The Performance Investigation of Bimodal Cation Exchange/Hydrophilic Interaction Liquid Chromatography–Electrospray Ionization Mass Spectrometry by Modifying Mobile Phase Composition in Amino Acid Separation

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Owing to its unique selectivity, mixed-mode chromatography (MMC) has been extended to the analysis of biological fluids. Among various MMC applications, ion exchange/hydrophilic interaction liquid chromatography (IEX/HILIC) is particularly beneficial for the analysis of polar compounds without derivatization or ion pairing. Nevertheless, no practical information about the retention behavior is discussed. In this study, the chromatographic behavior of amino acids in a CEX/hydrophilic interaction mixed-mode column via the modification of the mobile phase was investigated. A low buffer salt concentration led to increased retention; particularly, positively charged amino acids were not eluted by excessive interactions with the negatively charged stationary phase at a moderate salt concentration (<20 mM). An acid modifier of formic acid or acetic acid exhibited a similar effect on the separation, and a low acid concentration (<0.5%) led to peak broadening by additional ion exchange with a silanol group at high pH. With respect to the composition of organic solvents, aprotic solvents such as tetrahydrofuran or acetonitrile afforded appropriate retention. However, protic solvents led to the considerably reduced retention except positively charged amino acids. Novel, practical information for the optimization or development of applications with a CEX/HILIC column for polar analytes such as amino acids was obtained.

Keywords: Amino acid analysis, Cation exchange, Hydrophilic interaction liquid chromatography, Mixed-mode chromatography

Introduction

Recently, reversed-phase ultra-performance liquid chromatography (RP-UPLC)–electrospray/tandem mass spectrometry (ESI/MS/MS) has been frequently used for assay analyses in various research fields due to the high resolving power of the state-of-the-art LC systems and the inherently high selectivity and sensitivity of tandem mass spectrometers. However, RP chromatography is limited with respect to the retention and analysis of highly polar compounds due to its hydrophobic properties. For this reason, mixed-mode chromatography (MMC) has been introduced.

Typically, MMC can be categorized into cation exchange (CEX)/RP, anion exchange (AEX)/RP, CEX/hydrophilic interaction liquid chromatography (HILIC), and AEX/HILIC bimodal phases, respectively. These MMCs with bimodal ion exchange and a hydrophobic or hydrophilic interaction mechanism in a single stationary phase can retain and separate hydrophobic and hydrophilic compounds, which are not retained by typical reversed-phase high-performance liquid chromatography.[1,2] Diverse applications for MMC using various stationary phases in biological fluids have been reported.[3,4] Among these applications, Choi *et al.*[5] have reported the successful analysis of amino acids using embedded CEX/HILIC UPLC–ESI/MS/MS and demonstrated the simultaneous analysis, separation, and quantitation of amino acids without derivatization in human serum. In their study, amino acids were retained and

[Correction added on 19 July 2019 after first online publication: the duplicate corresponding author's email address has been deleted, and the running header and authors affiliations have been corrected.]

separated on CEX/HILIC under a mild, simple mobile phase condition compatible with MS detection.

From the comparison of the mobile phase and gradient conditions of RP, it is difficult to adjust the mobile phase composition of MMC to achieve optimal chromatographic results due to the complexity of several parameters.[6] Therefore, it is imperative to understand the relationship between the column and mobile phase composition to achieve the best chromatographic condition. For a given mixed-mode CEX/HILIC column, the predominant separation and retention mechanism apparently depends on the mobile phase composition. Choi *et al.*[5] have reported the use of a mobile phase condition comprising 0.1% formic acid in acetonitrile (ACN, A) and 50 mM ammonium formate in water (B). Table S1 (Supporting Information) summarizes the recommended mobile phase conditions by the manufacturers of the CEX/HILIC column used herein. Adjustment with 25–200 mM of ammonium formate and 0.1–0.5% of acetic acid and using the combination of ACN/tetrahydrofuran (THF) were suggested. The retention and separation of acidic and basic compounds on a single mixed-mode CEX/HILIC column are apparently carried out via the control of the mobile phase components such as pH, buffer and acid modifier concentrations, and type of organic solvent.

In the bimodal phase mechanism, it is difficult to adjust the mobile phase composition to achieve satisfactory chromatographic separation, peak shape, peak retention, and MS sensitivity because of their complex mechanism from multiple stationary phases and various mobile phase components. Furthermore, to the best of our knowledge, there is no detailed practical information regarding the role and effect of the mobile phase components in CEX/HILIC. Thus, it is imperative to investigate and understand the role and effect of the mobile phase components to control chromatographic peaks of the compounds separated by CEX/HILIC. A number of studies have reported the retention behavior and applications in the IEX/RP modes,[1,7] while studies using the IEX/HILIC MMC have been scarce. For the purpose of this study, amino acids are good model compounds because of properties, including zwitterion, as well as positive and negative charge properties.

In this study, the role and effect of mobile phase components were investigated, and information related to peak control was obtained. In addition, the types and concentrations of buffer, acid modifier, and organic solvent, on the CEX/HILIC column were investigated.

Experimental

Chemical and Reagents. Table S2 summarizes the amino acids. All amino acid standards were purchased from Sigma (St. Louis, MO, USA). The stock solution of each amino acid was prepared by dilution with 0.1 M HCl, and the sample mixture was prepared by dilution to 10 μg mL^{-1} of Glycine and Cysteine and 1 μg/mL of the others. All of the mobile phases used for chromatography were of high-performance liquid chromatography grade, and ACN, THF, and methanol (MeOH) were obtained from JT Baker (Phillipsburg, NJ, USA). Ethanol (EtOH) was purchased from Daejung Chemical (Daejeon, Korea), and water was purified from a Milli-Q purification system from Millipore (Bedford, MA, USA) and filtered over a nylon membrane filter from Whatman (Maidstone, England).

LC–MS Analysis of Amino Acids. Mass spectrometric analysis was carried out on an API 4000TM triple quadrupole mass spectrometer (Applied Biosystems, MDS Sciex, Toronto, Canada) with a Shimadzu UFLC liquid chromatography system (Tokyo, Japan). An Intrada amino acid column (50 mm length × 3.0 mm I.D., 3.0 μm particle size; Imtakt, Kyoto, Japan) was used for chromatographic separation, and each of the 2 μL of the standard mixture (100 ng/mL) sample was injected to investigate the chromatographic behavior. The mobile phase A was organic solvent such as ACN, THF, MeOH, or EtOH with acid modifier (formic acid or acetic acid), and mobile phase B was aqueous solvent containing buffer salt such as ammonium formate or ammonium acetate. Gradient elution was employed at a flow rate of 0.6 mL/min, and 14% of mobile phase B was held for 3.0 min (initial condition), ramped to 100% B over 7.0 min, and then maintained until 13.0 min. Subsequently, it was ramped for re-equilibration to 14% B over 0.1 min and held for 1.9 min, thereby yielding an overall runtime of 15 min.

An ion source was applied to the Turbo VTM electrospray source, and all of the amino acids were analyzed in the positive-ion mode. The spray voltage and ion source temperature were set to 5400 V and 650 °C, respectively. The gas pressures for source gases I and II, as well as for the curtain gas, were 60, 60, and 20 psi, respectively. Air was used in the ion source. Data acquisition was performed in the multiple reaction monitoring (MRM) mode. Table S2 summarizes the m/z values for the precursor and product ions as well as the collision energy. Most of amino acids were analyzed with different m/z value therefore no interference by co-elution of amino acid. Glutamine and lysine were set to same MRM transition, but they were separated well in most of mobile phase composition. Analyst 1.6 software was used for data acquisition and processing.

Results and Discussion

Owing to the complex binding and elution mechanism of bimodal CEX/HILIC, it is difficult to optimize the mobile phase composition to achieve the best chromatographic condition, which is known to be challenging. In continuing our efforts to understand the relationship between CEX/HILIC and the mobile phase composition, effects of the types and concentrations of the buffer, acid modifier, and organic solvents were investigated.

Effect of the Composition of Salt in the Mobile Phase on the Retention Behavior. To understand the effect of the composition of salt in the mobile phase, the salt concentration and species were investigated. Ammonium formate and

ammonium acetate in the mobile phase (B) were compared for the analysis of the amino acids, with concentrations of 0.1, 1, 10, 20, 50, and 100 mM. Figure S1 shows the changes in the retention time in the presence of ammonium formate, and Figure 1(a) shows the representative chromatograms of each group. In the case of all amino acids, with the decrease in the ammonium formate concentration, the retention time increased. In particular, the retention time drastically increased for positively charged amino acids (such as arginine, histidine, and lysine) at low salt concentration, and amino acids were not detected at less than 20 mM of ammonium formate. On the other hand, other groups (including hydrophobic, polar, and negatively charged groups) exhibited small changes in the ammonium formate concentration range of 10–100 mM, but their retention times considerably increased at a salt concentration of less than 10 mM. Therefore, amino acids in the hydrophobic and polar groups are not detected at a salt concentration of 0.1 mM. Negatively charged amino acids (such as aspartic acid and glutamic acid) were less affected by the salt concentration; hence, their peaks are observed in the presence of 0.1 mM of ammonium formate. The column stationary phase is regarded to be negatively charged, and the ammonium cation is thought to mainly interact with the stationary phase to elute the amino acids. This trend in the retention time shifts corresponded to the separation mechanism of

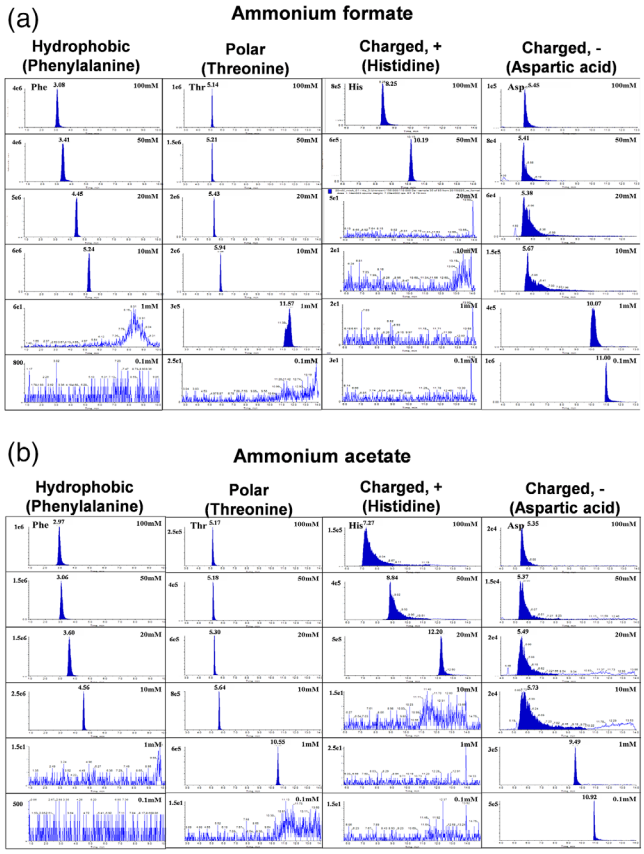

Figure 1. Representative chromatograms of amino acids in each group with two salt modifiers in the mobile phase. Each salt concentration ranged from 0.1 to 100 mM.

CEX chromatography. Hence, an ammonium formate concentration of greater than 10 mM is required for the separation and detection of amino acids; particularly, a higher concentration (>50 mM) is suggested for the separation of positively charged analytes.

A majority of the amino acids did not exhibit significantly different peak widths or shapes at greater than 10–50 mM (the concentration at which the retention time did not considerably shift). However, Aspartic acid exhibited a narrow peak width and good shape at a salt concentration of less than 1 mM (Figure 1(a)). Aspartic acid supposedly eluted via similar interactions with the HILIC column rather than with CEX at high salt concentration. However, interactions with an ion-exchange group on the stationary phase were predominant at low salt concentrations. Zhou *et al.* have already reported the peak broadening of Aspartic acidin HILIC.[8]

The use of ammonium acetate instead of ammonium formate as the mobile phase modifier revealed similar trends for the retention of amino acids. All amino acids exhibited increased retention times with the decrease in the ammonium acetate concentration; however, differences of retention times slightly decreased at a low concentration (<1 mM) compared to ammonium formate. Histidine exhibited poor separation and peak broadening at a high ammonium acetate concentration, but the peak width decreased at an ammonium acetate concentration of 20 mM (Figure 1(b)). In addition, aspartic acid exhibited a similar trend, and the peak width significantly decreased at an ammonium acetate concentration of less than 1 mM. This phenomenon was considered to be similar to the separation of aspartic acid using ammonium formate. Table S3 summarizes the retention times of amino acids at all concentrations of both salt compositions. The peak height of each amino acid was observed, and all amino acids, except arginine and glycine, exhibited higher intensity (Figure S2). Therefore, ammonium formate is suitable for the analysis of amino acids with better sensitivity and separation efficiency.

Effect of the Composition of the Acid Modifier in the Mobile Phase on the Retention Behavior. Typically, to achieve high ionization efficiency for electrospray ionization mass spectrometry, acidic conditions of the mobile phase are suggested; however, the optimized pH and acid composition are considerably dependent on the analyte or column characteristics. In this study, various concentrations (*i.e.*, 0.5, 0.1, 0.05, and 0.01%) of formic acid and acetic acid in mobile phase A were applied to investigate the effect of the acid modifier. Mobile phase B was fixed to 100 mM aqueous ammonium formate. Table S4 summarizes each of the retention times of amino acids, and cysteine was not detected under all conditions, only except with 0.5% of acetic acid. Cysteine is rapidly oxidized and dimerized to cystine during analysis[9,10]; therefore, cysteine is not detected under a majority of conditions.

With the decrease in the concentration of both acid modifiers, the retention times of all amino acids increased. However, the salt (*i.e.*, ammonium formate) was sufficiently provided by mobile phase B, and analytes were eluted from

the stationary phase by CEX. Hence, the shift in the retention time is not significant in the acid concentration range of 0.5–0.01%. For example, Ile exhibited a retention time increment of only ~0.5 min by decreasing concentration from 0.5% (132 mM) to 0.05% (13 mM), but a retention time increment of ~2.0 min was observed by changing the ammonium formate concentration from 100 to 10 mM (Figure 2). On the other hand, Ile did not exhibit significant changes in the peak width with the change in the salt concentration; however, peak broadening was observed with the decrease in the acid concentration. With the increase in the formic acid concentration from 0.5 to 0.01%, the peak width of isoleucine (full-width at half-maximum) changed from 0.2 to 0.5 min. Silanol groups on the stationary phase surface are thought to be ionized at high pH (low acid concentration), which provided additional retention by cation exchange by ionized silanol groups.[11,12] Hence, a sufficient amount of the acid modifier is required for the high-efficiency separation of analytes.

Effect of the Organic Solvent in the Mobile Phase. To investigate the separation efficiency in terms of the organic solvent in the mobile phase, various conditions of mobile phases were demonstrated. For a single-component solvent, MeOH, EtOH, isopropanol (IPA), THF, and ACN with 0.1% formic acid were considered for mobile phase A, and 100 mM aqueous ammonium formate was fixed as mobile phase B. Figure 3 shows the chromatograms of the amino acid mixture. The separation efficiency for amino acids followed the order of THF > ACN > IPA > EtOH > MeOH, and protic solvents (including IPA, EtOH, and MeOH) exhibited an overall poor separation efficiency and a short retention time, except positively charged groups such as arginine, histidine, and lysine. The trend of rapid elution by protic solvents is extremely similar to HILIC separation,[13,14] whereas arginine, histidine, and lysine exhibited strong interactions with cation exchange groups on the column; hence, the elution power is expressed mainly according to the ionic strength rather than the organic solvent composition. Hence, the multimode column is thought to separate analytes simultaneously by HILIC and CEX interactions, and the charge state of the analyte affects the separation efficiency and elution order.

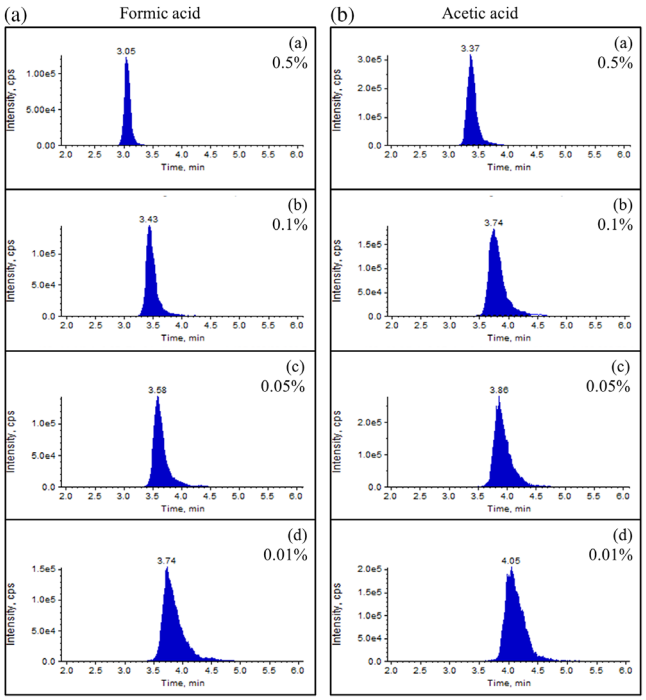

Figure 2. Retention behavior of isoleucine with respect to the acid modifier composition and concentration.

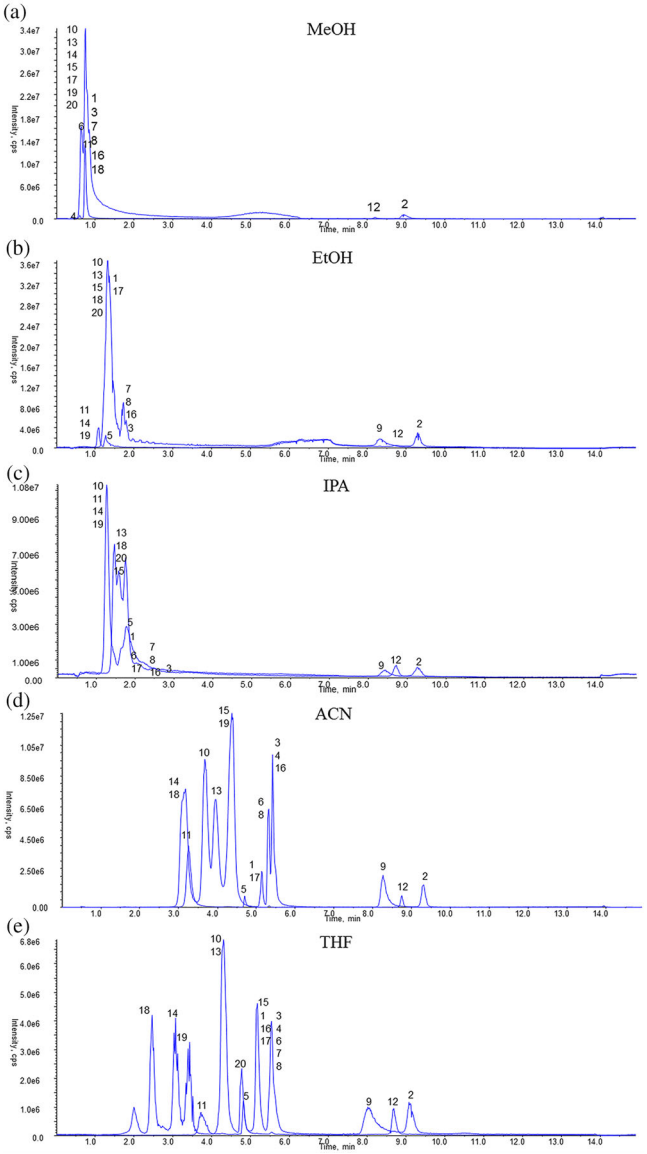

Figure 3. Total ion chromatogram (TIC) of (a) MeOH, (b) EtOH, (c) IPA, (d) ACN, and (e) THF 1: Alanine, 2: Arginine, 3: Asparagine, 4: Aspartic acid, 5: Cysteine, 6: Glutamic acid, 7: Glutamine, 8: Glycine, 9: Histidine, 10: Isoleucine, 11: Leucine, 12: Lysine, 13: Methionine, 14: Phenylalanine, 15: Proline, 16: Serine, 17: Threonine, 18: Tryptophan, 19: Tyrosine.

In addition, changes in the retention time under a mixed organic solvent composition were investigated. Each of the mobile phase A compositions was ACN:MeOH, ACN:

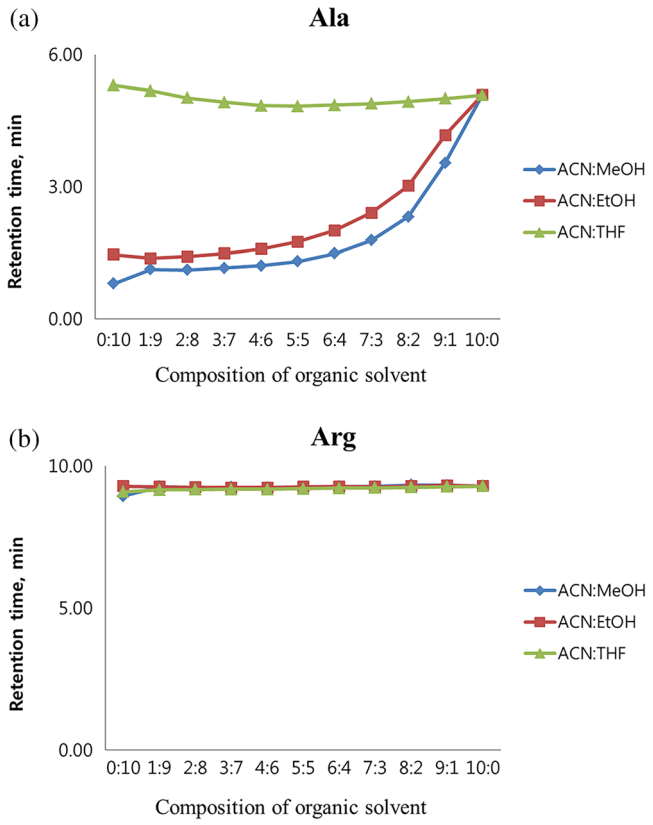

Figure 4. Changes in the retention time of (a) alanine and (b) arginine by the organic solvent composition.

EtOH, and ACN:THF with 0.1% formic acid, and the composition of mobile phase B was 100 mM ammonium formate. The ratio was changed from 0:10 to 10:0 with 10 stages. Retention time change trends were similar to the result expected from the single organic solvent composition. Figure 4 shows the changes in the retention times of alanine and arginine. For alanine, with the decrease in the MeOH or EtOH concentration, the retention times increased, but the addition of THF did not significantly affect the retention. Otherwise, Arginine exhibited constant retention times under any mobile phase condition; hence, positively charged amino acids are mainly eluted via the CEX mechanism, which can be controlled only by the concentration or species of salt additives. For the other amino acids (including hydrophobic, polar, and negatively charged amino acids), a good separation resolution was achieved when a protic solvent was not mixed in the mobile phase; however, the retention time decreased by the addition of protic solvents such as MeOH or EtOH for rapid elution.

As a result, we could identify that mobile phase conditions including salt, acid modifier and organic solvent are important factor to determine separation efficiency in CEX/HILIC column. We also observed the effects by column temperature at 35 °C and 50 °C but there was no significant difference in retention time or peak width. (data not shown). Our practical information will be helpful to modify separation condition to optimize separation efficiency rather than the manufacturer's guidelines.

Conclusion

The performance characterization of CEX/HILIC via the modification of the mobile phase was investigated using amino acids. In addition, effects of the types and concentrations of the buffer, acid modifier, and organic solvents, were investigated, and the composition of buffer salts and organic solvent was a key parameter to determine separation efficiency in bimodal CEX/HILIC. This study can provide information regarding the optimization of the analytical conditions to match the purpose and useful information to further applications.

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Supporting Information. Additional supporting information may be found online in the Supporting Information section at the end of the article.

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