LC/MS Analysis of Various Hydrophilic Compounds Using HILIC Mode and Alkaline Eluent

Introduction

Components of pharmaceutical and food products often include high polar compounds. Those compounds are hardly retained under reversed phase mode, the most often HPLC separation mode. To overcome the problem, pre-column derivertization or ion-pair reagents are often used. However, the use of derivertization adds additional steps in the analysis while the use of ion-pair regent increases background level from the ion-pair reagent residues on the column and the flow-lines.

Shodex HILICpak VG-50 series used in this application is a set of polymer-based amino columns which effectively separate various saccharides. The packing material consists of a polyvinyl alcohol base with a hydrophilic functional group, a modified tertiary amine (Fig. 1).

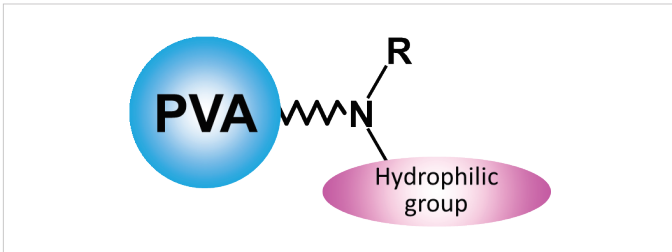


Fig. 1. Schematic diagram of VG-50 packing material

With some columns, reducing sugars form Schiff base with the packing material and are retained in the column. This does not occur with the HILICpak VG-50 series columns, leading to the series' ability to achieve a high recovery rate. Moreover, column bleeding (elution of column packing material related debris) that is often observed with silica-based amino columns is rarely found with the HILICpak VG-50 series columns, and consequently the related problems of increased background and/or ion suppression in MS are less likely to occur. Another advantage of the column over the silica-based amino column is that the HILICpak VG-50 series columns can be used under alkaline conditions (working pH range, 2 to 13). This lets a high sensitivity analysis of saccharides using negative mode in ESI-MS. Also, anionic compounds, such as organic saccharides, tend to be retained on the column when previously available methods are used. Alkaline conditions on the VG-50 series will elute, allowing for the analysis of organic saccharides.

This application introduces analysis of not only saccharides, but includes simultaneous analysis of saccharides, organic acids, and amino acids using a semi micro size column, Shodex HILICpak VG-50 2D, with LC/MS alkaline gradient conditions.

Experimental

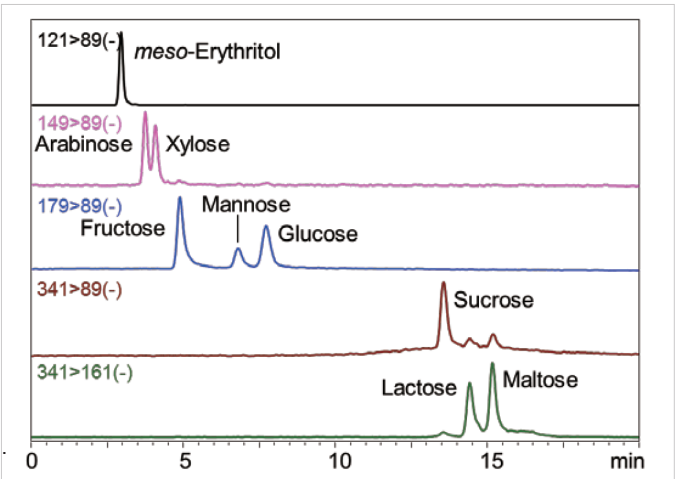
The LC/MS system used was Shimadzu Nexera/LCMS-8030 Plus. The column used was Shodex HILICpak VG-50 2D (2.0 mm I.D. x 150 mm; particle size 5 µm; pore size 100 Å). Specific analytical conditions used for each analysis are mentioned with their results. It should be noted that the pH of 0.5 % ammonia water used as an eluent is about 11.5. The LC/MS system used in the experiment was durable against the alkaline condition up to pH 13.

Results and Discussion

1. LC/MS analysis of sugars

1.1 Neutral saccharides

Figure 2 shows the chromatograms for meso-erythritol, arabinose, xylose, fructose, mannose, glucose, sucrose, lactose, and maltose. Gradient elution of 0.1 % ammonia water/acetonitrile was used. The neutral saccharides analyzed in this experiment can also be separated using water/acetonitrile as an eluent and results in similar resolution. However, addition of ammonia (analyzing under alkaline condition) promotes deprotonation during ESI which increases the sensitivity of negative ion detection. The peak height observed using ammonia water /acetonitrile eluent was three times higher than that of using water/acetonitrile eluent. The pH of 0.1 % ammonia water is about 11, thus most silica-based LC columns cannot be used under this condition. This emphasizes an advantage of VG-50 2D, packed with polymer-based packing material, as it well-tolerates against the high pH eluent like the one used in this experiment.

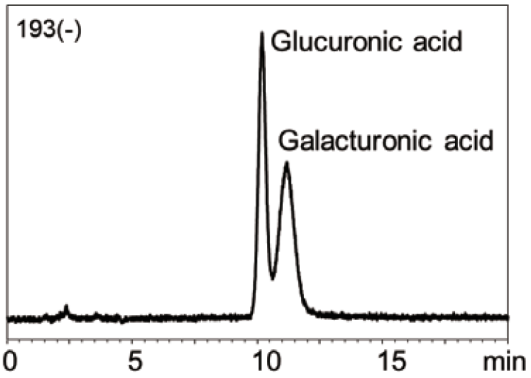


Sample : 50 ng/mL each in H_2O/CH_3CN = 1/9, 5 µL
Column : Shodex HILICpak VG-50 2D
Eluent : (A) 0.1 % NH_3 aq./(B) CH_3CN
Linear gradient
95 % B (0 - 5 min) → 80 % B (15 - 20
Flow rate min) : 0.3 mL/min
Detector : ESI-MS MRM(-)
Column temp.: 60 ºC

Fig. 2. Chromatograms of various neutral saccharides

1.2 Acidic saccharides

The use of water/acetonitrile eluent causes ionic adsorption effects, and thus retains the acidic saccharides in the column. The use of alkaline eluent prevents the dissociation of amino function groups on the stationary phase, and this prevents the saccharides from being kept retained in the column. Figure 3 shows the chromatograms of glucuronic acid and galacturonic acids. The degree of separation achieved here was better than that of previously available method using ion-exclusion chromatography. Using a high acetonitrile ratio is also advantageous for achieving a high sensitivity MS result.

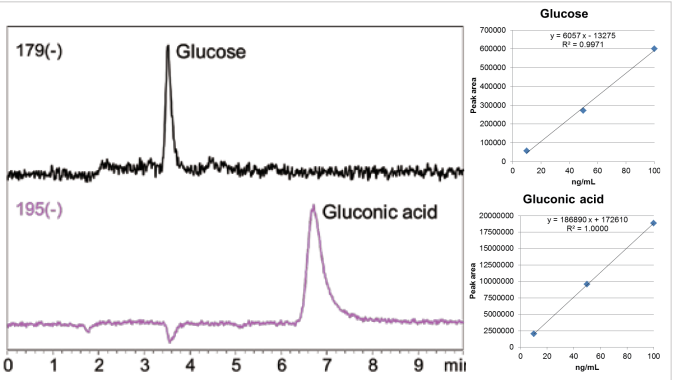


Sample : 10 ng/mL each in H_2O/CH_3CN = 1/4, 5 µL
Column : Shodex HILICpak VG-50 2D
Eluent : (A) 0.5 % NH_3 aq./(B) CH_3CN = 25/75
Flow rate : 0.2 mL/min
Detector : ESI-MS SIM(-)
Column temp.: 30 ºC

Fig. 3. Chromatograms of two acidic saccharides

1.3 Glucose and gluconic acid

Gluconic acid is generally converted from glucose, and thus simultaneous analysis of those two compounds is sometimes required. However, previously available method using ion-exclusion chromatography under acidic conditions did not provide an effective separation. Figure 4 shows chromatograms demonstrating a good separation of the two compounds. An additional advantage of the method described is that while acidic condition lactonises the gluconic acid which causes the peak tailing, but analysis under alkaline condition will not let the lactonization, and thus prevents tailing to occur. Thus, the use of an alkaline eluent improves the quantification of gluconic acid.

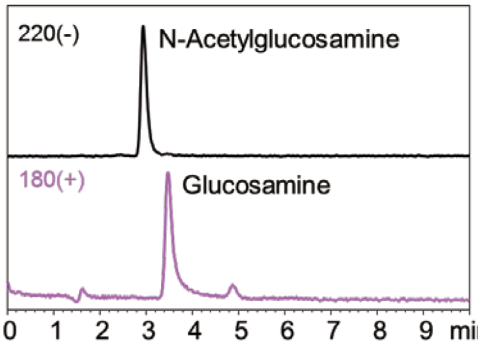


Sample : 10 ng/mL each in H_2O/CH_3CN = 1/4, 5 µL
Column : Shodex HILICpak VG-50 2D
Eluent : (A) 0.5 % NH_3 aq./(B) CH_3CN = 25/75
Flow rate : 0.2 mL/min
Detector : ESI-MS SIM(-)
Column temp.: 40 ºC

Fig. 4. Chromatograms of glucose and gluconic acid

1.4 Amino acids

Figure 5 shows chromatograms of N-acetylglucosamine and glucosamine. Since glucosamine contains an amino function group, higher sensitivity was achieved by monitoring protonated compound than monitoring its deprotonated compound. Amino sugars and their acetylated metabolites can also be analyzed under the alkaline condition (data not shown).



Sample : 100 ng/mL each in H_2O/CH_3CN = 1/4, 5 µL
Column : Shodex HILICpak VG-50 2D
Eluent : (A) 0.5 % NH_3 aq./(B) CH_3CN = 25/75
Flow rate : 0.2 mL/min
Detector : ESI-MS SIM(+/-)
Column temp.: 40 ºC

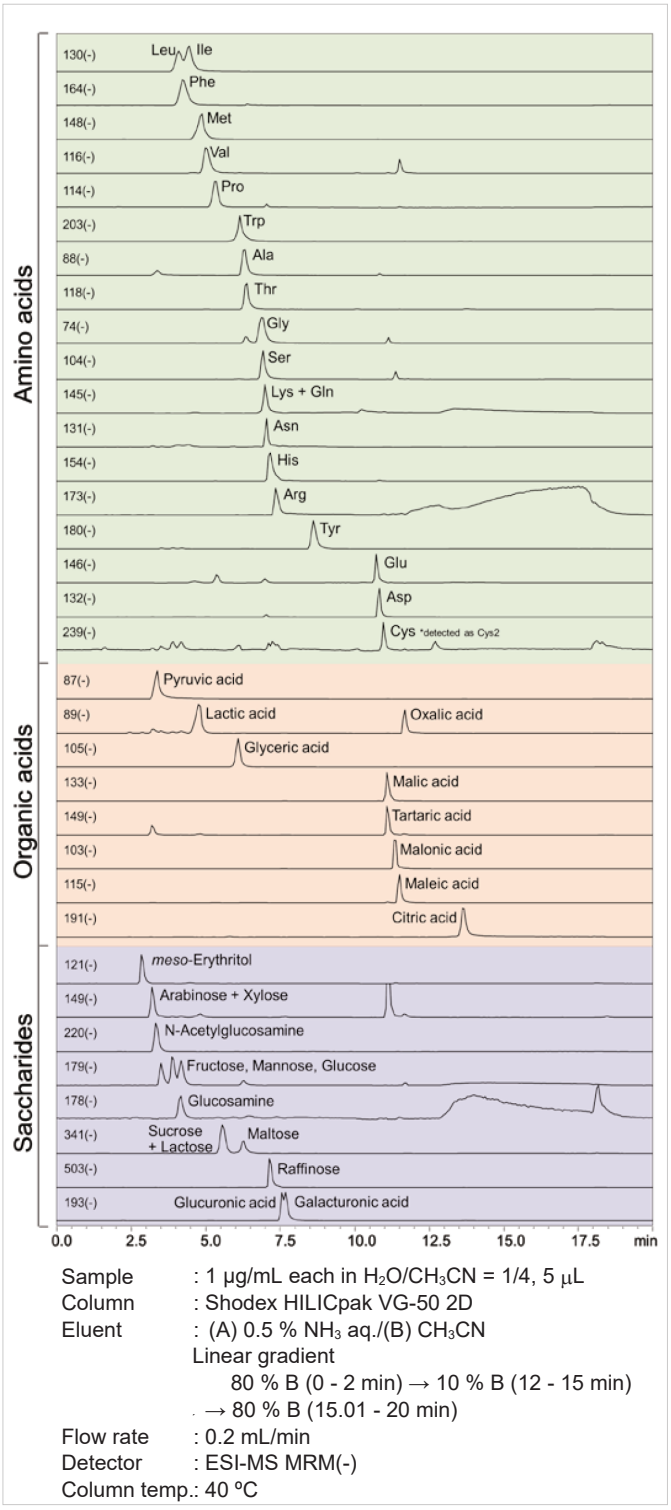
Fig. 5. Chromatograms of N-Acetylglucosamine and glucosamine

2. Simultaneous analysis of saccharides, organic acids, and amino acids

The successful method developed for the analysis of acidic saccharides in the alkaline condition was extended for the separation of organic acids and amino acids. Figure 6 shows the chromatograms of a mixture containing 14 saccharides, 9 organic acids, and 20 amino acids. A gradient method was used for the analysis.

The result demonstrates the VG-50 column's capability of analyzing organic acids. The elution order of the organic acids was mono, di, and tribasic acids. It required to use approximately 0.5 % ammonia to make the citric acid (a tribasic acid) to elute. Oxalic acid and citric acid are not retained well by ion-exclusion chromatography. The method developed here retains the acid peaks well, and allows the peaks to be less affected from early eluting impurities.

It also demonstrated the feasibility of analyzing amino acids. The chromatograms showed hydrophobic amino acids to have tendencies of eluting earlier while acidic amino acids to elute later.

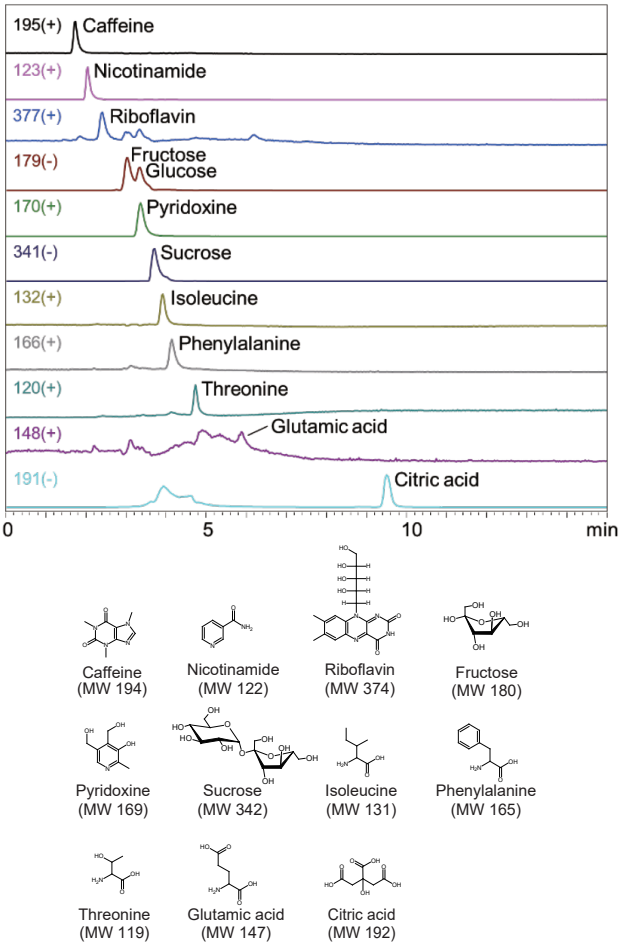


Sample : 1 µg/mL each in H_2O/CH_3CN = 1/4, 5 µL
Column : Shodex HILICpak VG-50 2D
Eluent : (A) 0.5 % NH_3 aq./(B) CH_3CN
Linear gradient
80 % B (0 - 2 min) → 10 % B (12 - 15 min)
→ 80 % B (15.01 - 20 min)
Flow rate : 0.2 mL/min
Detector : ESI-MS MRM(-)
Column temp.: 40 ºC

Fig. 6. Chromatograms showing the simultaneous LC/MS analysis of a mixture containing saccharides, organic acids, and amino acids

3. Application of the method for analyzing commercial energy drink

Figure 7 shows LC/MS analysis result of a commerciallyavailable energy drink. It demonstrated an effective simultaneous analysis of saccharides (fructose, glucose, sucrose), citric acid, and amino acids (isoleucine, phenylalanine, threonine, glutamic acid). Also, the method was feasible analyzing caffeine and water-soluble vitamins (nicotinamide, riboflavin, pyridoxine) present in the energy drink.



Sample : Commercial energy drink x100 dilution in H_2O/CH_3CN = 1/1, 2 μL
Column : Shodex HILICpak VG-50 2D
Eluent : (A) 0.5 % NH_3 aq./(B) CH_3CN
High pressure linear gradient
70 % B (0 min) → 10 % B (5 - 15 min)
Flow rate : 0.2 mL/min
Detector : ESI-MS MRM(-)
Column temp.: 40 ºC

Fig. 7. LC/MS analysis result of a commercial energy drink

Conclusions

A polymer-based amino column, Shodex HILICpak VG-50 2D, provides many advantageous analytical features when used under alkaline conditions. LC/MS with an ammonia water/acetonitrile gradient elution is effective in providing good separation and high sensitivity analysis of various hydrophilic compounds. The method is feasible for the analysis of saccharides, organic acids, and amino acids simultaneously, which was difficult by using previously available methods. This can be achieved without using pre-column derivatization nor addition of ion-paring reagent. The alkaline conditions promote deprotonation of saccharides which make it possible to monitor negative ions and contributes to the enhanced high sensitivity detection. The developed method showed its effectiveness in analyzing commercial energy drink. Not only monitoring saccharides, citric acid, and amino acids, the method demonstrated its ability to monitor caffeine and water soluble vitamins simultaneously.

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