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IMPROVED ANALYSIS OF ALDOSE ANOMERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON CATION-EXCHANGE COLUMNS

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SUMMARY

Aldose anomers were chromatographed on columns of a highly cross-linked, cation-exchange resin (Shodex DC-613) of sodium and calcium forms in combined partition and ligand-exchange mode. The pyranose anomers of D/L-arabinoses, D-lyxose, D-ribose, D-galactose, D-mannose, L-fucose and L-rhamnose were completely separated on the column of the sodium form with water–acetonitrile (20:80, v/v) as eluent below room temperature. Replacement of the counter-ion by the calcium ion changed the resolution pattern, giving complete separation of D/L-arabinoses, D-lyxose, D-xylose, D-altrose, D-galactose, D-glucose, D-mannose, L-fucose and L-rhamnose under the same conditions. Some aldoses also gave furanose peaks, but their anomers were not resolved. All aldose anomers were monitored sensitively by photometric detection after postcolumn labelling with 2-cyanoacetamide. Appropriate conditions for rapid, simultaneous determination of the pyranose anomers of D-glucose were also established and applied to the study of mutarotation and to the analysis of serum glucose.

INTRODUCTION

Recently Niki *et al.*[1] and Grodsky *et al.*[2] reported that α-D-glucopyranose specifically stimulates insulin secretion. Mutarotase was considered to control this stereospecific reaction by catalysing anomerization of D-glucose[3]. These findings suggest possible existence of similar specificity in biochemical reactions involving other aldoses.

Classical methods for the estimation of aldose anomers are based on the measurement of specific rotation or proton magnetic resonance (PMR), but they are insensitive. Enzymic methods involving the use of β-D-glucose oxidase (*e.g.* ref. 4) or a combination of β-D-glucose dehydrogenase and mutarotase[5] are superior in sensitivity and rapidity of analysis. Aldose anomers may be also analysed by gas chromatography (*e.g.* ref. 6). However, this method is inadequate in biochemical and kinetic studies, because anomers are not derivatized quantitatively to volatile compounds because anomerization changes their molar proportion. The use of liquid

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chromatography overcame this limitation. Ramnäs and Samuelson[7] separated anomers of some aldoses by partition chromatography on an anion-exchange column, but it took quite a few hours for complete separation. Although a moderate improvement was made in this type of chromatography by Oshima *et al.*[8], they examined the separation of only a limited number of aldoses. On the other hand Goulding[9] succeeded in separating aldose anomers on a cation-exchange column, but the number of saccharides separated was also a few. Better separation was achieved by Kahle and Tesařik[10], who used a column of aminopropylated silica gel, but the durability of this column is not satisfactory.

We describe here an improved method of anomer analysis on a highly cross-linked, cation-exchange resin of polystyrene base with post-column labelling using 2-cyanoacetamide[11]. We also present some applications, in particular to the analysis of D-glucose anomers.

MATERIALS AND METHODS

Chemicals

All aldoses were obtained from commercial sources. L-Arabinose, D-allose, D-altrose, D-gulose, D-idose and D-talose were from Sigma (St. Louis, MI, U.S.A.); D-xylose, D-galactose and α/β-D-glucoses from Kishida (Doshomachi, Higashi-ku, Osaka, Japan); D-lyxose and L-fucose from Tokyo Kasei Kogyo (Nihonbashi, Chuo-ku, Tokyo, Japan); D-arabinose, D-ribose, D-mannose and L-rhamnose from Wako (Doshomachi, Higashi-ku, Osaka, Japan). All other chemicals and solvents were of the highest grade commercially available.

PMR studies

The PMR spectra were obtained in [$^2H_3$]acetonitrile–^2H_2O (80:20, v/v) at 25°C using a JEOL FX-200 instrument. Signals were recorded in the δ-scale in parts per million from the proton signal of tetramethylsilane as the internal standard.

High-performance liquid chromatography

A Hitachi 635 high-performance liquid chromatograph was used for pumping eluents, and aldose samples were loaded as $2.5 \cdot 10^{-3}$ *M* solutions in water–acetonitrile (20:80, v/v) via a Rheodyne injector with a 20-μl loop. Prepacked Sodex RSPak DC-613 columns (150 × 6 mm I.D.) of sodium and calcium forms were jacketed and thermostated by circulating water, and eluted with aqueous acetonitrile at a flow-rate of 0.50 ml/min. This column packing is a spherical resin (particle diameter 6 μm) of sulphonated polystyrene, 55% cross-linked with divinylbenzene, and available from Showa Denko (Shiba-daimon, Minato-ku, Tokyo, Japan). To the eluate were added consecutively 0.50 *M* potassium borate buffer (pH 8.5) and an aqueous 1% 2-cyanoacetamide solution at the same flow-rate of 0.50 ml/min, by using Atto SF-2396 twin-piston pumps and two Y-shaped PTFE mixers, and the resultant effluent was passed through a PTFE reaction coil (5 m × 0.5 mm I.D.) set in an oven thermostated at 100 ± 1°C. After the reaction solution had been passed through a 1-m PTFE cooling coil of the same bore size, the absorbance at 280 nm was monitored by an Atto SF-1205-A photomonitor with a 8-μl quartz cell. Signals were recorded by a Rikadenki R-10 recorder (full scale, 2 mV) and integrated by an integrator built into the chromatograph.

Chromatographic conditions for the analysis of D-glucose anomers

The anomers of D-glucose were separated on the column of calcium form with water–acetonitrile (30:70, v/v) at a flow-rate of 0.90 ml/min at 4°C. The anomers in the eluate were monitored by the method described above.

Extraction of serum D-glucose

A 40-μl sample of serum from a 20-year-old normal man was freeze-dried immediately after collection without the addition of anticoagulants. The residue was extracted with 40 μl of cold water–acetonitrile (30:70, v/v), and a 20-μl portion of the extract was analysed under the conditions described above.

RESULTS AND DISCUSSION

Effect of column temperature on the separation of aldose anomers

Fig. 1a shows the changes of capacity factors (k') of the pyranose anomers of D-xylose and D-glucose on the column of the calcium form, together with separation factors (R_s) between their anomers, as a function of column temperature. The k' value continued to decrease as column temperature rose, and the R_s value fell drastically to reach *ca.* 0 at *ca.* 35°C (D-xylose) or 70°C (D-glucose). At these temperatures

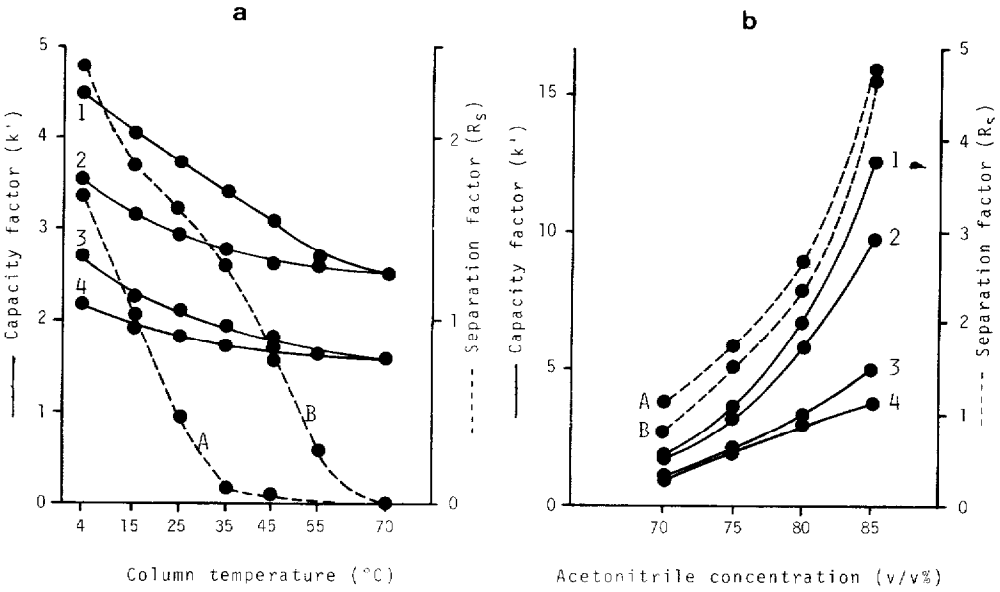

Fig. 1. Optimization of the separation of aldose anomers. (a) Effect of column temperature. Column, Shodex RSPak DC-613 (calcium form); column temperature, 4°C, 15°C, 25°C, 35°C, 45°C, 55°C and 70°C; eluent, aqueous acetonitrile (20:80, v/v); flow-rate, 0.50 ml/min; detection, 280 nm (after postcolumn labelling with 2-cyanoacetamide). Curves: 1 = α-D-glucopyranose; 2 = β-D-glucopyranose; 3 = α-D-xylopyranose; 4 = β-D-xylopyranose; A = α/β-D-xylopyranoses; B = α/β-D-glucopyranoses. (b) Effect of acetonitrile concentration. Column, Shodex RSPak DC-613 (sodium form); column temperature, 4°C; eluent: aqueous acetonitrile (30:70, 25:75, 20:80, 15:85, 10:90, v/v); flow-rates, 0.50 ml/min; detection, 280 nm (after postcolumn labelling with 2-cyanoacetamide). Curves: 1 = β-D-galactopyranose; 2 = α-D-galactopyranose; 3 = β-L-fucopyranose; 4 = α-L-fucopyranose; A = α/β-L-fucopyranoses; B = α/β-D-galactopyranoses.

TABLE I

CAPACITY FACTORS, SEPARATION FACTORS AND MOLAR RATIOS OF PYRANOSE ANOMERS OF ALDOSES

Columns, Shodex RSPak DC-613 (15 cm × 6 mm I.D.); column temperature, 4°C; eluent, water acetonitrile (20:80, v/v); flow-rate, 0.50 ml/min; detection, 280 nm (after postcolumn labelling with 2-cyanoacetamide).

Aldose anomer	*Sodium form*			*Calcium form*			*α/β Molar ratio (PMR)*	*Number of*		*Preferred conformation*
	Capacity factor (k')	*Separation factor (R_s)*	*α/β Molar ratio (peak integration)*	*Capacity factor (k')*	*Separation factor (R_s)*	*α/β Molar ratio (peak integration)*		*ax eq ax trihydroxyl groupings*	*ax-eq dihydroxyl groupings*	
α-D-Arabinose	4.36	2.63	0.61	4.46	2.44	0.57	0.59	0	1	*1C*
β-D-Arabinose	3.48			5.96				0	2	*1C ⇌ C1*
α-L-Arabinose	3.48	2.63	1.54	5.96	2.44	1.56	1.54	0	2	*1C ⇌ C1*
β-L-Arabinose	4.36			4.46				0	1	*1C*
α-D-Lyxose	2.43	1.23	0.31	2.26	6.03	0.18	0.34	0	1	*1C ⇌ C1*
β-D-Lyxose	3.26			6.73				0	2	*C1*
α-D-Ribose	5.04	2.98	3.32	nd*	–	–	2.87	1	3	*1C ⇌ C1*
β-D-Ribose	2.75			nd				0	2	*1C ⇌ C1*
α-D-Xylose	2.86	0.00	–	2.68	1.69	1.29	1.24	0	1	*C1*
β-D-Xylose	2.86			2.20				0	0	*C1*
α-D-Allose	5.14	0.92	3.55	nd	–	–	3.31	1	3	*C1*
β-D-Allose	4.84			4.26				0	2	*C1*
α-D-Altrose	4.08	0.53	–	3.70	2.11	1.47	1.43	0	1	*1C ⇌ C1*
β-D-Altrose	3.66			5.36				0	2	*C1*
α-D-Galactose	5.66	2.33	2.03	7.20	2.32	1.99	2.13	0	2	*C1*
β-D-Galactose	6.67			5.42				0	1	*C1*
α-D-Glucose	4.90	0.38	1.35	4.50	2.42	1.45	1.43	0	1	*C1*
β-D-Glucose	5.00			3.56				0	0	*C1*
α-D-Gulose	4.54	0.00	–	nd	–	–	4.13	1	2	*C1*
β-D-Gulose	4.54			4.74				0	1	*C1*
α-D-Idose	3.32	0.23	–	4.12	0.00	–	1.64	0	1 ⇌ 0	*1C ⇌ C1*
β-D-Idose	3.44			4.12				0	1	*C1*
α-D-Mannose	3.86	5.37	0.35	3.62	4.27	0.39	0.38	0	1	*C1*
β-D-Mannose	6.73			5.76				0	2	*C1*
α-D-Talose	5.40	0.00	–	nd	–	–	0.38	1	2	*C1*
β-D-Talose	5.40			nd				1	3	*C1*
α-L-Fucose	2.93	2.56	1.82	3.60	1.77	2.16	1.97	0	2	*1C*
β-L-Fucose	3.24			2.88				0	1	*1C*
α-L-Rhamnose	1.61	4.42	0.37	1.65	2.41	0.22	0.22	0	1	*1C*
β-L-Rhamnose	2.69			2.65				0	2	*1C*

* nd: Peaks were not detected owing to strong retention on the column.

the peaks of α- and β-anomers of both aldoses were completely superimposed. This temperature dependence is considered to be due to acceleration of anomerization as the temperature increases; at high temperatures anomers are so rapidly interconverted that they are eluted as a fused peak. As a result, the best separation of pyranose anomers was obtained at the lowest temperature examined, *i.e.* 4°C, for both aldoses. For the column of the sodium form, however, peak resolution was also affected, though only slightly, by solvent diffusibility, and the optimum temperature was raised to 25°C. The effects of column temperature observed for D-xylose and D-glucose were common to all aldoses.

Effect of solvent composition on the separation of anomers

Fig. 1b shows the effect of solvent composition on the retention and resolution of the anomers of D-galactose and L-fucose on the column of the sodium form. An increase in the acetonitrile concentration delays the elution of these anomers and increases their resolution. Similar effects of solvent composition were observed for other aldoses, irrespective of column form.

Separation of anomers of various aldoses

Table I summarizes the retention and resolution data for the anomers of all pento- and hexoaldoses of the D-series, together with a few popular L-sugars, as obtained on the columns of both forms with aqueous acetonitrile (20:80, v/v) at 4°C. It also presents the molar ratios based on peak integration. In this experiment each aldose sample was equilibrated in the eluent before injection. Peaks were assigned by comparing the molar ratios based on peak integration with those obtained by PMR measurement.

The column of the sodium form completely separated the pyranose anomers of D/L-arabinoses, D-lyxose, D-ribose, D-galactose, D-mannose, L-fucose and L-rhamnose. The anomers of D-allose were almost also completely resolved. The column of the calcium form had a similar resolvability; the aldoses whose pyranose anomers were completely resolved include D/L-arabinoses, D-lyxose, D-xylose, D-altrose, D-galactose, D-glucose, D-mannose, L-fucose and L-rhamnose. It is noticeable that the pyranose anomers of D-glucose, the most important aldose from the biochemical viewpoint, could be completely resolved. Some aldoses gave the peaks of furanose sugars, but their anomers were not resolved. Fig. 2 shows chromatograms for typical aldoses.

Chromatography on cation-exchange resins with polar solvents such as aqueous alcohols and aqueous acetonitrile is thought to proceed in the partition mode[12,13]. However, the characteristic change of resolution pattern by alteration of the metal ion suggests that there must be another interaction between polyhydric structures of aldose anomers and metal ions (Na^+ and Ca^{2+}) on the stationary phase. Goulding[9] postulated that this was a ligand exchange interaction between the water molecules in the aquated metal ions and the hydroxyl groups in aldose molecules. Although the results in Table I were obtained with aqueous acetonitrile, rather than *i.e.* distilled water, as used by Goulding, an analogous mechanism should have been exerted, together with partition. According to Goulding, trivalent complexation by three adjacent hydroxyl groups should be the most advantageous for the retention of aldoses by forming stable tridentates, especially with the calcium ion. This pre-

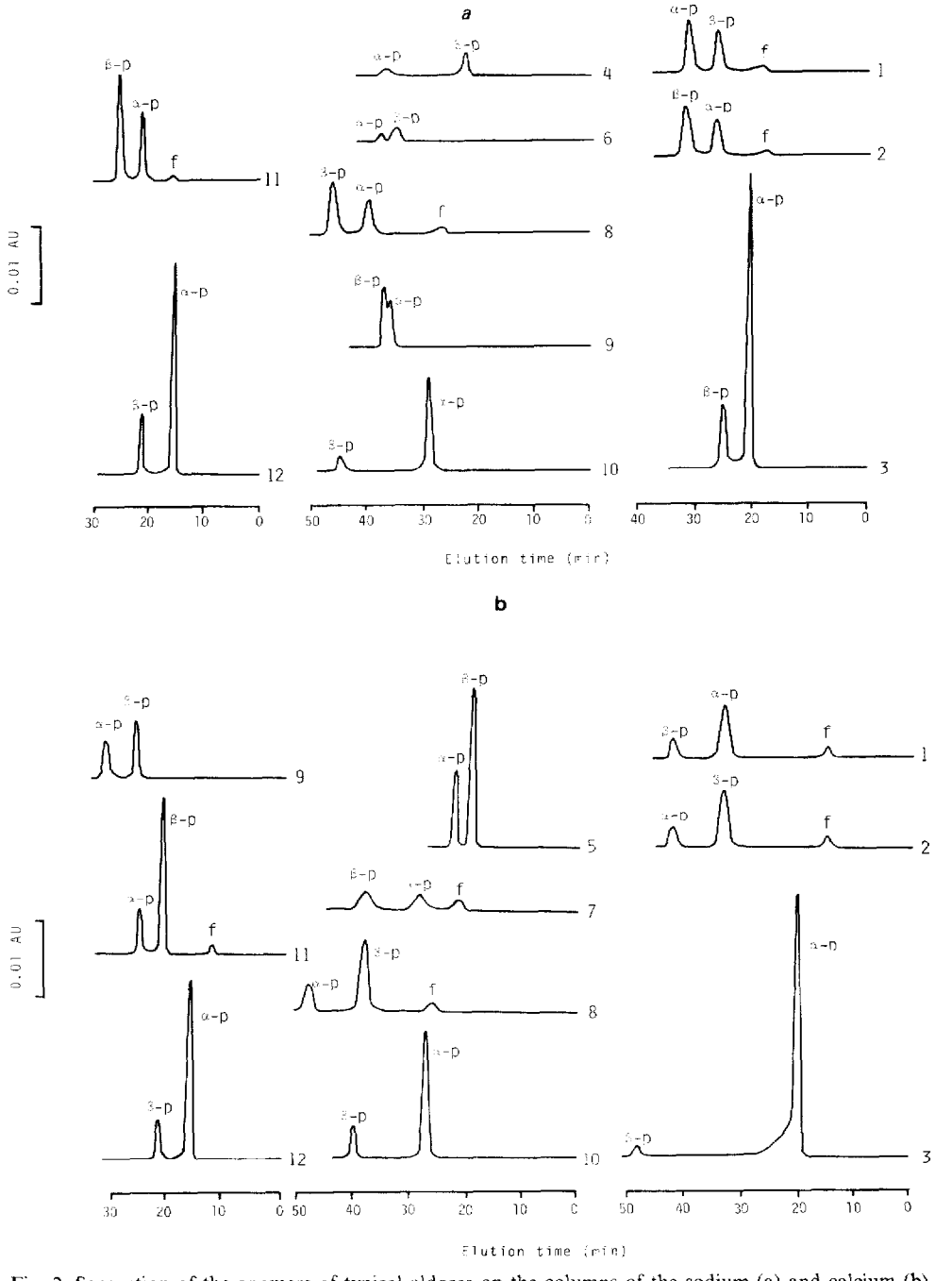

Fig. 2. Separation of the anomers of typical aldoses on the columns of the sodium (a) and calcium (b) forms. Sample scales, 50 nmol each. The analytical conditions are as described in Table I. Peaks: f = furanose; p = pyranose. Curves: 1 = D-arabinose; 2 = L-arabinose; 3 = D-lyxose; 4 = D-ribose; 5 = D-xylose; 6 = D-allose; 7 = D-altrose; 8 = D-galactose; 9 = D-glucose; 10 = D-mannose; 11 = L-fucose; 12 = L-rhamnose.

diction came true in the analysis presented here, because all aldose anomers with axial–equatorial–axial configuration (α/β-D-riboses, α-D-allose, α-D-gulose and α/β-D-taloses, *cf.* Table I) were strongly retained on the column of the calcium form. The axial–equatorial vicinal hydroxyl groups will be also favorable for retention of aldoses by forming bidentates, and the stability constants of the chelates will be greater, as the number of this functional group is increased. Comparison of the k' values between anomers indicates that this is also true with all aldoses, except for those with an axial–equatorial–axial trihydroxyl grouping as mentioned above, in the case of he column of the calcium form. Aldoses whose α-anomers have a larger number of axial–equatorial dihydroxyl groupings include L-arabinose, D-xylose, D-galactose, D-glucose and L-fucose. With all of these aldoses the α-anomer gave the larger k' value. For D-arabinose, D-lyxose, D-altrose, D-mannose and L-rhamnose, whose α-anomers have smaller numbers of axial–equatorial dihydroxyl groupings, the elution order of anomers was reversed. The α-anomer of D-idose is known to exist in an equilibrated state between the *1C* and *C1* conformations, which have one and no axial–equatorial dihydroxyl grouping, respectively. Chelation will occur with the *1C* conformer, and the *C1* conformer will be converted into the *1C* conformer to supplement the amount consumed by chelation. As a result, both anomers of D-idose will give chelates with approximately the same ease. Actually both anomers gave the same k' value.

The foregoing discussion completely vindicates Goulding's view for the column of the calcium form. For the column of the sodium form the aldose anomers with an axial–equatorial–axial trihydroxyl grouping also gave relatively large k' values. However, there was no clear relationship between the elution order of anomers and their steric status for other aldoses. The contribution of the ligand-exchange mechanism will in any case be smaller in the sodium form.

Simultaneous determination of D-glucose anomers

Further optimization studies established the best conditions for the analysis of D-glucose anomers. By using the column of the calcium form at 4°C and water–acetonitrile (30:70, v/v) as eluent at a flow-rate of 0.90 ml/min, the pyranose anomers of D-glucose were completely resolved in 12 min. The elution is performed at 4°C, the lowest accessible temperature in practical analysis, to minimize the interconversion of anomers. The calibration graph of the α-anomer showed good linearity, at least for samples in the range 10–350 nmol, and the coefficient of variation (n = 10) at the 18 nmol level was 1.4%. The linearity range of the β-anomer was at least 10–650 nmol, and the coefficient of variation was 1.0% at the 32 nmol level.

Figs. 3a and b illustrate the course of anomerization of α- and β-D-glucoses, respectively, in water at 27°C, as estimated under the above conditions. Because the total response of both peaks was constant throughout measurement, the monitoring with 2-cyanoacetamide is considered to have the same sensitivity to both anomers. Equilibration was reached after 2 h, whichever anomer was used as the starting material. The β/α molar ratio was 1.75 at the equilibrium state. The molar ratio changed during chromatographic analysis only to a negligible extent.

Fig. 4 shows a chromatogram obtained for human serum. Analysis of the serum extract indicated that both pyranose anomers were almost completely resolved without significant interference by the contaminant substances, giving a value of 1.78

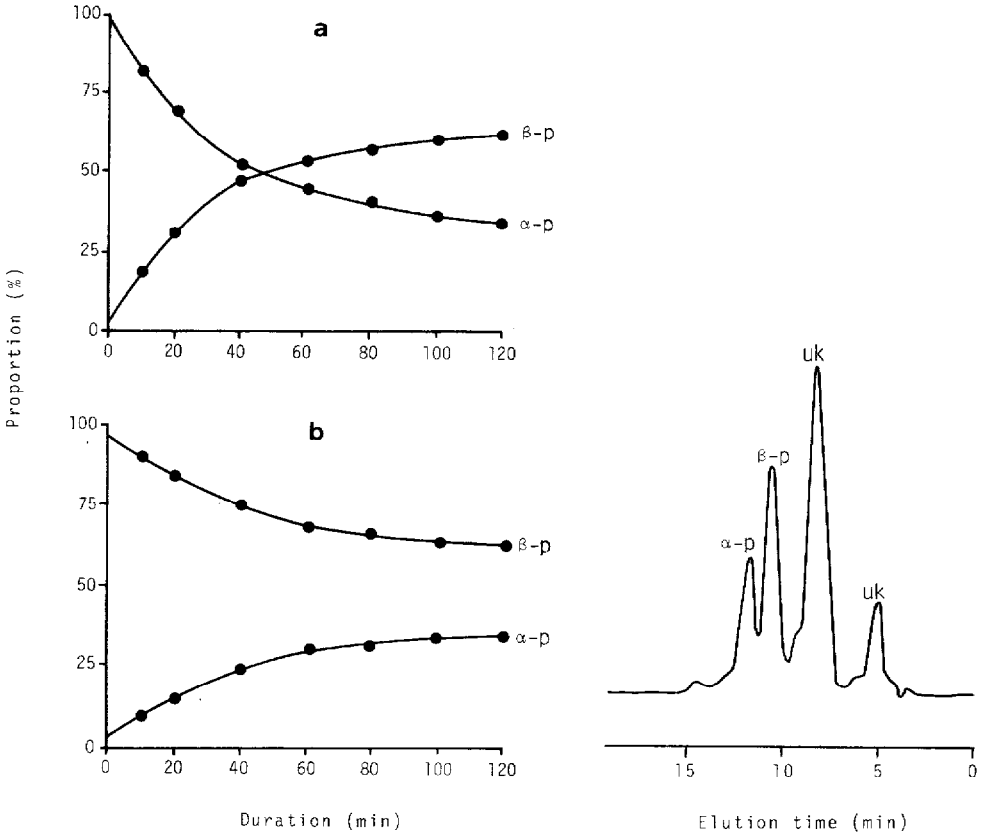

Fig. 3. Course of anomerization of (a) α- and (b) β-D-glucoses in distilled water at 27°C. Column, Shodex RSPak DC-613 (calcium form); column temperature, 4°C; eluent, aqueous acetonitrile (30:70, v/v); flow-rate, 0.90 ml/min; detection, 280 nm (ater postcolumn labelling with 2-cyanoacetamide). p = Pyranose.

Fig. 4. Analysis of D-glucose anomers in human serum. Sample scale, equivalent to 20 μl of serum. The analytical conditions are as described in Table I. Peaks: p = pyranose; uk = unknown substance.

for the β/α molar ratio. This value is not significantly different from that obtained for an equilibrium mixture of the standard sample of D-glucose.

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