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Short Communication

Analyses of isomeric mono-O-methyl-D-glucoses, D-glucobioses and D-glucose monophosphates by high-performance anion-exchange chromatography with pulsed amperometric detection

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ABSTRACT

To investigate the contribution of each hydroxyl group of D-glucose to retention on pellicular quaternary amine-bonded resins and to the pulsed amperometric detector response, all sets of isomeric mono-O-methyl-D-glucoses and D-glucobioses and three isomeric D-glucose monophosphates were analysed by high-performance anion-exchange chromatography under alkaline conditions with pulsed amperometric detection. The results showed that the reduction of retention time on mono-O-methylation follows the order 2-OH > 3-OH > 6-OH ≥ 4-OH > 1-OH. Although the elution order of 1-, 3- and 6-phosphates was the same as obtained for the corresponding methyl esters, in the case of glucobioses the elution position was somewhat affected by the configurational contribution and hydrophobic interaction. The pulsed amperometric detector response was little affected by the acidity of each hydroxyl group of D-glucose. The suppression of the pulsed amperometric detector response by substitution of hydroxyl group seemed to be due to the inhibitory effect of the substituent group on the vicinal hydroxyl groups and, as a rule, the degree of suppression caused by 6-O-substitution was the smallest.

INTRODUCTION

Recently it has been shown that an accurate and sensitive analysis of carbohydrates can be performed using high-performance anion-exchange chromatography (HPAEC) coupled with triple-pulse amperometric detection (PAD) at a gold electrode, and this method has become a powerful tool in carbohydrate research [1–3]. In HPAEC, strong alkaline solutions are used as eluents and the hydroxyl groups of carbohydrates can be ionized under these conditions. It is known that there are subtle differences in the pK_a of the hydroxyl groups in carbohydrates [4–5].

In this work we analysed all sets of isomeric mono-O-methyl-D-glucoses and D-glucobioses to investigate the contribution of each hydroxyl group of D-glucose to the elution position during HPAEC and to PAD response [6]. In addition, the chromatographic behaviour of three isomeric D-glucose monophosphates on this HPAEC system was examined.

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EXPERIMENTAL

Materials

Methyl β-D-glucoside [7] was prepared by methylation and deacetylation of tetra-O-acetyl-α-D-glucosyl bromide [8]; m.p. 111°C, $[\alpha]_D^{22}$ −32.7° ($c=2$, H_2O).

2-O-Methyl-D-glucose [9] was prepared by methylation and deacetylation of 1,3,4,6-tetra-O-acetyl-α-D-glucose [10] (m.p. 98°C); m.p. 157–158°C.

For the preparation of 3-O-Methyl-D-glucose [9], 3-O-methyl-1,2:5,6-diisopropylidene-D-glucofuranose (prepared by methylation according to Hakomori's method [11] from 1,2:5,6-diisopropylidene-D-glucofuranose [12]) was deisopropyridenated with Amberlite IR-120B (H^+) to give 3-O-methyl-D-glucose; m.p. 166.5–168°C.

4-O-Methyl-D-glucose was prepared by methylation and deacetylation of 1,2,3,6-tetra-O-acetyl-β-D-glucose (m.p. 127–127.5°C, $[\alpha]_D^{22}$ −33.5°) [13]; chromatographically pure syrup.

6-O-Methyl-D-glucose [9] was prepared by methylation and deacetylation of 1,2,3,4-tetra-O-acetyl-β-D-glucose [14] (m.p. 118–119°C, $[\alpha]_D^{22}$ +8.3°); m.p. 146–147°C.

Trehalose (α,α-1,1), maltose (α-1,4), cellobiose (β-1,4), isomaltose (α-1,6) and gentiobiose (β-1,6), were of the highest grade commercially available. Kojibiose (α-1,2), sophorose (β-1,2), nigerose (α-1,3) and laminalibiose (β-1,3) were all gifts. These glucobioses were purified by high-performance liquid chromatography (HPLC) on a YMC-Pack PAMN column (250 × 10 mm I.D.) (YMC, Kyoto, Japan) with 60–75% acetonitrile before use.

Neotrehalose (α,β-1,1) and isotrehalose (β,β-1,1) were synthesized by glucosylation of a mixture of 2,3,4,6-tetra-O-acetyl-α- and β-D-glucopyranoses with tetra-O-acetyl-α-D-glucopyranosyl bromide in the presence of mercuric cyanide according to the procedure of Helferich and Weis [15]. The reaction product was separated by column chromatography on a Lobar prepacked column, LiChroprep Si 60 (40–63 μm), size C (Merck, Darmstadt, Germany) with benzene–acetone (7:1). The peracetylated disaccharide fraction obtained was deacetylated, and two isomeric trehaloses were separated by HPLC on Asahipak NH2P-50 (250 × 10 mm I.D.) (Asahi Kasei, Tokyo, Japan) with acetonitrile–water (75:25) (Fig. 1). HPLC analyses of two fractions, I and II, on a graphitized carbon column, Hypercarb (100 × 4.7 mm I.D.) (Shandon Scientific, Cheshire, UK), with acetonitrile–water (3:97) showed that I was neotrehalose containing traces of trehalose and II was pure isotrehalose (Fig. 2). Fraction I was purified by repeating chromatography under the same conditions as in Fig. 2. Characterization of neotrehalose and isotrehalose was performed by ^{13}C NMR spectroscopy [16].

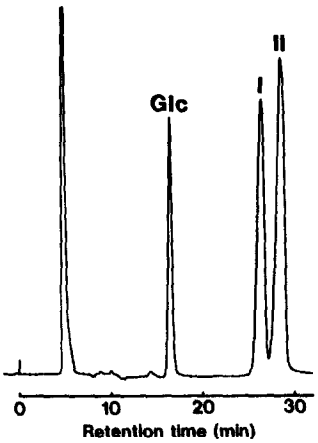

Fig. 1. Separation of two isomeric trehaloses in chemically synthesized products on an Asahipak NH2P-50 column. Peaks: Glc = glucose; I = neotrehalose containing small amounts of trehalose; II = isotrehalose. Chromatographic conditions: eluent, acetonitrile–water (75:25); flow-rate, 2.4 ml/min; temperature, ambient; detector, Shodex RI SE-61.

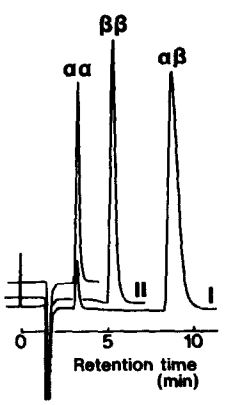

Fig. 2. Analysis of fractions I and II in Fig. 1 on a graphitized carbon column, Hypercarb. Peaks: αα = trehalose; αβ = neotrehalose; ββ = isotrehalose. Chromatographic conditions: eluent, acetonitrile–water (3:97); flow-rate, 1 ml/min; temperature, 35°C; detector, Shodex RI SE-61.

α-D-Glucose 1-phosphate disodium salt hydrate ($C_6H_{11}O_9PNa_2 \cdot 3H_2O$) and D-glucose 6-phosphate disodium salt hydrate ($C_6H_{11}O_9PNa_2 \cdot 3.5H_2O$) were purchased from Nacalai Tesque (Kyoto, Japan) and Sigma (St. Louis, MO, USA), respectively, and were of the highest grade. They were used as such, because they are very labile, decomposed on lyophilization, and lost unsettled water of crystallization on vacuum drying. D-Glucose 3-phosphate was a gift.

Apparatus and column

HPAEC was conducted with a Dionex BioLC Model 4000i system and a Model PAD 2 pulsed amperometric detector. The column used was a Dionex HPIC-AS6 (250 × 4 mm I.D., the same type of column as CarboPac PA-1) equipped with an AG6 guard column (50 × 4 mm I.D.) (all from Dionex, Sunnyvale, CA, USA). A Chromatopac C-R3A digital integrator (Shimadzu, Kyoto, Japan) was used to calculate peak areas.

Chromatographic conditions and measurements

The following pulse potentials (E) and durations (t) were used at range 2 (sampling period, 200 ms): $E_1 = 0.10$ V ($t_1 = 300$ ms); ($E_2 = 0.60$ V ($t_2 = 120$ ms); $E_3 = -0.80$ V ($t_3 = 300$ ms). The response time of the PAD 2 detector was set to 1.0 s. The eluents for monomethyl ethers and monophosphates of D-glucose, and glucobioses were 50 and 200 mM sodium hydroxide solutions, respectively, which were prepared by dilution of carbonate-free 50% sodium hydroxide solution in 18 MΩ cm deionized water, purified using a NANO-pure II (Barnstead, Newton, MA, USA). For control of retention, sodium acetate of analytical reagent grade was added to the eluent. Eluents containing sodium acetate were filtered through a 0.2-μm membrane filter. Eluents prepared daily were degassed by sonication under bubbling of helium gas and kept under a stream of helium. All purified samples of monomethyl-D-glucoses and glucobioses were lyophilized. Samples obtained in the amorphous state were dried as completely as possible in a desiccator over phosphorus pentoxide and were then weighed with the utmost caution to avoid taking up water during weighing. Each sample solution of $5 \cdot 10^{-4}$ M prepared using 18 MΩ cm deonized water was filtered through a 0.2-μm membrane filter. The sample size used for an analysis was 50 μl. All HPAEC analyses were carried out at ambient temperature with a flow-rate of 1 ml/min.

RESULTS AND DISCUSSION

Contribution of each hydroxyl group of D-glucose to retention

The retention times (t_R) of five positional isomers of monomethyl-substituted D-glucose with 50 mM sodium hydroxide solution are listed in Table I. Methyl β-D-glucoside, with its most acidic hydroxyl group occupied by a methyl group, was eluted much earlier than the positional isomers. Roberts *et al.* [4] reported the acidities of hydroxyl groups in methyl α- or β-D-glucopyranoside to be 2-OH > 6-OH > 3-OH > 4-OH when the sodium hydroxide concentration is ≤ 0.1 M. According to Rendleman's review [5], the acidity of hydroxyl groups in methyl D-glucopyranoside decreases in the order 2-OH ≫ 6-OH > 3-OH > 4-OH, and substitution for the hydroxyl group at C-2 should increase the acidities of the 3- and 4-hydroxyl groups. It is easily understandable that masking of the more acidic hydroxyl group by a methyl group should result in a greater reduction in t_R. However, the elution order of mono-O-methyl-glucoses was 4-methyl, 6-methyl, 3-methyl and 2-methyl derivatives, and the differences in their t_R values were small. This result indicates that the presence of a free 1-OH group reduces the effect of differences in acidities of the

TABLE I

RETENTION TIMES (t_R) AND RELATIVE DETECTOR RESPONSES (RDR)/OH OF MONOMETHYL-D-GLUCOSES

The analytical conditions were as described in the text.

Monomethyl-D-glucose	t_R (min)	RDR/OH[a]
1-Methyl	1.7	0.59
2-Methyl	5.4	0.64
3-Methyl	4.8	0.60
4-Methyl	3.9	0.65
6-Methyl	4.0	0.88

[a] The ratio of (molar response of monomethyl-D-glucose)/4 to (molar response of D-glucose)/5 was used as the reference standard.

other hydroxyl groups, and other effects such as hydrophobic interaction might prevail.

The results of the analysis of glucobioses are summarized in Table II. In glucobioses, the linkage positions influence the elution position for the same reason as described above, but the situation is more complicated. Masking of the most acidic 1-OH by a glucosidic linkage should result in the weakest retention and, in fact, 1,1-linked trehaloses were earlier eluted than the other glucobioses, but the t_R values of three trehaloses with different linkage configuration differed from each other. Of the reducing glucobioses, kojibiose (α-1,2), having a masked 2-OH group, whose acidity is said to be the highest of the hydroxyl groups other than 1-OH, eluted with a relatively short t_R, whereas sophorose (β-1,2), which also has a masked 2-OH group had the highest t_R value of all glucobioses. Except for 1,4-linked isomers (maltose and cellobiose), the α-linked isomers generally moved faster than their β-linked counterparts. This tendency is similar to that of their retention on a graphitized carbon column [17], on which planar molecules are generally retained more than non-planar molecules [18]. The elution order of three trehaloses was trehalose (αα), isotrehalose (ββ) and neotrehalose (αβ), and was the same as that on the graphitized carbon column (Fig. 2), while on the octadecylsilane (ODS) column neotrehalose eluted faster than isotrehalose (Fig. 1). Nevertheless, it has previously been indicated on the basis of the retention order of cyclodextrins [19] that there are some hydrophobic interactions on this HPIC column. Honda *et al.* [20] also pointed out that the partition mode should be considered for the separation of neutral sugars on this column. They studied the relationship between capacity factor (k') and partition coefficient (K) for 1-butanol, and concluded that the k' of neutral sugars with a larger K was larger, for example the K of maltose (17.9) was larger than that of cellobiose (10.0), and therefore maltose eluted more slowly than cellobiose. Previously we investigated HPAEC of each homologous series of D-gluco-oligo- and -polysaccharides on an HPIC-AS6 column using a 150 m*M* sodium hydroxide solution containing 100 m*M* sodium acetate as the eluent [21]. Under these conditions the elution order of the smallest member in each series, glucobiose, was the same as that under the present conditions, except that β-1,3 and β-1,2 were eluted at the same time, whereas comparison of the t_R values of glucotrioses showed a change in the elution order of β-1,2, α-1,4 and α-1,3, that is their t_R values increased in that order, and moreover, in the cases of degrees of polymerization ≥8 (gluco-octaose), β-1,2, eluted faster than β-1,4.

TABLE II

RETENTION TIMES (t_R) AND RELATIVE DETECTOR RESPONSES (RDR)/OH OF D-GLUCOBIOSES

The analytical conditions were as described in the text.

D-Glucobiose	t_R	RDR/OH[a]	
	(min)	1[b]	2[c]
Trehalose (α,α-1,1)	2.9	0.55	0.60
Neotrehalose (α,β-1,1)	4.2	0.59	0.59
Isotrehalose (β,β-1,1)	4.0	0.58	0.58
Kojibiose (α-1,2)	6.4	0.73	0.74
Sophorose (β-1,2)	13.6	0.90	1.03
Nigerose (α-1,3)	10.7	0.72	0.79
Laminalibiose (β-1,3)	12.7	0.68	0.74
Maltose (α-1,4)	11.0	0.66	0.71
Cellobiose (β-1,4)	7.8	0.80	0.83
Isomaltose (α-1,6)	5.7	0.67	0.72
Gentiobiose (β-1,6)	7.7	0.82	0.88

[a] The ratio of (molar response of D-glucobiose)/8 to (molar response of D-glucose)/5.

[b] Eluent: 200 m*M* sodium hydroxide.

[c] Eluent: 200 m*M* sodium hydroxide containing suitable amounts of sodium acetate (40–75 m*M*) for control of t_R to around 4 min.

We next investigated the separation of three positional isomeric monophosphates of D-glucose which are important compounds for biosynthesis and biodegradation of starch and glycogen. Sugar monophosphates are bivalent acidic compounds and, consequently, they are strongly retained on the anion-exchange resin. To elute them from the column, addition of considerable amounts of sodium acetate to the eluent was necessary. Fig. 3 shows an elution profile of three isomeric monophosphates of D-glucose by isocratic elution with 50 m*M* sodium hydroxide solution containing 300 m*M* sodium acetate. Within 7.5 min adequate separation was achieved. When the ionic strength of the eluent was increased and the t_R of the last peak was 5 min, baseline separation of the three isomeric monophosphates of D-glucose was still possible. The elution order of 1-, 3- and 6-phosphates was the same as that of the corresponding methyl ethers. Any

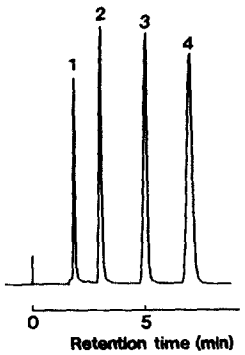

Fig. 3. Elution profile of glucose monophosphates on an HPIC-AS6 column. Peaks: 1 = glucose; 2 = glucose 1-phosphate; 3 = glucose 6-phosphate; 4 = glucose 3-phosphate. Chromatographic conditions: eluent, 50 m*M* sodium hydroxide solution containing 300 m*M* sodium acetate; flow-rate, 1 ml/min; temperature, ambient; detector, PAD 2.

eluent which is strong enough to elute monophosphates caused glucose to elute with or very near the front.

Contribution of each hydroxyl group of D-*glucose to the PAD response*

As PAD detects the electric current produced by the oxidation of oxyanions, transformed from the hydroxyl groups of carbohydrates with strong alkali in the eluent, it is thought that the PAD response is proportional to the number of oxyanions. If all free hydroxyl groups of glucose and glucose derivatives are in the same situation, the responses of PAD for monosubstituted glucose derivatives (four free OH groups) and glucobioses (eight free OH groups) should be 4/5 and 8/5 of that for glucose (five free OH groups), respectively. Consequently, relative detector responses (RDR, relative to glucose) were compared in one OH equivalent.

Table I shows the RDR/OH of monomethyl-D-glucoses. The presence of methyl ether suppressed the PAD response. 6-Methyl had the least effect and the effects of the other methyls were about the same. The results shown in Table I suggest that the PAD response was little affected by the acidity of each hydroxyl group of D-glucose and the presence of a methyl group might hinder the interaction between the vicinal hydroxyl groups and the electrode of the detector. In the way, it was confirmed that the lowest alkali concentration of eluent which gave sufficient detector response for D-glucose derivatives was 50 m*M*.

The relative detector responses of D-glucobioses are summarized in Table II. To correct the difference in peak areas arising from the large difference in t_R values, the t_R values of all glucobioses were adjusted to about 4 min by addition of suitable amounts of sodium acetate to the eluent. Comparison of RDR/OH of eleven D-glucobioses showed that those of the three 1,1-linked trehaloses were almost the same, and masking of the 1-OH with another glucosyl residue resulted in the largest reduction in the PAD response. In other cases, the response was affected by the linkage configuration rather than the linkage position, β-linked isomers generally showing a larger response than α-linked isomers. This phenomenon may be explained on the basis of interaction between hydroxyl groups in the same molecule, for example the 3-OH and 2′-OH groups of maltose (α-1,4), form a hydrogen bond and they are somewhat protected against oxidation, whereas the two glucose residues of cellobiose (β-1,4), are favourably located and all hydroxyl groups of cellobiose are available for oxidation on the electrode.

Glucose 1- and 6-phosphates gave values of 0.72 and 0.99 relative to the response for glucose in one OH equivalent, respectively. Townsend *et al.* [22] reported that the presence of phosphate suppresses the PAD response. However, it is thought that the suppression is a result of masking of hydroxyl group(s), and RDR/OH was actually little affected by phosphate substituted on the 6-OH group of glucose, though 1-phosphate suppressed the PAD response somewhat.

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