Retention of Fluorescent Amino Acid Derivatives in Ion-pairing Reversed-phase Liquid Chromatography

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Recent studies have shown that pillar array columns enable fast and quantitative analysis of amino acids. However, hydrophilic amino acids still cannot be retained on pillar array columns since they have limited retention ability. Ion-pairing liquid chromatography is a promising means of increasing analyte retention. In this study, the effects of ion-pairing reagents on the retention of eight hydrophilic amino acids (histidine, asparagine, glutamine, serine, arginine, aspartic acid, glycine, and glutamic acid) derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) under reversed-phase conditions on a conventional ODS column were studied. Among the ion-pairing reagents investigated, tetraheptylammonium bromide proved to be the most effective for increasing analyte retention. With a mobile phase consisting of 20 mM citrate buffer (pH 6.3)-acetonitrile (100:40, v/v) and 2 mM tetraheptylammonium bromide, the retention times of the eight NBD-amino acids—except NBD-arginine—were longer than 19.4 min, which was the retention time of NBD-valine when eluted without an ion-pairing reagent. As NBD-valine was well retained on pillar array columns, the chromatographic conditions may thus be applied in the analysis of hydrophilic amino acids using pillar array columns.

Keywords Hydrophilic amino acids, quaternary ammonium ions, fluorescence, 4-fluoro-7-nitro-2,1,3-benzoxadiazole

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Introduction

Amino acids play particular important roles in many biological processes, both as building blocks of proteins and as intermediates in metabolism. Because of their biological significance, the determination of amino acids has attracted great interest in many areas of science, such as biochemistry, clinical science, and food science.

So far, many analytical methods for amino acids using high-performance liquid chromatography (HPLC) instruments coupled to various detection systems have been developed.1–8 However, these methods, which mainly use conventional particle-packed columns, have the disadvantages of long separation time and high analyte consumption. Despite major research efforts and improvements in column packing, the barrier of minimal plate height cannot be surpassed due to the irregular structure of the mobile phase zone.

In the last decades, after micromachining technology from the microelectronics industry was integrated in chemistry, chip-based pillar array columns were produced and used as novel chromatographic columns. Pillar array columns with perfectly ordered structures can shorten separation times significantly without lessening the quality of the separation.9–16 However, pillar array columns filled with non-porous pillars have limited retention ability since only the outer surface of the pillars is available for separation. Therefore, until now, only fluorescent derivatives of hydrophobic amino acids, such as valine (Val), phenylalanine, and tyrosine, derivatized with the fluorogenic reagent, 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), were retained and separated by pillar array columns.14–16 The analysis of hydrophilic amino acids still cannot be performed on pillar array columns because of their weak retention ability.

The addition of an ion-pairing reagent into reversed-phase chromatography might be a promising solution to the poor retention ability of pillar array columns. An ion-pairing reagent with an ionic group and a hydrophobic component in its structure can make analytes neutral and hydrophobic, and thus increase their retention on the nonpolar stationary phase.

Before applying ion-pairing reversed-phase chromatography to pillar array columns, it is necessary to investigate the effect of ion-pairing reagents with conventional columns. The retention behavior of NBD-amino acids in pillar array column is in accordance with the one in conventional columns.16,17 Therefore, the results of conventional columns might be used in the prediction of retention behavior of analytes on pillar array columns.

In our previous study,17 NBD-derivatives of eight amino acids, histidine (His), asparagine (Asn), glutamine (Gln), serine (Ser), arginine (Arg), aspartic acid (Asp), glycine (Gly), and glutamic acid (Glu), were shown to be less retained on a nonpolar stationary phase under reversed-phase conditions than NBD-derivatives of other amino acids. Thus, in this study, we investigate the retention of the eight amino acid NBD-derivatives in ion-pairing reversed-phase chromatography. By examining suitable chromatographic conditions for retaining fluorescent amino acid derivatives (except that of Arg) on a pillar array column, this study will enable the analysis of all kinds of amino acids on pillar array columns.

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Experimental

Reagents and chemicals

NBD-F was obtained from Dojindo Laboratories (Kumamoto, Japan). Amino acids, tetratetramonium bromide (HPLC grade), and tetratetramonium bromide (HPLC grade) were purchased from Sigma Aldrich (St. Louis, MO). Tetratetramonium bromide (HPLC grade) was acquired from Nacalai Tesque (Kyoto, Japan). Methylamine was obtained from Wako Pure Chemical (Osaka, Japan). Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany). Water was purified using a Milli-Q system from Merck Millipore (Darmstadt, Germany).

Fluorescence derivatization

The amino acids in 0.1 M HCl solution (20 μL) and 10 mM NBD-F in acetonitrile (40 μL) were mixed with 180 μL of 0.2 M borate buffer (pH 9.0). The mixture was heated in a water bath at 60°C for 5 min. After cooling the reaction solution in ice water, 120 μL of 0.1 M HCl solution was added to the aliquot to stop the reaction.

Instruments for HPLC-fluorescence detection and chromatographic conditions

The HPLC system consisted of a pump (PU-980, Jasco, Tokyo, Japan), a ternary gradient column (LG-1500-02, Jasco), a degasser (DG-980-50, Jasco), an autosampler (AS-1550, Jasco), an intelligent column thermostat (CO-1560, Jasco), and a fluorescence detector (RF-20A, Shimadzu, Kyoto, Japan). Inertsil ODS-4V (250 × 3.0 mm i.d., 5 μm, GL Sciences, Tokyo, Japan) was used as an analytical column. Chromato-Pro (Run Time Corporation, Kanagawa, Japan) software was used to analyze chromatograms. The column temperature was set at 40°C. Fluorescence detection was carried out at an emission wavelength of 530 nm with excitation at 470 nm. The NBD-amino acids were separated under isocratic elution conditions using different mobile phases, at a flow rate of 0.6 mL/min.

Results and Discussion

Necessity of ion-pairing reagent addition

According to our previous study, NBD-Val is retained on pillar array columns when water-acetonitrile-trifluoroacetic acid (TFA) is used as the mobile phase. Hence, the retention time of NBD-Val on pillar array columns could be used as a reference. The retention time of NBD-Val on a conventional ODS column with water-acetonitrile-TFA (100:40:0.12, v/v/v) mobile phase was 19.4 min. Thus, it was reasoned that when the retention times of the eight NBD-amino acids were longer than 19.4 min on the same conventional ODS column, the derivatives should be as effectively retained on pillar array columns as NBD-Val. It should be noted that, for all the experiments in this study, chromatographic conditions other than mobile phase composition (such as flow rate and column temperature) were the same as stated in the experimental section. The objective of this study was to find a suitable mobile phase composition under which the retention times of the eight NBD-amino acids are longer than 19.4 min.

First, we investigated whether it was possible to achieve the objective without adding an ion-pairing reagent to the mobile phase of 20 mM citrate buffer (pH 6.3)–acetonitrile (100:40, v/v). The retention times of the eight NBD-amino acids were less than 3 min (Fig. 1(a)). To increase their retention, in addition to decreasing the ratio of organic solvent to water, lowering the pH of the buffer is also useful. We calculated pK<sub>a</sub> of NBD-amino acids by MarvinSketch (ChemAxon, Budapest, Hungary). Most of the NBD-amino acids are neutral under pH 3.0 and are anionic under pH 6.3 (Table 1). Therefore, the following three mobile phase conditions were further investigated: 1) 20 mM citrate buffer (pH 3.0)-acetonitrile (100:40, v/v); 2) 20 mM citrate buffer (pH 6.3)-acetonitrile (100:10, v/v); and 3) 20 mM citrate buffer (pH 3.0)-acetonitrile (100:10, v/v). As shown in Figs. 1(b)–1(d), even under 20 mM citrate buffer (pH 3.0)-acetonitrile (100:10, v/v), most NBD-amino acids had shorter retention times than 19.4 min. This clearly indicated that addition of an ion-pairing reagent into the mobile phase was necessary to further increase the retention of the NBD-amino acids.

Optimization of type and concentration of ion-pairing reagent

Quaternary ammonium is a typical ion-pairing reagent for acidic samples. In this study, tetratetramonium bromide (TBAB), tetratetramonium bromide (TPeAB), and tetraheptylammonium bromide (THAB) were used as ion-pairing reagents. The retention of eight NBD-amino acids with a mobile phase of 20 mM citrate buffer (pH 6.3)-acetonitrile (100:40, v/v) and 2 mM each ion-pairing reagent was investigated. As shown in Table 2(a), adding 2 mM THAB largely increased retention of NBD-amino acids while TPeAB or TBAB did not. The chromatogram of the eight NBD-amino acids with the mobile phase containing 2 mM THAB is shown in Fig. 1(e). The retention times of all the NBD-amino acids except NBD-Arg were longer than 19.4 min. Different concentrations of TPeAB (5, 10, 15, and 20 mM) were also investigated. When the TPeAB concentration increased, retention of NBD-amino acids also increased. Even when the TPeAB concentration was increased to 20 mM, the retention times of five NBD-amino acids were still less than 19.4 min. Accordingly, 2 mM THAB was selected as the most suitable ion-pairing reagent to achieve sufficiently long retention times for hydrophilic amino acids derivatized with NBD-F.

The separation after the addition of 2 mM THAB can be explained as follows. For NBD-Arg, it is uncharged at pH 6.3 and does not undergo the ion-pairing effect, and thus it was less retained. For NBD-His, even though the main charge number is also 0 at pH 6.3, 36% of NBD-His are negatively charged, and thus it was more retained than NBD-Arg. As shown in Table 1, the main charge numbers of NBD-Asp and NBD-Glu at pH 6.3 are −2, and due to the strong ion-pairing effect, they were more retained than the NBD-derivatives ([NBD-Gly, -Asn, -Gln, and -Ser] of which the main charge numbers are −1. Concerning NBD-Gly, -Asn, -Gln, and -Ser, similar order of the peaks was observed, which indicated that THAB simply increased the retention of the compounds.

Effects of pH on retention in ion-pairing liquid chromatography

As shown in Table 1, the charge states of NBD-amino acids vary with pH and the NBD-amino acids investigated are neutral or cationic at pH 3.0. To study the interaction between the ion-pairing reagent and positively-charged or neutral analytes, the retention behavior of the eight NBD-amino acids with a 20 mM citrate buffer (pH 3.0)-acetonitrile (100:40, v/v) with 2 mM THAB were investigated. As shown in Fig. 1(f) and Table 2(b), all the NBD-amino acids except NBD-Arg and NBD-His were more retained at pH 3.0 than at pH 6.3. The shorter retention times for NBD-Arg and NBD-His at pH 3.0 are probably because they become positively-charged at pH 3.0 which is similarly charged as the ion-pairing reagent. The other six
NBD-amino acids mainly become negatively-charged at pH 6.3 and have no charge at pH 3.0. It is known that the charge and aliphatic chains of ion-pairing reagents have different effects on retention. The ion-pairing effect increases analyte retention, while longer aliphatic chains of ion-pairing reagents enhance their absorption onto the stationary phase and hence increase analyte retention. The reason why the six NBD-amino acids were more retained at pH 3.0 might be that THAB, with a long aliphatic chain, increases the hydrophobicity of the stationary phase, and thus even neutral solutes are more retained. To confirm the hypothesis, the retention time of NBD-methylamine, a neutral compound, was investigated under ion-pairing reversed-phase chromatographic conditions. The result proved that adding THAB (2 mM) into the elution system increased NBD-methylamine retention (from 8.6 to 12.5 min). This showed that the ion-pairing reagent with a long aliphatic chain increased the hydrophobicity of the stationary phase.

### Conclusions

In this study, retention behaviors of eight NBD-amino acids on a conventional ODS column were investigated under various mobile phase conditions with different pH, organic solvent concentration, and ion-pairing reagent. A mobile phase...
consisting of 2 mM THAB in 20 mM citrate buffer (pH 6.3)-acetonitrile (100:40, v/v) achieved sufficient retention for the eight NBD-amino acids except NBD-Arg. This study will contribute to realization of analysis of hydrophilic amino acids on pillar array columns.

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