol was chosen as organic modifier since it is one of the typically used organic modifier in RPLC and is very polar and hydrogen bond acidic (donating) in nature so that one can see feasibly how intermolecular hydrogen bonding between the chiral selector and analyte molecule influence retention and selectivity.[24]

In ion-exchange processes type of buffer or counter ions in the mobile phase are to influence retention of analytes as buffer anions compete with amino acid anions for the anionic exchange sites of the quinine ring and thus changing buffer type and concentration cause to change retention. A typical chromatogram for the resolution of racemic DNP-valine in 60:40 (v/v %) methanol/0.1 M Na_2HPO_4 buffer (pH 5.0) is shown in Figure 1. Figure 2 shows enantioselectivity (α), resolution (R_s) (shown as bars), and retention factors (k_1) (shown as symbols) for the first eluting enantiomers in 80/20 (v/v %) methanol/0.1 M aqueous buffer (pH 5.0) composed of different buffer material. Amino acids are retained longer in acetate buffers than in phosphate and citrate buffers, in which retention for most of the amino acids are about the same. Best enantioselectivities are obtained in the eluent of phosphate buffer for most of amino acids except Glu, Asp and Phg. Resolution is highest in the

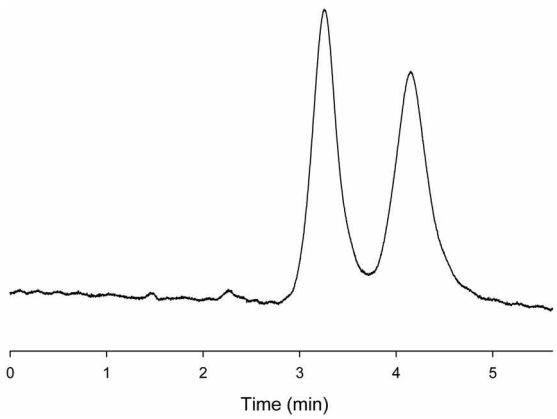

Figure 1. Chromatogram for the separation of racemic DNP-valine in 60:40 (v/v %) methanol/0.1 M Na_2HPO_4 buffer (pH 5.0).

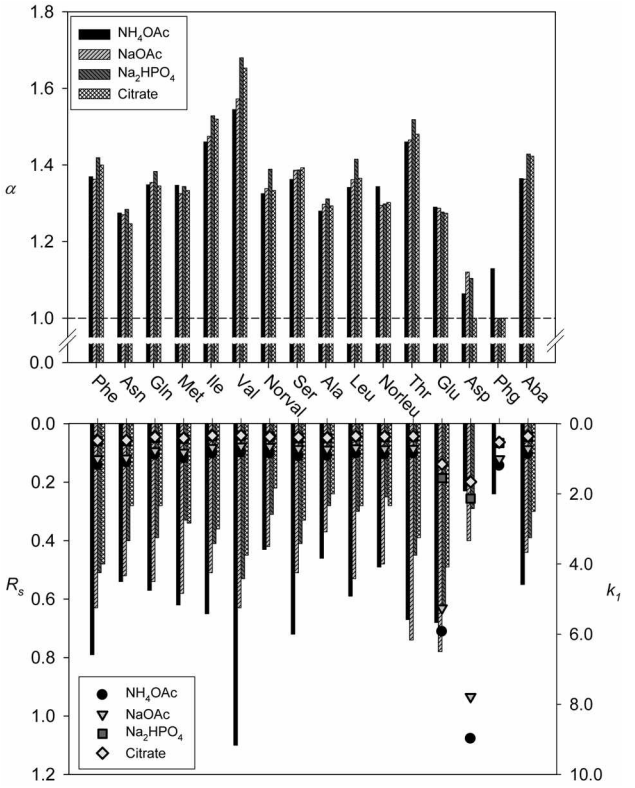

Figure 2. Enantioselectivity (α) and resolution (R_s) and retention factors (k_1) for the first eluting enantiomers in 80/20 (v/v %) methanol/0.1 M aqueous buffer (pH 5.0) composed of different buffer material. *Aba* is acronym for aminobutyric acid.

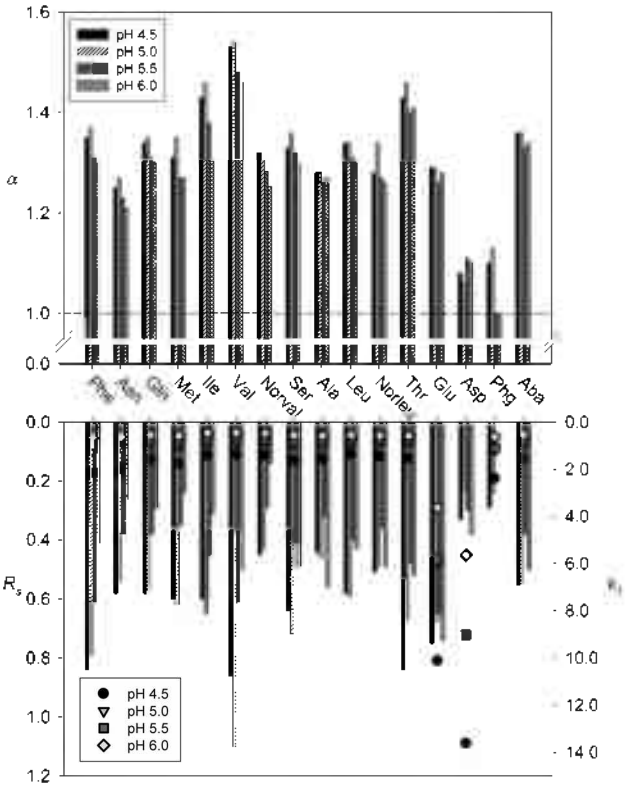

Figure 3. Enantioselectivity (α) and resolution (R_s) and retention factors (k_1) for the first eluting enantiomers in 80/20 (v/v %) methanol/0.1 M ammonium acetate buffer with different pH.

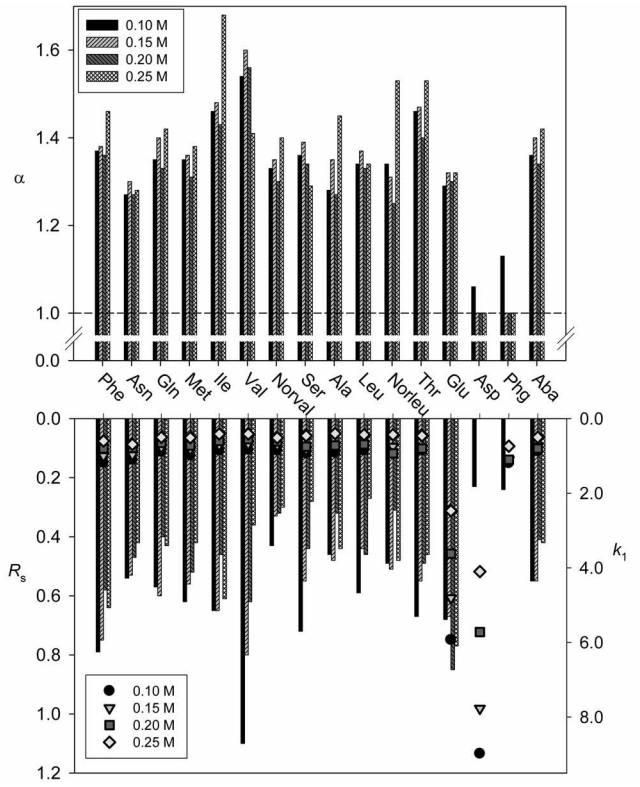

Figure 4. Enantioselectivity (α) and resolution (R_s) and retention factors (k_1) for the first eluting enantiomers in 80/20 (v/v %) methanol/ammonium acetate buffer (pH 5.0) with varying buffer concentration.

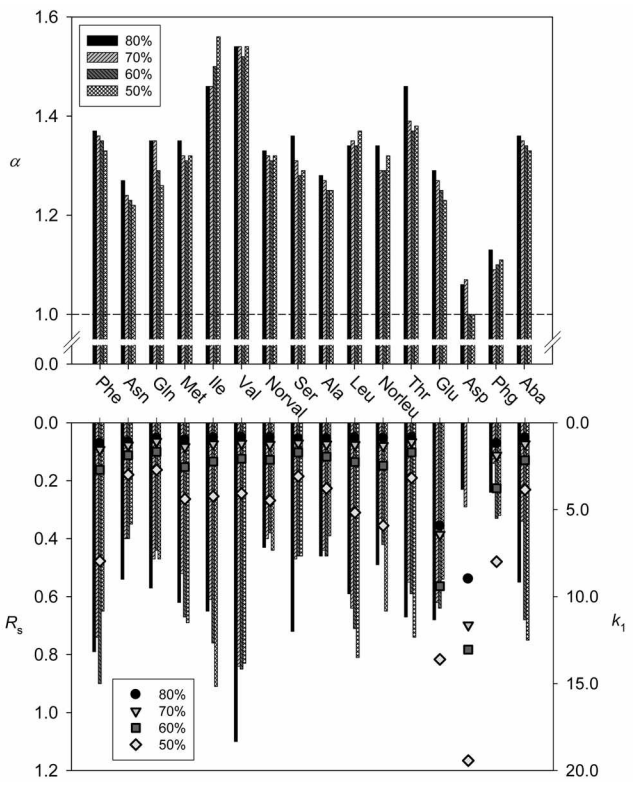

Figure 5. Enantioselectivity (α) and resolution (R_s) and retention factors (k_1) for the first eluting enantiomers in methanol/0.1 M ammonium acetate buffer (pH 5.0) with varying amount of methanol.

eluent of ammonium acetate buffer and decreases in the order, sodium acetate > sodium phosphate > citrate. It follows that with a well-packed column the mobile phase of phosphate buffer would provide the best overall separation efficiency as retention is short and yet the best enantioselectivity is obtained.

Effect of mobile phase pH on α, R_s and k_1 is shown in Figure 3. The mobile used was 80/20 (v/v %) methanol/0.1 M ammonium acetate buffer. Retention is strongly dependent on the eluent pH, decreasing with pH, whereas enantioselectivity does not vary monotonically with pH. For most of amino acids studied best a values are observed at pH 5.0. Variation of resolution is also not monotonic with pH and best R_s values are also observed for most of amino acids at pH 5.

Figure 4 shows variations of α, R_s and k_1 in methanol/ammonium acetate buffer (pH 5.0) with buffer concentration. Concentration of buffer is also to influence retention in ion exchange processes. Buffer anions compete with amino acids anions for the anionic exchange sites of the quinine ring and thus increasing buffer concentration causes decrease in analyte retention. Best resolution is obtained for most of amino acids at buffer concentration of 0.1 M. Enantioselectivity seems to increase with buffer concentration but the extent of variation is not great. On the whole the lower buffer concentration provides best overall separation efficiency as retention does not increase significantly with buffer concentration.

Effect of organic modifier content on α, R_s and k_1 in methanol/0.1 M ammonium acetate buffer (pH 5.0) with varying amount of methanol is shown in Figure 5. Retention decreases with increasing methanol content, showing typical RPLC retention behavior. Enantioselectivity decreases in general with decreasing methanol content but the extent of its decrease is rather small in the range of methanol content from 70 to 50%. This indicates that one can find the optimum retention window by adjusting methanol content without significant loss of enantioselectivity. Resolution does not vary monotonically with methanol content. For some amino acids such as Asn, Gln, Norval and Val, R_s increases with increasing methanol content while for others R_s in general increases with decreasing methanol content. For amino acids with short retention one can use the eluent with lower methanol content to obtain enantioseparation with good resolution.

It can be summarized based on the above observations that overall best enantioseparation of *N*-FMOC amino acids could be obtained in 80/20 (v/v %) methanol/0.1 M ammonium acetate buffer at pH 5.0. One can further control retention for a specific *N*-FMOC amino acid to obtain optimum enantioseparation by manipulating type and concentration of buffer, pH and content of organic modifier in the mobile phase.

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References

1. Schreier, P.; Bernreuther, A.; Huffer, M. *Analysis of Chiral Organic Molecules*; Walter de Gruyter: New York, 1995.
2. Greene, T. W.; Wuts, P. G. M. *Protective Groups in Organic Synthesis*, 3rd Ed.; Wiley: New York, 1999.
3. *Modern Derivatization Methods for Separation Sciences*; Toyooka, T., Ed.; Wiley: New York, 1999.
4. Einarsson, S.; Folestad, S.; Josefsson, B.; Lagerkvist, S. *Anal. Chem.* **1986**, *58*, 1638-1643.
5. *A Practical Approach to Chiral Separations by Liquid Chromatography*; Subramanian, G., Ed.; VCH: New York, 1994.
6. Zukowski, J.; Pawlowska, M.; Armstrong, D. W. *J. Chromatogr.* **1992**, *623*, 33.
7. Tang, Y.; Zukowski, J.; Armstrong, D. W. *J. Chromatogr. A* **1996**, *743*, 261.
8. Ekborg-Ott, K. H.; Liu, Y.; Armstrong, D. W. *Chirality* **1998**, *10*, 434.
9. Piccinini, A.-M.; Schmid, M. G.; Pajpanova, T.; Pancheva, S.; Grueva, E.; Gubitz, G. *J. Biochem. Biophys. Methods* **2004**, *61*, 11.
10. Oi, H.; Kitahara, H.; Aoki, F.; Kisu, N. *J. Chromatogr. A* **1995**, *689*, 195.
11. Pirkle, W. H.; Lee, W. *Bull. Korean Chem. Soc.* **1998**, *19*, 1277.
12. Knoche, B.; Milosavljev, S.; Gropper, S.; Brunner, L. A.; Powell, M. L. *J. Chromatogr. B* **1997**, *695*, 355.
13. Li, Y. H.; Lee, W. *J. Sep. Sci.* **2005**, *28*, 2057.
14. Li, Y. H.; Baek, C.-S.; Jo, B. W.; Lee, W. *Bull. Korean Chem. Soc.* **2005**, *26*, 998.
15. Xiong, X.; Baeyens, W. R. G.; Aboul-Enein, H. Y.; Delanghe, J. R.; Tu, T.; Ouyang, J. *Talanta* **2007**, *71*, 573.
16. Krawinkler, K. H.; Maier, N. M.; Sajovic, E.; Lindner, W. *J. Chromatogr. A* **2004**, *1053*, 119.
17. Krawinkler, K. H.; Maier, N. M.; Ungaro, R.; Sansone, F.; Casnati, A.; Lindner, W. *Chirality* **2003**, *15*, S17-S29.
18. Mandl, A.; Nicoletti, L.; Lammerhofer, M.; Lindner, W. *J. Chromatogr. A* **1999**, *858*, 1.
19. Laemmerhofer, M.; Lindner, W. *J. Chromatogr. A* **1996**, *741*, 33.
20. Hôrvath, C. G.; Preiss, B. A.; Lipsky, S. R. *Anal. Chem.* **1967**, *39*, 1422.
21. Scott, R. P. W.; Kucera, P. *J. Chromatogr.* **1976**, *125*, 251.
22. Ishii, D. In *Introduction to Microscale High-Performance Liquid Chromatography*; Ishii, D., Ed.; VCH: Weinheim, 1988; Chapter 1.
23. Bodansky, M.; Bodansky, A. *The Practice of Peptide Synthesis*; Springer: New York, 1984.
24. Park, J. H.; Jang, M. D.; Kim, D. S. *J. Chromatogr.* **1990**, *513*, 107.