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RAPID COMMUNICATION

A rapid method for separation of anomeric saccharides using a cyclodextrin bonded phase and for investigation of mutarotation

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HPLC separation of anomeric sugars using a β-cyclodextrin bonded phase and an eluent ethyl acetate/methanol/water has been achieved. HPLC is a useful method for analytically separating the anomers of sugars in a mutarotated system and is a good supplement to the nuclear magnetic resonance. By means of this technique investigations of the mutarotation of D-glucose gave 36% α-form and 64% β-form. The pH dependence of mutarotation in an organic solvent was examined and discussed. Further mono- and disaccharides were separated in anomers. Furanoid forms of the sugar can also be determined by HPLC.

INTRODUCTION

Classical methods for the determination of sugar anomers are based on the measurement of the specific rotation and nuclear magnetic resonance (NMR) (Angyal, 1968; Angyal & Dawes, 1968; Angyal, 1984, Angyal, 1991), but the NMR is expensive and difficult to work with. Moreover polarimetry is a non-specific method and the proportion of the furanose forms cannot be determined in this way. Anomers may be also analysed by gas chromatography (e.g. Sweeley *et al.*, 1963; Paez *et al.*, 1987). However this method requires a derivatisation step. The determination of some anomers is possible using stereospecific enzymes, e.g. glucose oxidase (Vogel *et al.*, 1988). Occasionally liquid chromatography has been used (Honda *et al.*, 1984; Kahle & Tesarik, 1980; Armstrong & Jin, 1989; Oshima *et al.*, 1980). Honda *et al.* (1984) separated anomers on a highly cross-linked cation-exchange resin with a polystyrene base, but the procedure was comparatively lengthy (20–60 min). Kahle & Tesarik (1980) achieved good separation at 0°C, using a column of amino-propylated silica gel in the sulphate form, but the durability of this column is not satisfactory.

Armstrong & Jin (1989) separated anomeric forms of saccharides with cyclodextrin bonded phases, but they separated mostly sugar derivatives. We describe here an improved method of anomer analysis on a cyclodextrin bonded phase and the application of this method for investigation of the mutarotation of D-glucose in an organic solvent.

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EXPERIMENTAL

Materials

D-arabinose (Fluka), D-fructose (Belger), D-galactose (Leidholdt), α-D-glucose (Serva), β-D-glucose (Sigma), α-D-lactose (Serva), β-D-maltose (Merck), α-D-melibiose (Sigma), L-rhamnose (L. Light & Co. GmbH), D-ribose (Arcochemie), D-xylose (Berlin Chemie).

The solvents were research grade or better and were obtained from a variety of sources.

High performance liquid chromatography

The chromatographic system consisted of Shimadzu LC9A pump, rheodyne injector with a 20 μl loop, a Shimadzu RID-6A differential refractometric detector and a Shimadzu C-R4AX integrator. The analytical column (200 × 4.0 mm I.D.) used throughout was packed with 5 μm particle diameter β-cyclodextrin bonded silica gel (Nucleodex β-OH, Macherey Nagel). The mobile phases tested were methanol/water (40/60, 60/40, 90/10), acetonitrile/water (50/50, 80/20, 90/10) and ethyl acetate/methanol/water (40/30/30, 60/25/15, 70/20/10, 75/15/10, 80/14/6, 80/16/4). The mobile phase

was isocratic and pumped at 0.4–1.7 ml/min. Different temperatures were used: 10, 23, 35°C.

Adjustment of mutarotation equilibrium:

Sugars were dissolved in ethyl acetate/methanol/water (70/20/10, v/v/v) and allowed to stand overnight at room temperature, or were dissolved in dimethyl sulfoxide, heated at 90°C for 5 min and allowed to stand overnight at room temperature. pH values were adjusted with acetic acid and triethylamine.

Determination of anomer composition:

The sugars were dissolved in water and the change of optical rotation was measured with polarimeter from Carl Zeiss Jena using a mercury lamp (578 nm line).

RESULTS AND DISCUSSION

The Nucleodex β-OH column is a chiral stationary phase containing native β-cyclodextrin. It is possible to use the sterically fixed hydroxyl groups at positions 2 and 3 as a high density diol phase with specificity for saccharide structures.

Separation of D-glucose anomers

Methanol/water and acetonitrile/water mixtures are not suitable for the separation of the anomers. However the use of the relatively non-polar ethyl acetate at a concentration of about 70% results in a separation of the anomeric forms of D-glucose. Retention time and spreading of the HPLC peaks increase with ethyl acetate concentration.

In this investigation glucose was dissolved in a solvent mixture (ethyl acetate/methanol/water 70/20/10/, v/v/v), since the HPLC of aqueous solutions of glucose changed the eluent profile and prevented a separation of anomers. Also, the rate of mutarotation is lower in the organic solvent (Angyal, 1968). Polarimetric measurements indicate that the position of the mutarotation equilibrium of glucose in this solvent mixture is not altered.

Nucleodex β-OH, supplied by Macherey Nagel, gave the best resolution with the eluent ethyl acetate/methanol/water (80/14/6, v/v/v) (Fig. 1, chromatogram A). The resolution R is a parameter to judge the quality of a separation, where t_{ms1} and t_{ms2} are the retention times of peaks 1 and 2, respectively, w_1 and w_2 are the peak widths (at the base of each peak) for components 1 and 2, respectivcly.

$$R = \left[\frac{2(t_{ms2} - t_{ms1})}{(w_1 + w_2)}\right]; t_{ms2} > t_{ms1} \qquad (1)$$

Values of the resolution parameter are given in Table 1. It is known that, sometimes in HPLC, a lowering of

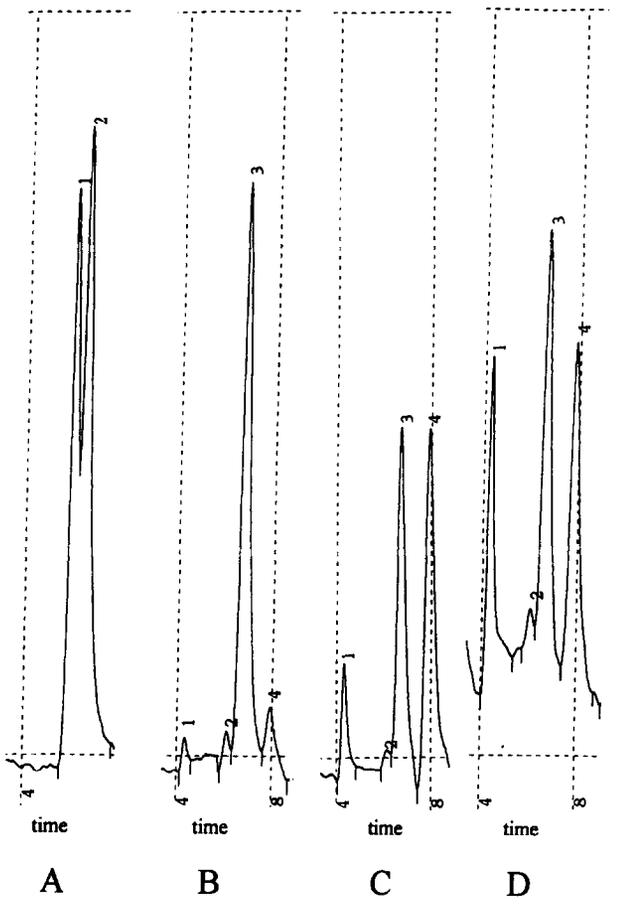

Fig. 1. HPL-chromatographic separation of the anomeric D-glucose (A) and D-galactose (B–D); (A) 1: α-D-glucopyranose, 2: β-D-glucopyranose; (B–D) 1 and 2: furanoide forms, 3: α-D-galactopyranose, 4: β-D-galactopyranose; dissolved in different solutions (B: ethyl acetate/methanol/water, fresh solved; C: ethyl acetate/methanol/water, standing overnight; D: dimethyl sulfoxide, standing overnight).

temperature produces an increase in resolution, but it was not possible to improve the resolution by reducing the column temperature to 10°C. In general an increase in temperature has the effect of decreasing the viscosity, reducing peak width and particularly improved resolution. However, in the present case an increase of column temperature has impaired the separation.

A change in flow rate in the range 0.4 –1.7 ml/min has no effect on resolution. High speed and resolution are essential for the separation of saccharides that undergo mutarotation. All subsequent separations were carried out at the flow rate of 1.7 ml/min and the temperature of 23°C. In a pH range between 3.5 and 8.0 the retention time of anomers (ethyl acetate/methanol/water) was independent of pH value or the addition of phosphate ions to a maximum of 0.01 mol/litre.

Investigations of the mutarotation of D-glucose

As is generally known, D-glucose mutarotates in aqueous solution to give an equilibrium mixture of α-D-glucose (36%) and β-D-glucose (64%). The rate of anomerization depends on the reaction medium.

Table 1. Chromatographic data of the separation of some saccharides

Compound	Pyranose forms									Furanose forms[c]			
	t_s		R	%[a]		%[b]				%[a]	%[b]		
	α-	β-		α-	β-	α-	β-						
Arabinose	3.2	3.7	0.8	49.0	46.7	62	29	43.8	NMR GC	4.3	8.8	5.4	NMR GC
Galactose	5.2	6.4	2.0	38.0	54.2	30.2	63.8	62.6	NMR GC	7.8	6.3	5.4	NMR GC
Glucose	4.8	5.3	1.2	36.0	64.0	36.9	63.1	60.2	NMR GC	0	0	0	NMR GC
Rhamnose	1.8	2.3	1.1	77.5	22.5					0			
Ribose	2.2	2.5	0.6	40.9	59.1	21.5	58.5		NMR	0	20.0		NMR
Xylose	2.6	3.2	1.1	44.5	55.5	36.5	63.0	55.2	NMR GC	0	< 1.0	3.4	NMR GC
Lactose	20.9	23.0	0.6	39.1	57.3					—			
Maltose	17.5	19.6	0.8	38.1	61.9	44.9	55.1		GC	—			
Meliblose	20.0	25.0	0.45	42.3	57.3					—			

t_s —net retention time (min); R —resolution; [a] —percentage peak area (this work); [b] —percentage anomer distribution in references [NMR (Angyal, 1994), GC (Sweeley *et al.*, 1963)]; [c]the percentage distribution of anomer furanose is given by addition.

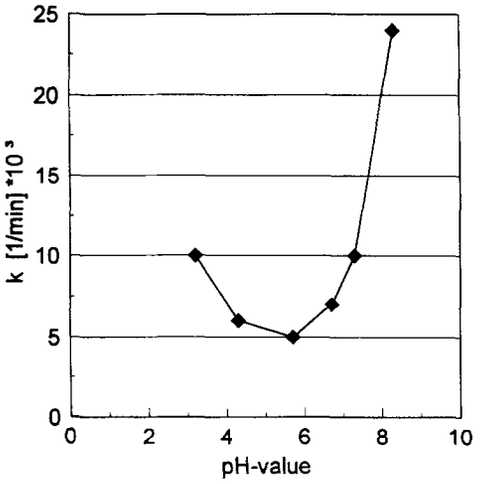

Fig. 2. The rate constant of formation of β-D-glucose from α-D-glucose as a function of pH.

Figure 2 shows the first order rate constant for the formation of β-D-glucose from the α-form as a function of pH determined by the HPLC procedure developed here. As expected, the mutarotation rate increased both in acid and alkaline solution. The same pH-profile was obtained when the reaction was started from the α- or β-anomers. A plot of the logarithm of concentration against the reaction time results in a straight line indicating a first-order reaction. We were unable to confirm the observation of Armstrong & Jin (1989) showing a marked decrease of the α-D-glucose concentration in the first 15 min of the reaction. The mutarotation equilibrium of α- and β-D-glucose in ethyl acetate/methanol/water was found to be identical to that in water.

Mutarotation and separation of other saccharides

For the cases where standards were not available the anomers were determined using polarimetry. The optical rotation was greater for D-arabinose and L-rhamnose and lower for D-galactose and D-xylose. As reported by Rauen (1964), D-arabinose exists in the β-form and D-galactose, L-rhamnose and D-xylose exist in the α-form. Table 1 shows data for the equilibrium ratio of anomers of some selected saccharides in comparison with values reported in the literature. Apart from investigated pentoses and hexoses the mutarotated equilibrium of disaccharides (D-lactose, D-maltose and D-melibiose) was determined with the HPLC. In general, the α-forms eluted before the β-forms, suggesting smaller interaction of this anomeric form with the stationary phase. Depending upon the method used, different anomeric distributions were obtained, except for D-glucose. In comparison with previous work Armstrong & Jin (1989) we can show the existence of the furanoide forms of the sugars, which is consistent with investigations in dimethyl sulfoxide. Perlin (1966) and Angyal (1994) showed that there is a greater proportion of the furanose forms for some saccharides in dimethyl sulfoxide. The percentage ratios of α-(α-p) to β-pyranose (β-p) and furanose (f) in dimethyl sulfoxide for D-galactose and D-arabinose are 39,1% (α-p): 35,0% (β-p) and 25,9% (f) and 53,5% (α-p): 24,0% (β-p) 22,5% (f), respectively. For D-glucose, D-ribose and D-xylose there was no measured difference in the number and the relation of peak areas. For these saccharides Angyal (1994) determined with NMR small changes of composition in dimethyl sulfoxide. Figure 1 (chromatogram B, C and D) also shows the chromatograms of freshly dissolved D-galactose in ethyl acetate/methanol/water (chromatogram B), after standing overnight at room temperature in ethyl acetate/methanol/water (chromatogram C) and in dimethyl sulfoxide (chromatogram D). In the fresh dissolved sample (chromatogram B) D-galactose dominated the α-form, the mutarotated equilibrium adjusted after standing overnight (chromatogram C and D) at which the α- and β-pyranoide forms (peak 3 and 4) are about equal distributed and one furanoide form (peak 1) is the prefered form. The increased proportion of the furanose forms of galactose can be explained by the instable axial hydroxyl group located at C-4 (Pigman & Isbell, 1968; Angyal, 1994).

For the ketohexose, D-fructose, four peaks were observed, but they were only moderately well separated. In the literature, conflicting statements are found for the isomeric and anomeric distribution of D-fructose (Pigman & Isbell, 1968; Cockman *et al.*, 1987). The reason may be the more complex process of fructose mutarotation and the strong temperature dependent anomer distribution (Cockman *et al.*, 1987). D-ribose showed only two peaks in spite of the fact that four isomers (Perlin, 1966; Cockman *et al.*, 1987; Angyal, 1994) are known. These two peaks are not quantitatively changed upon longer standing in our solvent mixture or in dimethyl sulfoxide.

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