Simultaneous LC/MS Analyses of Phosphorylated Saccharides

Introduction

Phosphorylated saccharides take the important role as intermediates in various metabolic pathways of animals. For example, during glycolysis process, glucose is first converted to glucose-6-phosphate, then to other phosphorylated saccharides (such as fructose-6-phosphate and fructose-1,6-bisphosphate) and finally to pyruvate to produce ATP, the energy source of cell processing. Through glycogenolysis process, glucose-1-phosphate is produced from breakdown process of glycogen branches. In this process, glucose-6-phosphate is also produced as an isomer, and the product goes through a glycolysis process via different pathway. High sensitive analysis of phosphorylated saccharides in vivo is important for understanding the biological phenomena and early detection and treatment of the related diseases. One such case is glycogenosis, which stores abnormal amounts of glycogen in the body, and is possibly related to genetic glycogen enzymes deficiencies. Newborn glucose-6-phosphate concentration monitoring is considered an effective tool for the early detection of the disease.

However, phosphorylated saccharides generally have low UV absorbance, and thus high-sensitive detection of them is difficult. The high hydrophilic nature of the phosphorylated saccharides result in very low retention when analyzed by reversed phase mode, typically used in LC analyses. To overcome the problem, derivertization or ion-pair reagents are often used, however they add additional steps in the analysis.

In this application, we used a combination of Shodex HILICpak columns and LC/MS for simultaneous and high sensitive analysis of various phosphorylated saccharides without the use of ion-pair reagent nor derivatization.

Experimental

The system used was Shimadzu Nexera UHPLC system with LCMS 8030 Plus. The column used was either Shodex HILICpak VT-50 2D (2.0 mm I.D. x 150 mm, particle size 5 µm, pore size 100 Å) or Shodex HILICpak VG-50 2D (2.0 mm I.D. x 150 mm, particle size 5 µm, pore size 100 Å). Phosphorylated saccharides form metal complexes easily. Thus, we employed PEEK column housings for both VT-50 and VG-50, and this avoids the adsorption of saccharides on the columns and prevents the consequence peak tailing. ESI was used as an ion source of MS. Either SIM(-) or MRM(-) mode was used for the detection. PEEK tubings were used for the LC system as much as possible. A 0.1 mm I.D. PEEK tubing was attached to the column and connected directly to the MS. Specific analytical conditions, flow rate and eluent compositions etc., of each analysis are mentioned in their corresponding result sections.

Figure 1 shows schematic diagrams of the packing materials used in VT-50 and VG-50. Both columns use porous polyvinyl alcohol as their base materials. The gels for VT-50 2D are modified with quaternary ammonium and that of VG-50 2D are modified with amino groups.

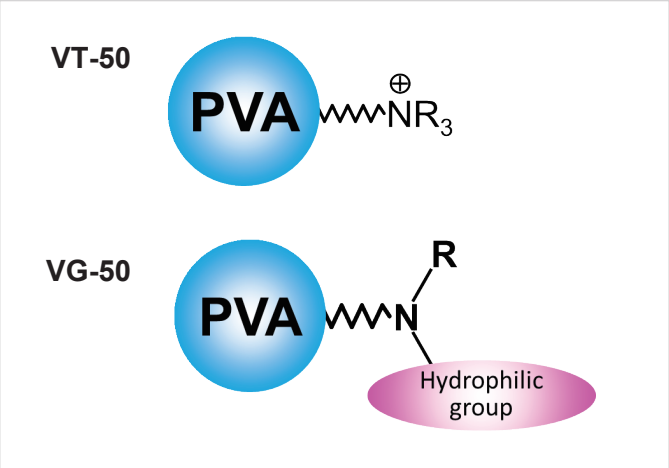


Fig. 1. Schematic diagrams of VT-50 and VG-50 packing materials

Results and Discussion

1. Analysis of Phosphorylated Saccharides Using HILICpak VT-50 2D

We worked on developing a method for analyzing six phosphorylated saccharides; glucose-1-phosphate (G1P), glucose-6-phosphate (G6P), fructose-1-phosphate (F1P), fructose-6-phosphate (F6P), fructose-1,6-bisphosphate (F1,6bP), and uridine diphosphate glucose (UDP-Glc). Structures of phosphorylated saccharides analyzed in this study are shown in figure 2.

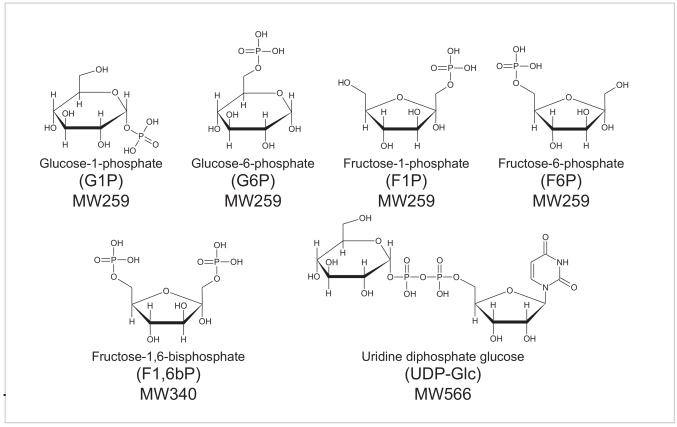


Fig. 2. Structures of phosphorylated saccharides studied

G1P, G6P, F1P, and F6P are the isomers, having the same molecular weights. Since their product ions monitored by MRM are also the same, they need to be separated by chromatography prior to the MS detection. Good separations of saccharide isomers were obtained by using an eluent mixture of 25-mM ammonium formate/acetonitrile = 80/20 (Fig. 3). A superior sensitivity was achieved by using MRM mode compared to SIM mode. Good linearities were obtained for the calibration curves in the concentration range of 0.005 to 0.5 µM. Under this low-acetonitrile condition, phosphorylated saccharides are assumed to be separated by anion-exchange mode rather than by HILIC mode. The cause of different elution timings of the four isomers are not clear, but it can be possible that they are separated by their differences in hydrated ion radii. In addition, although it is not a phosphorylated saccharide, but dihydroxyacetone phosphate, an intermediate of the glycolysis process, can also be analyzed using the same method (it elutes after F1P).

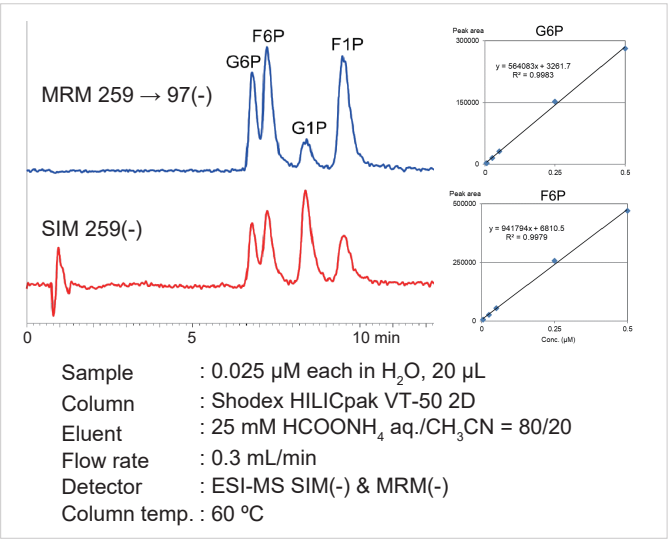


Fig. 3. Simultaneous LC/MS analysis of G1P, G6P, F1P, and F6P by HILICpak VT-50 2D

The saccharides with two phosphate functional groups (F1,6bP and UDP-Glc) are strongly retained and difficult to eluted under this condition. Therefore, a gradient method with increased ammonium formate concentration was used. Obtained chromatogram demonstrates the method's feasibility for the simultaneous analysis of six phosphorylated saccharides (Fig. 4).

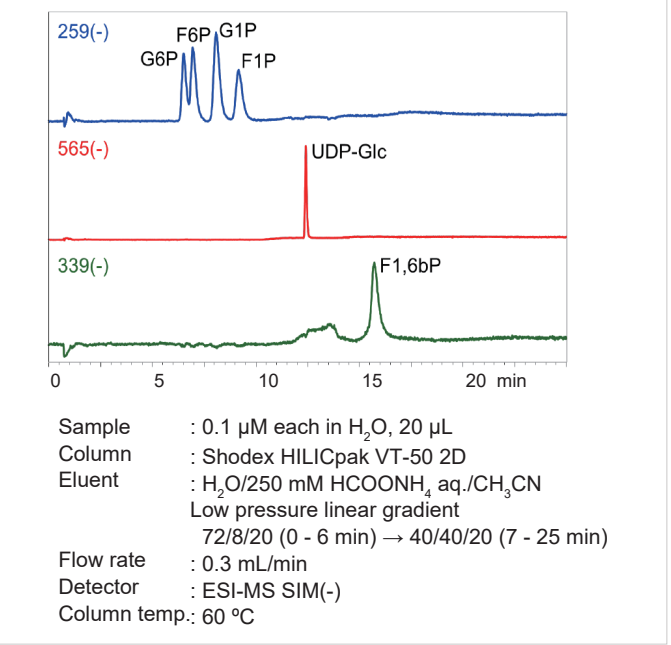
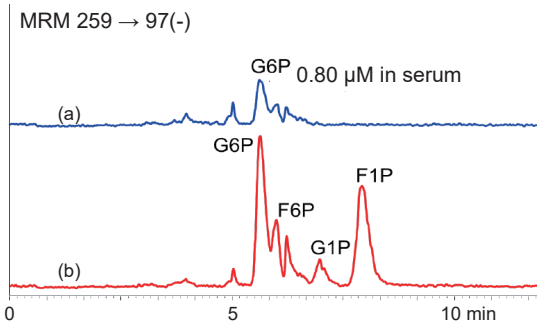


Fig. 4. Simultaneous LC/MS analysis of G1P, G6P, F1P, F6P, and UDP-Glc using HILICpak VT-50 2D

Figure 5 shows the analysis of G6P in guinea pig serum. The sample was prepared by diluting the serum 50 times with ultrapure water and removing protein by ultrafiltration. This simple sample pretreatment allowed the detection of G6P and its quantification. A mixed standard containing 0.025-µM each of G1P, G6P, F1P, and F6P was added to the 50x diluted serum. The recovery rate of G6P was 120 %, confirming that the sample was not influenced by ion suppression caused by proteins and other components. This suggests the method's capability for the analysis of other biological samples (plasma, urine, tissue extract etc.).



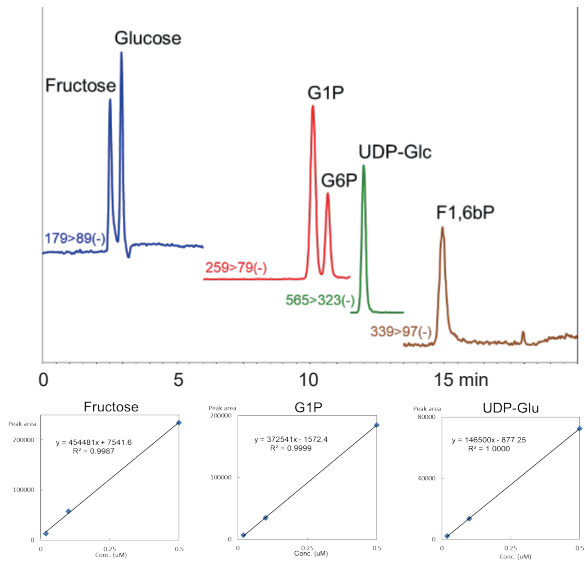
Sample : (a) Guiniea pig serum 50x diluted with H_2O
(b) 0.025 µM each G1P, G6P, F1P, F6P in (a), 20 µL
Column : Shodex HILICpak VT-50 2D
Eluent : 25 mM $HCOONH_4$ aq./CH_3CN = 80/20
Flow rate : 0.3 mL/min
Detector : ESI-MS MRM(-)
Column temp.: 60 ºC

Fig. 5. Simultaneous LC/MS analysis of G6P in guinea pig serum using HILICpak VT-50 2D

2. Analysis of Phosphorylated Saccharides Using HILICpak VG-50 2D

Phosphorylated saccharides can also be analyzed using VG-50 2D under alkaline conditions. Anionic components are separated by appropriately suppressing the dissociation of amino functional groups modified to the stationary phase (Refer to Shodex Technical Article No.3: LC/MS Analysis of Various Hydrophilic Compounds Using HILIC Mode and Alkaline Eluent).

The eluent used was a mixture of 0.5 % ammonium water/ acetonitrile. A simultaneous analysis of phosphorylated saccharides and other non-phosphorylated saccharides were achieved by using a gradient method; starting with a high acetonitrile concentration (80 %) then increased the ammonium water ratio over time (Fig. 6).



Sample : 0.1 µM 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
High pressure linear gradient
80 % B (0 min)
→ 60 % B (5 - 8 min)
→ 10 % B (13 - 16 min)
→ 80 % B (16.01 - 20 min)
Flow rate : 0.3 mL/min
Detector : ESI-MS SIM(-)
Column temp.: 35 ºC

Fig. 6. A simultaneous LC/MS analysis of fructose, glucose, G1P, G6P, UDP-Glc, and F1,6bP using HILICpak VG-50 2D

By using a high concentration of acetonitrile eluent, non-phosphorylated saccharides (fructose and glucose) were retained and separated by HILIC mode. This high acetonitrile eluent condition provides advantages in accelerating the ESI ionization. Moreover, the alkaline condition also accelerates the deprotonation of saccharides during the ionization process, and this increases the detection sensitivity. The phosphorylated saccharides will elute by increasing the ammonium water ratio, i.e., increasing the eluent pH, once non-phosphorylated saccharides are eluted. Good linearities were confirmed for the calibration curves of all compounds in the concentration ranges 0.02 to 0.5 µM. Although under this condition, the elution times of F1P, F6P, G1P, and G6P are very close and it is difficult to separate them. Also, it should be noted that the LC system must be alkaline tolerant, up to about pH12. Depending on the target components and the available system, a suitable column between VT-50 2D and VG-50 2D should be selected.

Conclusions

In this application, we demonstrated highly sensitive LC/ESI-MS analysis methods for phosphorylated saccharides. The use of simple analytical conditions, which do not require ion-pair reagent nor derivatization, were achieved by using Shodex HILICpak VT-50 2D, a polymer based quaternary ammonium HILIC column, and Shodex HILICpak VG-50 2D, a polymer based amino group HILIC column

Using its anion exchange mode, VT-50 2D is feasible to separate four monophosphorylated saccharides studied in this application. Use of salt gradient makes the method applicable for the analysis of diphosphorylated saccharides also. There is a publication on a high sensitivity analysis of various phosphorylated saccharides related to glycolysis in a biological samples and pentose phosphate pathway.[1]

Under alkaline conditions, the VG-50 2D mainly provides HILIC mode and allows separations of phosphorylated saccharides as well as enables a simultaneous analysis of phosphorylated and non-phosphorylated saccharides. There is a report using ammonium bicarbonate to alkalize the eluent and performed a high sensitivity analysis of inositol phosphate in biological samples.[2]

The separation patterns of phosphorylated saccharides and optimal eluent conditions differ between the two columns. Highly sensitive and simultaneous analyses of phosphorylated saccharides related to energy metabolisms of organisms can be achieved by selecting either VT-50 2D or VG-50 2D column, based on the compositions of the target samples and the system availability.

References

1. Shinkichi Ishikawa et al., Multi-omics analysis: Repeated exposure of a 3D bronchial tissue culture to whole-cigarette smoke, Toxicology in Vitro, 54 (2019) 251-262

2. Masatoshi Ito et al., Hydrophilic interaction liquid chromatography–tandem mass spectrometry for the quantitative analysis of mammalian-derived inositol poly/pyrophosphates, Journal of Chromatography A, 1573 (2018) 87–97

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