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On-column isomerization of sugars during high-performance liquid chromatography: analysis of the elution profile

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Abstract

Four monosaccharides (glucose, galactose, mannose and fructose) and one disaccharide (maltose) were subjected to high-performance liquid chromatography with UV or refractive index detection. Various profiles such as broad, tailed and splitted peaks were produced, depending on column temperature and eluent flow-rate because these saccharides underwent isomerization. In contrast, α-methylglucoside, a non-converting derivative, always produced a sharp peak. By analyzing these profiles kinetic constants of the isomerization were obtained and compared with the literature data.

1. Introduction

In earlier studies we have demonstrated through the analysis of HPLC profiles that the immunosuppressive drugs, cyclosporins (cyclic peptides) and FK506 (a macrolide), undergo reversible conversion between two isomers [1,2]. However, this was still not confirmed because the real structures of the isomers were not determined and because no reference data were found on the isomerization kinetics. Furthermore, examples of chromatograms of isomerizing compounds were rarely presented or analyzed. Thus we have investigated HPLC profiles of sugars because the isomerization kinetics and real structures of some sugars are already known.

For the quantitation of isomeric sugars, the column is usually heated to 70–90°C because these sugar molecules undergo isomerization at a high frequency at high temperatures. They migrate in the column at an apparently constant velocity to produce one peak. When eluted at a lower temperature, they produce complex profiles because isomerization occurs at a lower frequency, randomly but at a certain probability. On a column cooled to 1.5–4°C, α- and β-anomers of some sugars are separated from each other because essentially no molecules undergo α–β conversion at that low temperature as reported by Baker and Himmel [3] and Honda et al. [4]. Not only column temperature but also eluent flow-rate could be a parameter determining the profile, and combination of these two parameters could be a key to profile analysis.

2. Experimental

A Hitachi HPLC system (Tokyo, Japan), consisting of an L-5000LC controller, 655A pump, 655A variable-wavelength UV detector, was used. A Tosoh 8012 refractive index (RI) detector (Tokyo, Japan) was also used. Peak

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area and retention time were measured by a Hitachi D-2500 integrator, but the areas corresponding to the converted or non-converted molecules on the profiles in Fig. 2 were measured by cutting out and weighing the paper. The separation was made in the ligand-exchange mode on a Ca^{2+}-form Aminex HPX-87C column (300 × 7.8 mm I.D., sulfonated polystyrene packing) (Bio-Rad Laboratories, Tokyo, Japan) with pure water as eluent. The column was encased in a Hitachi column oven or in a cooled water bath.

A 25-μl solution of sugar (usually 2 mg/ml in water) was injected into the column. All sugars were obtained from Wako Pure Chemicals (Tokyo, Japan), i.e., D-glucose, D-galactose, D-mannose, D-fructose, maltose and 1-α-methyl-D-glucoside.

3. Results and discussion

The elution profiles of glucose changed depending on column temperature (Fig. 1): a broad peak with a little distorted shape at 75°C; two peaks with a bridge in between at 45°C; two peaks with a low bridge at 25°C. Fig. 1 also shows the dependency of the profile on the eluent flow-rate. Despite the enormous change in the profile, the integrated area was constant at all the temperatures (75, 60, 50, 45, 40, 30, 25°C at a 1.0 ml/min flow-rate with a C.V. of 3%) and all the flow-rates (1.2, 1.0, 0.8, 0.6, 0.5, 0.4 ml/min at 45°C with a C.V. of 5%). The first and second peak on the profiles at 45°C and 25°C could be assigned to the six-membered ring pyranose of β- and α-glucose, respectively, because the α-anomer assumes the *cis*-vicinal OH configuration at positions C1 and C2 of the glucose molecule to interact with Ca^{2+} on the stationary phase [3]. Peaks of the other sugars could also be assigned to their isomers by comparing the number of *cis*-vicinal OH as shown later. The bridge, the so-called "reaction zone", should represent the elution of molecules that have undergone isomerization at least once during elution.

The zones representing the converted and

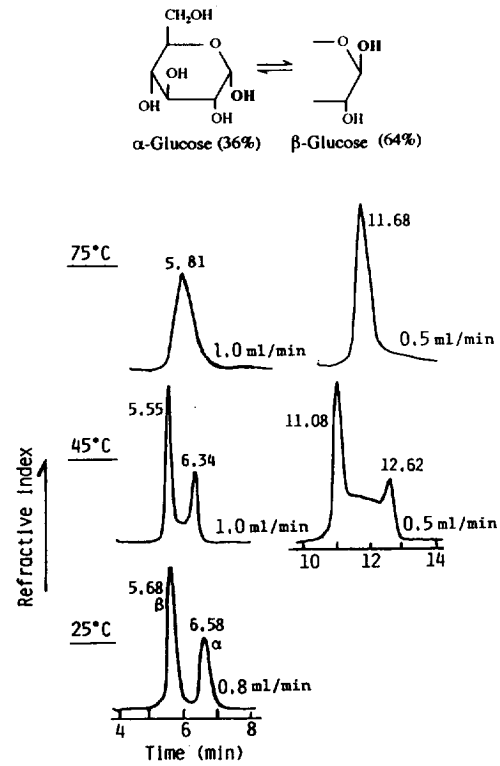

Fig. 1. Chromatograms of glucose. Column: Ca^{2+}-form Aminex HPX-87C at 75, 45 or 25°C. Eluent: pure water at a 1.0, 0.8 or 0.5 ml/min flow-rate. Detection: refractive index.

non-converted glucose anomers were fused with each other when eluted at 45° or 60°C (Fig. 2). But the fused areas could be separated by bordering them on the assumption that the non-converted anomers produce symmetrical peaks as shown by the dashed lines in the profiles of Fig. 2. The area of each zone on the copy of the profile was measured by cutting along the border line. Areas of both first and second peaks, which should correlate with the amounts of the non-converted anomers, were smaller at the lower flow-rate due to the higher chances of conversion during the longer stay in the column, whereas the bridge areas increased with lower flow-rates. Plots of the areas of the non-converted anomers vs. retention time were linear on a semi-logarithm graph, indicating that the reaction follows first-order kinetics (Fig. 3). The half-lives were determined from these lines, though they

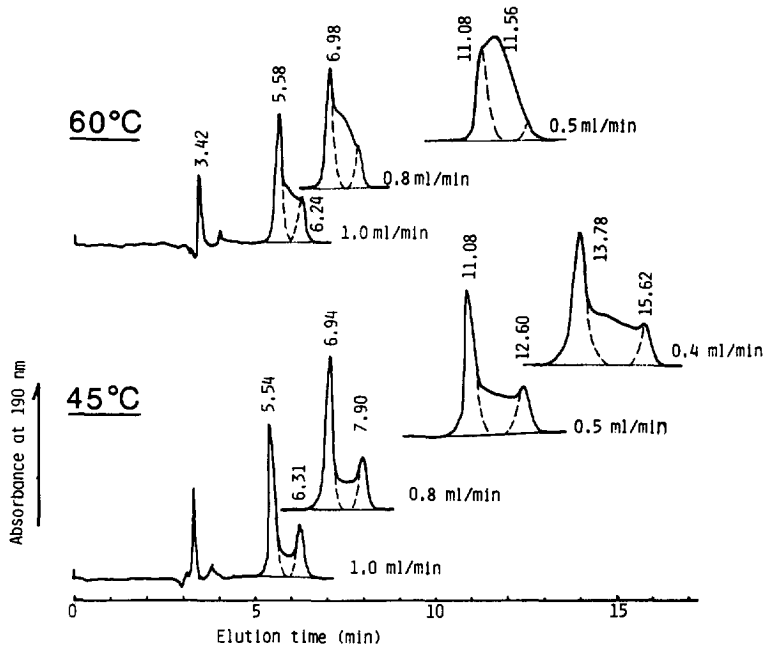

Fig. 2. Chromatograms of glucose. Detection: UV absorbance at 190 nm. Other HPLC conditions were the same as described in Fig. 1. The dashed lines show the borders between the zones of non-converted and converted molecules.

might not be the same as those in solution. Either anomer had a longer half-life at a lower temperature; for example, the β-anomer had a half-life of 24 min at 45° and 9.4 min at 60°C.

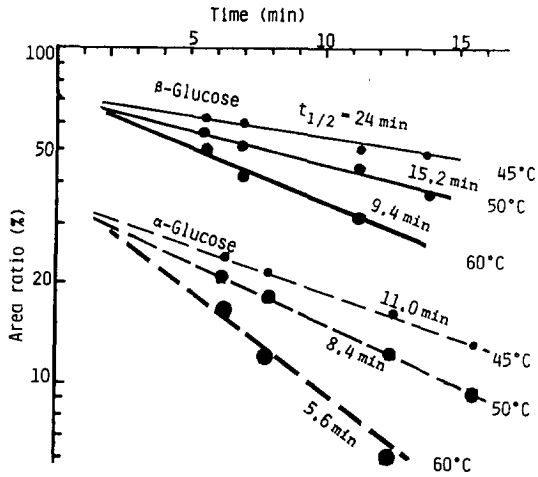

Fig. 3. Relationship between the retention time and the peak area ratio of each glucose anomer to the total area; time course of the non-converted α-anomers (dashed line) and β-anomers (solid line) in the column.

The β-anomer had a longer half-life than the α-anomer at a given temperature, for example, 15.2 min and 8.4 min, respectively, at 50°C. The existence ratios were estimated to be ca. 70% and 35% for the β- and α-anomer at time zero. This ratio was close to that found in the literature: 64% for β-pyranose, 36% for α-pyranose and a negligible existence for the five-membered ring furanose (less than 0.5%) and open-chain aldehyde form (about 0.02%) in aqueous solution at ambient temperature [5,6]. The profiles obtained by RI detection (shown in Fig. 1) were similar to those obtained with UV detection at 190 nm (shown in Fig. 2), indicating that both detectors showed an identical response to the two anomers.

α-Methylglucoside, a non-converting glucose derivative, was eluted as a reference compound and produced always one symmetrical peak (Fig. 4). The theoretical plate number of the peak at flow-rates of 1.0 and 0.5 ml/min was: 6400 and 10 000 at 75°C; 4500 and 6500 at 45°C; 2600 and 4000 at 25°C. The highest separation efficiency would be obtained at a flow-rate lower than 0.4

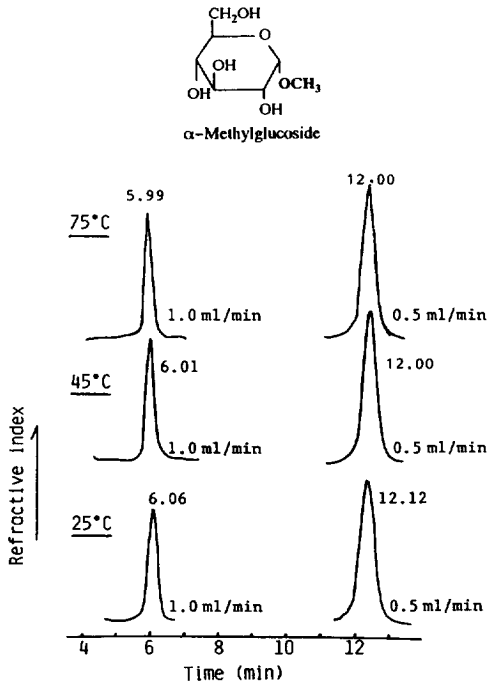

Fig. 4. Chromatograms of α-methylglucoside. HPLC conditions as described in Fig. 1.

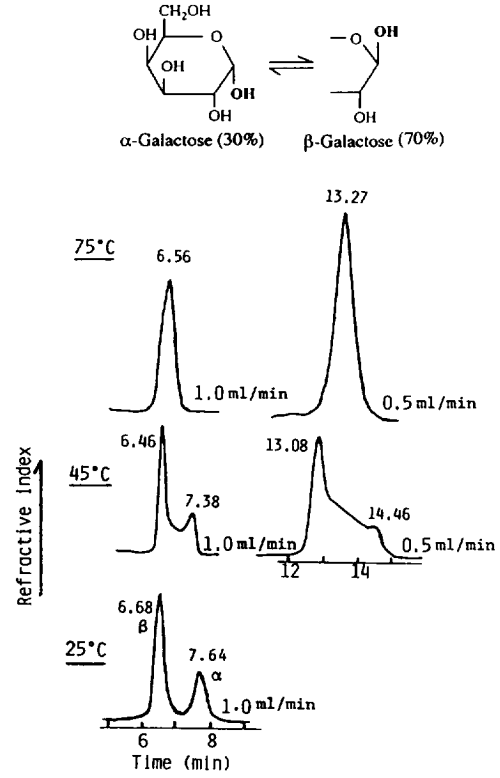

Fig. 5. Chromatograms of galactose. HPLC conditions as described in Fig. 1.

ml/min, which could be due to the slow equilibration of the solute between stationary and mobile phases in this separation mode. The plate numbers of the α-methylglucoside peaks agreed with those of the glucose anomer peaks under all given HPLC conditions (Fig. 2), indicating that the anomers and the methyl derivative were chromatographed in the same separation mode.

The profiles of galactose were similar to those of glucose (Fig. 5). The first and second peaks at low column temperature were assigned to the β- and α-anomer, respectively, and the half-lifes were estimated to be 18.5 and 6.5 min at 50°C, and the existence ratios at time zero were 68% and 32%, which is close to the literature data of 70% and 30% [5,6].

Mannose produced profiles similar to those of galactose and glucose (not shown). In contrast to the other sugars, the first peak should represent the α-anomer because the other anomer, β-mannose, has a *cis*-vicinal OH configuration at positions C1 and C2. The ratios at time zero were 62% and 38% for α- and β-mannose, respectively, being fairly consistent with the literature data of 67% and 33% [5,6].

The profiles of fructose were a little different from the others (Fig. 6). The retention times of the two major isomers were far apart, which contributed to the extremely broad peak with the column kept at 45°–75°C. A linear slope was produced as the reaction zone rather than a horizontally linear bridge. The ratios of the first and second peak areas at time zero were 18% and 82%, which is in agreement with the literature that an aqueous fructose solution contains about 20% of the furanose form [5]. The first peak obtained with the cooled column was overlapped by some unidentified minor peaks, probably because fructose undergoes both α–β and pyranose–furanose conversions and exists even in an open-chain form in appreciable amounts [5,6].

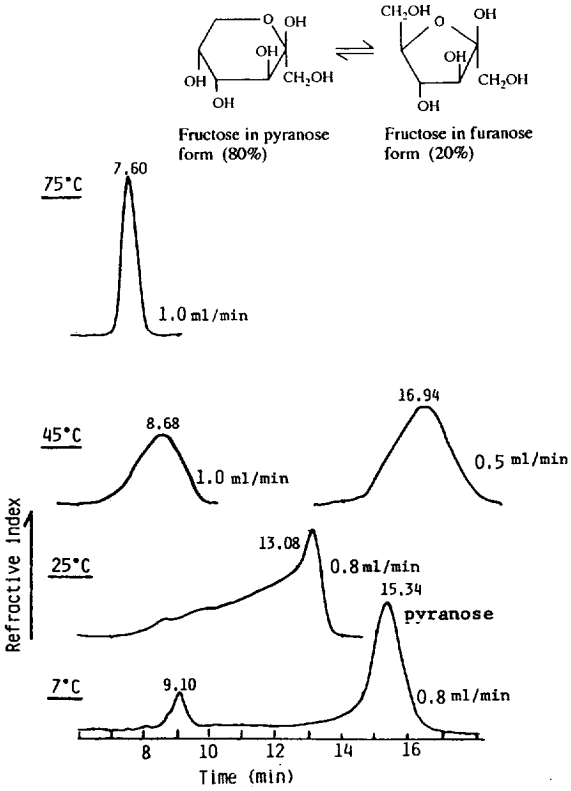

Fig. 6. Chromatograms of fructose. HPLC conditions as described in Fig. 1.

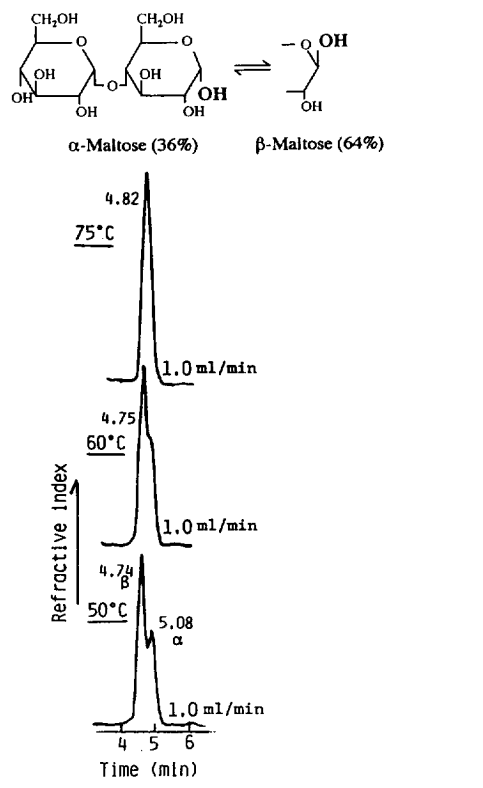

Fig. 7. Chromatograms of maltose. HPLC conditions as described in Fig. 1.

Table 1
Comparison between experimental and calculated retention times of a fused peak

Sugar (flow-rate)	Retention time (min)	
	Experimental	Calculated from the equation[a]: $t = (r_p/t_p + r_q/t_q)^{-1}$
Glucose (0.5 ml/min)	11.68 at 75°C	$11.59 = (0.64/11.08 + 0.36/12.62)^{-1}$ at 45°C
Galactose (0.5 ml/min)	13.27 at 75°C	$13.47 = (0.70/13.08 + 0.30/14.46)^{-1}$ at 45°C
Mannose (1.0 ml/min)	6.75 at 75°C	$6.86 = (0.67/6.62 + 0.33/7.63)^{-1}$ at 35°C
Fructose (1.0 ml/min)	7.60 at 75°C	$9.53 = (0.2/7.02 + 0.8/10.46)^{-1}$ at 25°C
Maltose (1.0 ml/min)	4.82 at 75°C	$4.86 = (0.64/4.74 + 0.36/5.08)^{-1}$ at 50°C

[a] The existence ratios (r_p and r_q) are quoted from the literature [5,6]. The retention times (t_p and t_q) were obtained at the column temperature described below each equation.

Maltose also produced two peaks at a lower temperature (Fig. 7). However, because their retention times were too close the peaks overlapped, which made profile analysis difficult.

We derived the following equation to predict the retention time (t) of a molecule converting between forms P and Q, as explained in the previous report [2]:

$$1/t = (r_p/t_p) + (r_q/t_q)$$

where t_p and t_q are the retention times of P and Q, r_p and r_q are the existence ratios ($r_p + r_q = 1$). Using the ratios found in the literature, the calculated retention times t agreed well with the experimental values for glucose, galactose, mannose and maltose, indicating this equation to be valid (Table 1). Only fructose gave a slightly different value probably because the real reaction was more complex than the postulated one.

Such unusual chromatographic behaviour of the sugars or immunosuppressants has sometimes been explained by other mechanisms such as column deterioration at low temperature, viscosity of the eluent or of sugars, the presence of impurities in the sample, the appearance of new isomers, and variable sensitivity of detection. These mechanisms might partly be valid but could never be the main contributors to the behaviour of these compounds.

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