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Simultaneous Enantioseparation of Monosaccharides Derivatized with L-Tryptophan by Reversed Phase HPLC

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Three reducing monosaccharides (glucose; Glc, galactose; Gal, and mannose; Man) were derivatized with L-tryptophan (L-Trp) under alkaline conditions. The DL-Gal and DL-Man derivatives were chirally resolved by HPLC with an acidic mobile phase, but the DL-Glc derivative was not. All of the three DL-monosaccharide derivatives were simultaneously enantioseparated using HPLC with a SunShell RP-AQUA column (C28) and a basic mobile phase. The optimum mobile phase conditions consisted of 5 mM phosphate and 25 mM tetraborate buffer (pH 9.6) at 20°C. With this system, resolution of D- and L-isomers of the Glc, Gal and Man derivatives were approximately 1.7, 2.2 and 2.4, respectively. When the three monosaccarides were derivatized with L-phenylalanine instead of L-Trp, DL-Gal and DL-Man were enantioseparated under both acidic and basic conditions, but DL-Glc was not. It was observed that enantiomer elution orders of the three monosaccharides derivatized with L-Trp were reasonably reversed when derivatized with D-Trp. It was also revealed that borate anions were required for simultaneous enantioseparation with HPLC.

Keywords Enantioseparation, monosaccharide, HPLC, borate, diastereomer

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Introduction

Carbohydrates are one of the major nutrients and also physiologically important mediators of various cellular events, such as recognition, differentiation, proliferation and signal transduction.[1-5] Since they consist of monosaccharides alone or monosaccharide polymers containing oligosaccharides and polysaccharides, it is important to determine the composition and configuration of carbohydrates. Gas chromatography with flame ionization detection[6] and mass spectrometry[7] have been used for the analysis of monosaccharides that were converted into their corresponding per-*O*-methylsilyl or per-*O*-methylsilylated oxime derivatives. Since monosaccharides lack inherent chromophore or fluorophore groups, direct analysis of mono- and di-saccharides, and sugar alcohols has been conducted with high performance liquid chromatography (HPLC) with refractive index detection[8] and evaporative light scattering detection.[9] To analyze monosaccharides by HPLC with UV and fluorescent detection, numerous methods have been developed for derivatization of carbohydrates.[10,11] They can be classified into four categories involving reductive amination, amine coupling *via* glycamine, hydrazine formation and condensation with the active methylene group. Among these four methods, reductive amination is the most frequently used derivatization method. Monosaccharides derivatized with 2-aminopyridine,[12] 2-aminobenzoic acid[13] and 2-aminobenzamide[14] as reagents for reductive amination have been analyzed by HPLC with UV detection. Capillary electrophoresis (CE) with UV or fluorescent detection for analysis of derivatized monosaccharides has been also studied as reviewed.[15,16]

Aldohexoses have 16 isomers, of which half are D-isomers and the rest are L-isomers. In nature, so-called D-monosaccharides that are represented by D-Glc occupy the major portion of sugars. On the other hand, L-arabinose is native form and D-arabinose is a minor isomer. It is also important to analyze minor enantiomers of monosaccharides. Stefansson and Novotny firstly reported that several monosaccharides, which were derivatized by reductive amination with 2-aminopyridine, 5-aminonaphthalene-2-sulfonic acid or 4-amino-5-hydroxy-naphthalene-2,7-disulfonic acid, were enantioseparated by CE as complexes with borate and linear or cyclic dextrins.[17] Noe and Freissmuth achieved a single-run separation of 16 sugar enantiomers derivatized with (*S*)-1-phenylethylamine by CE.[18] Some monosaccharides, which were derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP), were optically resolved by micellar electrokinetic chromatography with (*S*)- or (*R*)-dodecoxycarbonylvalines.[19] We also reported chiral resolution of monosaccharides derivatized with 8-aminonaphthalene-1,3,6-trisulfonate by ligand exchange CE using borate as a central ion of the chiral selector.[20] Recently, enantioseparation of monosaccharides, which were derivatized with 2,3-naphthalene-diamine, by CE with sulfated-α-cyclodextrin as the chiral selector has been reported.[21] CE is a fast, cost-effective and high-resolution technique. However, identification of peaks of

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monosaccharide enantiomers was conducted only by the migration time. Since carbohydrates have a number of isomers and their related compounds that structurally resemble each other, peaks of minor enantiomers of monosaccharides should be identified by methods other than the migration time, such as mass and/or NMR spectra. Using HPLC methods, components of peaks that were fractionated could be identified by the above methods. Lopez and Gaspar reported a direct separation method of enantiomers, anomers and structural isomers of monosaccharide by HPLC with a Chiralpak AD-H column and refractive index detection.[22] Separation of diastereomer by HPLC with an achiral column is often feasible after derivatization with chiral molecules. Bai *et al*.[23] reported enantioseparation of per-*O*-methylated monosaccharides derivatized with cesium salt of (+)-2-methyl-2-β-naphtyl-1,3-benzodioxole-4-carboxylic acid (MNB carboxylic acid) by normal phase HPLC. But this derivatization method requires three steps (per-*O*-methylation, chlorination and finally MNB derivatization). Recently, Tanaka *et al*.[24] reported enantioseparation of aldose enantiomers derivatized with L-cysteine methyl ester and *O*-tolyl isothiocyanate by HPLC with UV detection. Each monosaccharide was successfully enantioseparated, but epimers could not be well separated. Wang *et al*.[25] reported a simultaneous enantioseparation of monosaccharides derivatized with L-cysteine methyl ester and phenyl isothiocyanate by ultra-performance liquid chromatography (UPLC) with mass spectrometry, where enantiomeric pairs of three hexoses (Glc, Gal and allose), three pentoses and four deoxy-D-hexoses were simultaneously separated. But it is difficult to achieve simultaneous separation of these epimer derivatives by the UPLC method with UV detection.

The aim of the present study was to develop a convenient and simultaneous enantioseparation method for monosaccharides (Glc, Gal and Man), derivatized with L-Trp using HPLC with UV detection.

Experimental

Reagents and chemicals

L-Glc and L-Man were obtained from Tokyo Kasei (Tokyo, Japan). D-Glc, D-Gal, L-Gal, D-Man, L-Trp, D-Trp, L-phenylalanine, L-tyrosine, dimethylamine borane and other chemicals (analytical grade) were obtained from Wako Pure Chemicals (Osaka, Japan).

HPLC apparatus and chromatographic conditions

The HPLC system consisted of a JASCO (Tokyo, Japan) Model PU-2080 pump, a JASCO Model UV-2075 detector, a Rheodyne (Cotati, CA) manual injector, a Taitec (Saitama, Japan) cooling water circulator Model CL-150 coolnit and a Flom (Tokyo, Japan) degasser Model AG-14. Inertsil ODS-3 (4.6 mm i.d. × 150 mm, GL Sciences, Tokyo, Japan), InertSustain C18 (4.6 mm i.d. × 150 mm, GL Sciences), Mightysil RP-18 GP (6.0 mm i.d. × 150 mm, Kanto Kagaku, Tokyo, Japan), Kaseisorb LC ODS Super (4.6 mm i.d. × 150 mm, Tokyo Kasei), SunShell C-18 (4.6 mm i.d. × 100 mm, ChromaNik, Osaka, Japan) and SunShell RP-AQUA (4.6 mm i.d. × 100 mm, ChromaNik) columns were used. An acidic mobile phase consisted of 1% phosphoric acid and 4% acetonitrile. Basic mobile phases were 25 mM tetraborate (pH 9.2) or 5 mM phosphate and 25 mM tetraborate (pH 9.6). Elution was carried out at a flow rate of 1.0 mL/min at 20 or 30°C. Analytes were detected at 220 nm. Data acquisition and processing were conducted with a Chromato-PRO (Runtime Instruments Co., Kanagawa, Japan).

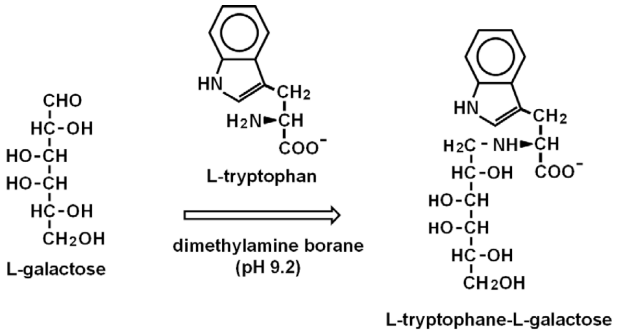

Fig. 1 Reductive amination of D-Gal with L-Trp.

Preparation of monosaccharide derivatized with L-tryptophan

Stock solutions (100 mM) of each enantiomer of Glc, Gal and Man were separately prepared with purified water, stored at –15°C and diluted to 2 mM before use. L-Trp (400 mM) was dissolved in a mixture of 1 mL of methanol and 2 mL of 100 mM tetraborate solution. Dimethylamine borane (2 M) was prepared with purified water before use. The monosaccharide (100 µL), L-Trp (300 µL) and dimethylamine borane (100 µL) solutions were mixed and were incubated at 40°C for 4 h. The reaction mixture was diluted 10-fold with the mobile phase and the 10 µL aliquot was analyzed by HPLC.

Results and Discussion

Derivatization of monosaccharides with L-Trp

Generally, reductive amination of monosaccharides with derivatization reagents such as 2-aminopyridine has been carried out in solutions containing acetic acid.[15,16] We also tried to derivatize DL-Glc, DL-Gal and DL-Man with L-Trp and dimethylamine borane in the presence of acetic acid. However, peaks of these monosaccharide derivatives were not detected by HPLC. The reaction of reductive amination starts with an attack of the lone pair of amino groups of derivatization reagents to the carbon of carbonyl groups of reducing sugars, yielding a Schiff base (imine derivative). The Schiff base is reduced with a reducing agent to a stable secondary amine. The stoichiometry of labeling is one label per saccharide. The pK_a values of primary amines of 2-aminobenzamide, 4-aminobenzoate ethylester and 8-aminonaphthalene-1,3,6-trisulfonate, which have been well used as derivatization reagents, were 2.92, 2.11 and 2.51, respectively.[26] Amino groups of the above reagents were not protonated or only partially protonated during the derivatization process in the presence of acetic acid. However, the pK_a value of the primary amino group of L-Trp was 9.39. Since the amino group is fully protonated in the presence of acetic acid, monosaccharides could not be derivatized with L-Trp under acidic conditions. We tried to derivatize monosaccharide enantiomers with L-Trp under basic conditions using 40 mM tetraborate (pH 9.2). As a result, monosaccharide derivatives were detected by HPLC (Fig. 1).

Mobile phase condition for enantioseparation of DL-Glc, DL-Gal and DL-Man

Chiral resolution of monosaccharides derivatized with L-Trp was studied by HPLC with acidic and basic mobile phases. When the derivatized monosaccharides were analyzed with an

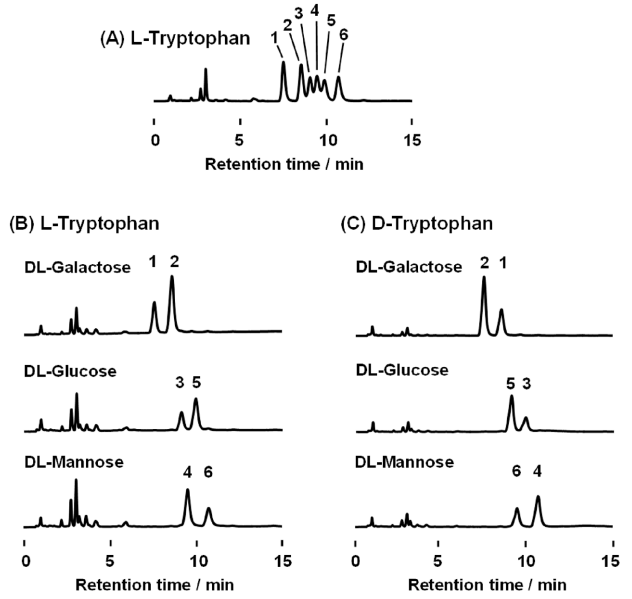

Fig. 2 Chromatograms of DL-monosaccharide derivatives by HPLC using a SunShell RP-AQUA column with a mobile phase containing 5 mM phosphate and 25 mM tetraborate (pH 9.6) at 20°C. (A) Mixtures of DL-monosaccharide derivatized with L-Trp. (B) Each DL-monosaccharide derivatized with L-Trp. (C) Each DL-monosaccharide derivatized with D-Trp. Peaks are monosaccharide derivatives of D-Gal (1), L-Gal (2), D-Glc (3), L-Man (4), L-Glc (5) and D-Man (6).

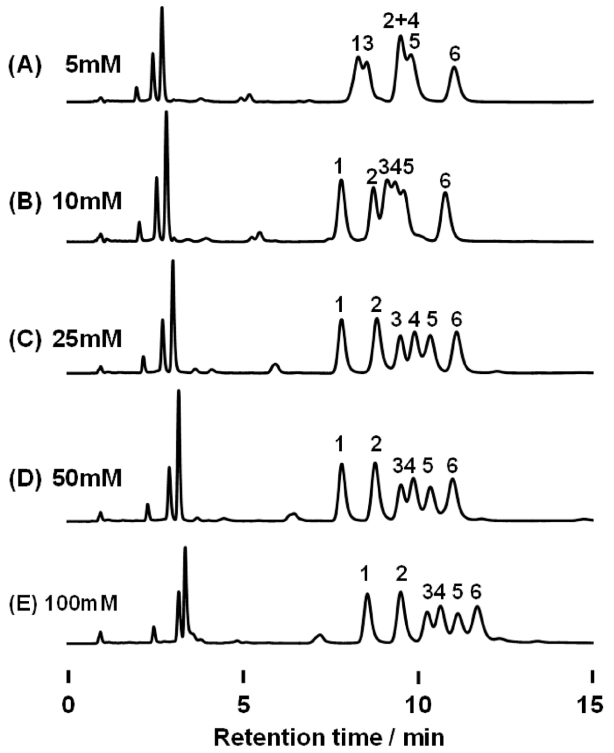

Fig. 3 Effect of the concentration of tetraborate on the retention time and resolution of monosaccharide derivatives. The mobile phase (pH 9.6) consisted of 5 mM phosphate and 5 – 100 mM tetraborate. Peaks are monosaccharide derivatives of D-Gal (1), L-Gal (2), D-Glc (3), L-Man (4), L-Glc (5) and D-Man (6).

InertSustain C18 column and a mobile phase containing 1% phosphoric acid and 4% acetonitrile, DL-Gal and DL-Man were enantioseparated, but DL-Glc was not. The same results were obtained by HPLC using Inertsil ODS-3, Mightysil RP-18 GP or Kaseisorb LC ODS Super columns instead of an InertSustain C18 column. Then, enantioseparation of the monosaccharide derivatives was carried out in alkaline pH conditions with an InertSustain C18 column, which was the only column usable under alkaline conditions among above the ODS columns. All the three monosaccharides derivatized with L-Trp were enantioseparated by an HPLC with the InertSustain C18 column and a basic mobile phase containing 25 mM tetraborate (pH 9.2). The retention times of the D- and L-Gal derivatives were shorter than those of the other monosaccharide derivatives. But peaks of the D- and L-Glc derivatives overlapped with those of the L- and D-Man derivatives, respectively. Among natural amino acids, three amino acids (L-Trp, L-phenylalanine, and L-tyrosine) have an aromatic group, which shows UV absorption and can be retained by ODS columns. Since L-tyrosine was not dissolved in the derivatization solution, monosaccharides derivatized with L-phenylalanine were analyzed with the above HPLC systems. As a result, the DL-Gal and DL-Man derivatives were enantioseparated under both acidic and basic conditions, but the DL-Glc derivative was not.

Recently, DeStefano *et al.*[27] reported that columns of fused-core particles, so-called "core-shell column", exhibited very high efficiency at higher mobile phase velocities. Using a SunShell C18 column, which is a core-shell column, chromatographic patterns of enantiomers of the three monosaccharide derivatives obtained by using acidic and basic mobile phases were the same as those obtained with an InertSustain C18 column. On the other hand, the three monosaccharide derivatives could be simultaneously enantioseparated with a SunShell RP-AQUA column, which introduced octacosyl groups (C28), and a basic mobile phase (Fig. 2-A). The concentration of L-monosaccharides was set at twice that of D-monosaccharides to distinguish the peaks of the L- and D-forms (Fig. 2-B). Enantiomer elution orders of the Gal, Glc and Man derivatives were DL, DL and LD, respectively. These orders were reasonably reversed by derivatizing the monosaccharide enantiomers with D-Trp instead of L-Trp (Fig. 2-C).

Optimization of HPLC conditions for enantioseparation of monosaccharides

It was reported that the addition of borate in the electrolyte on CE analysis changed the selectivity of separation between epimers of monosaccharides (Glc, Gal, Man and so on) due to the complex formation with vicinal hydroxyl moieties of the saccharides.[28,29] The effect of tetraborate concentration (5 – 100 mM) in the mobile phase on the retention time and resolution of the monosaccharide derivatives was evaluated (Fig. 3). The resolution was little affected by the tetraborate concentration. The retention times of the Gal and Man derivatives were not significantly affected by the tetraborate concentration, but a decrease in the concentration of tetraborate brought about a decrease in the retention times of the D- and L-Glc derivatives. Therefore, it was suggested that adjustment of the concentration of tetraborate was required for the enantioseparation of the L-Trp-monosaccharide derivatives in the proposed HPLC system, and the optimum tetraborate concentration for the simultaneous separation of all the derivatives was determined as 25 mM. When the monosaccharide derivatives were analyzed repeatedly with a mobile phase containing only 25 mM tetraborate without phosphate, their

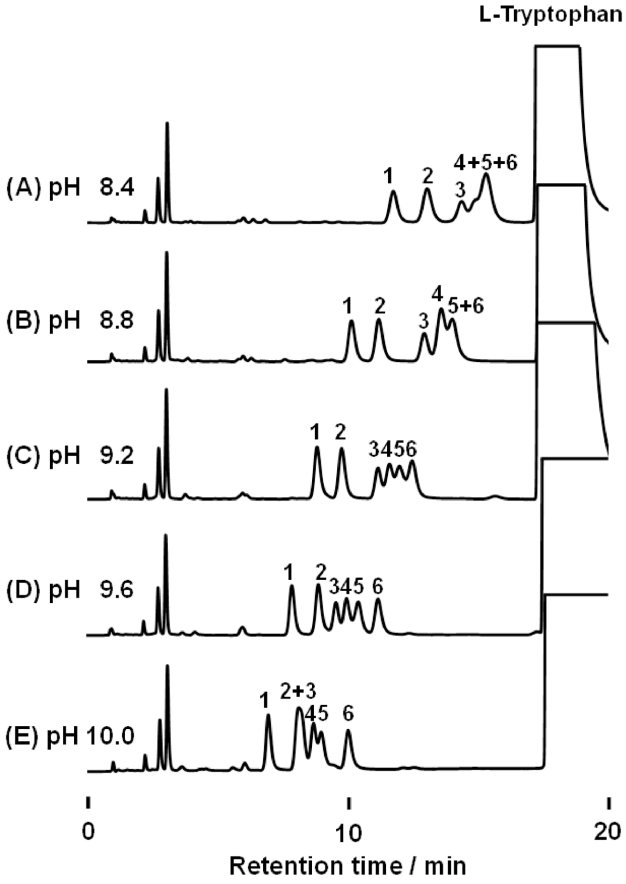

Fig. 4 Effect of pH of the mobile phase on the retention time and resolution of monosaccharide derivatives. The mobile phase (pH 8.4 – 9.6) consisted of 5 mM phosphate and 25 mM tetraborate. Peaks are monosaccharide derivatives of D-Gal (1), L-Gal (2), D-Glc (3), L-Man (4), L-Glc (5) and D-Man (6).

peak shapes gradually deteriorated. But the derivatives could be stably analyzed by the addition of 5 mM phosphate in the mobile phase. This may suggest that phosphate suppresses the adsorption of borate on a SunShell RP-AQUA column.

By changing the pH of the mobile phase containing 25 mM tetraborate and 5 mM phosphate, the retention time of residual L-Trp was kept constant, but the retention times of all the monosaccharide derivatives decreased (Fig. 4). This could suggest that an increase in pH of the mobile phase brought about an increase in anionic complex formation of monosaccharide derivatives with borate anion. Although increasing the pH did not affect the resolution of the DL-Gal and DL-Glc derivatives, it caused a gradual increase in the resolution of the D- and L-Man derivatives.

The effect of the column temperature (15 – 40°C) on the retention time and resolution of the monosaccharide derivatives was studied. A lower column temperature caused decreases in both the resolution and the retention times of all the monosaccharide derivatives. Therefore, the optimum mobile phase conditions were determined as 5 mM phosphate and 25 mM tetraborate buffer (pH 9.6) at 20°C. With this system, resolution of D- and L-isomers of the Glc, Gal and Man derivatives were approximately 1.7, 2.2 and 2.4, respectively.

Conclusions

Three monosaccharides (DL-Gal, DL-Glc and DL-Man) could be derivatized with L-Trp under alkaline conditions, but not under acidic conditions. DL-Monosaccharides derivatized with L-Trp were simultaneously enantioseparated by HPLC using a SunShell RP-AQUA column with an alkaline mobile phase, but not with an acidic mobile phase. Further studies are needed to widen the separation window to analyze many monosaccharides.

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