Simultaneous Determination of 2-Deoxy-D-glucose and D-Glucose in Rat Serum by High-Performance Liquid Chromatography with Post-Column Fluorescence Derivatization

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A simple high-performance liquid chromatographic method for the determination of 2-deoxy-D-glucose and D-glucose in rat serum is described; this method is based on a post-column fluorescence derivatization. The sugars are automatically converted into fluorescent derivatives by reaction with *meso*-1,2-bis(4-methoxyphenyl)ethylenediamine in an alkaline medium after their separation on a strong anion exchanger column (TSK gel Sugar AXG). The detection limits ($S/N=3$) for 2-deoxy-D-glucose and D-glucose in rat serum are 0.52 and 0.56 nmol/ml, respectively.

Keywords 2-deoxy-D-glucose; D-glucose; post-column fluorescence derivatization; *meso*-1,2-bis(4-methoxyphenyl)ethylenediamine; anion exchanger column; high-performance liquid chromatography

2-Deoxy-D-glucose is a synthetic reducing sugar and acts as an antagonist toward D-glucose: dosed 2-deoxy-D-glucose is converted by hexokinase into 2-deoxy-D-glucose 6-phosphate (DG6P) *in vivo* similarly to D-glucose, but DG6P cannot be metabolized further, and the accumulated DG6P exericises a feedback inhibition on the hexokinase-mediated phosphorylation of D-glucose to cause accumulation of D-glucose. Therefore, 2-deoxy-D-glucose has antiviral activity. Its effects on influenza virus multiplication, and herpes simplex and human genital herpes infection have been investigated.[1-3] On the other hand, a method for the determination of 2-deoxy-D-glucose has become a very useful tool in the study of D-glucose metabolism in brain.[4]

2-Deoxy-D-glucose and D-glucose have been measured separately. 2-Deoxy-D-glucose in serum has been determined by colorimetric[5] and fluorometric methods[6,7] using 3,5-diaminobenzoic acid, which serves as both a chromogenic and a fluorogenic reagent, and by a high-performance liquid chromatography (HPLC) method with ultraviolet absorption detection (195 nm).[8] These methods are neither very sensitive nor selective. The quantification of D-glucose in serum has been carried out by various methods.[9] Thus, a sensitive and selective method that permits simultaneous determination of 2-deoxy-D-glucose and D-glucose is still required.

We previously reported a method for the determination of reducing sugars in human serum and urine by means of borate-complex anion-exchange HPLC with post-column fluorescence derivatization using *meso*-1,2-bis(4-methoxyphenyl)ethylenediamine (*p*-MOED), a fluorogenic reagent for reducing sugars including deoxy sugars.[10,11] Although the chemical structures of the fluorescent products formed from reducing sugars remain unknown, pyrazine derivatives are possibly formed by reaction of vicinal diamino groups of the reagent with the α-ketol group of the sugars. This study was designed to establish a sensitive HPLC method for simultaneous determination of 2-deoxy-D-glucose and D-glucose in serum from rat dosed with 2-deoxy-D-glucose by using the above-mentioned HPLC technique. L-Fucose was used as an internal standard. Serum levels of 2-deoxy-D-glucose and D-glucose after administration of 2-deoxy-D-glucose were determined by using the method developed.

Experimental

Chemicals *p*-MOED was synthesized as described previously.[12] The reducing sugars were purchased from Wako Pure Chemicals (Osaka,

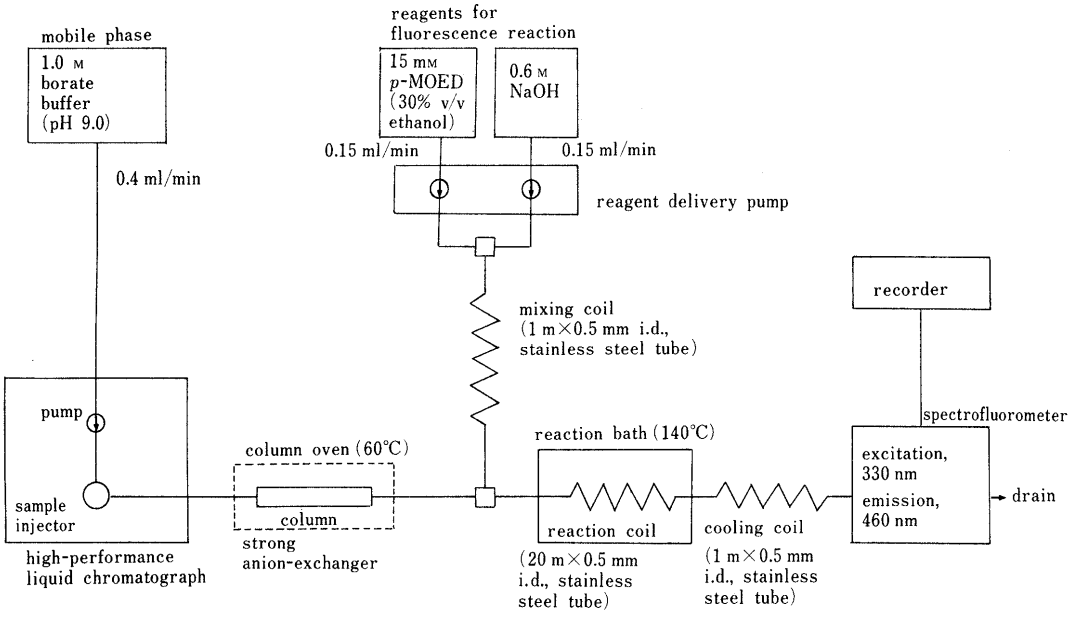

Fig. 1. Flow Diagram of the HPLC System for Deoxy Sugars

Japan) or Nacalai Tesque (Kyoto, Japan). All other chemicals were of reagent grade. Deionized and distilled water was used. *p*-MOED solution (15 mM) was prepared in aqueous 30% (v/v) ethanol.

Apparatus and HPLC Conditions A flow diagram of the HPLC system is shown in Fig. 1. Borate complexes of sugars were separated on a strong anion-exchanger (a trimethylammonium-bonded styrene-divinylbenzene copolymer resin) column (TSK gel Sugar AXG, 150 × 4.6 mm i.d.; Tosoh, Tokyo, Japan) and the column temperature was kept at 60 °C in a Tosoh HLC-805 column oven. Borate buffer (1.0 M, pH 9.0) as the mobile phase was delivered at a flow rate of 0.4 ml/min by means of a Tosoh CCPM pump equipped with a Rheodyne 7125 syringe sample-loading injector valve (100-μl loop).

The effluent from the column was mixed with a stream of a mixture of the *p*-MOED solution and 0.6 M sodium hydroxide solution, both of which were delivered at 0.15 ml/min by an SSP PM-2M 1024 pump (Sanuki Kogyo, Tokyo, Japan) having two pump heads. The mixture was then heated in a reaction coil (20 m × 0.5 mm i.d.; stainless steel tube) placed in a Shimadzu (Kyoto, Japan) CRB-6A reaction bath (140 °C). The effluent from the reaction coil was passed through an air-cooling coil (1 m × 0.5 mm i.d.; stainless steel tube) and finally the fluorescence was monitored at 460 nm with excitation at 330 nm, using a Tosoh FS-8000 spectrofluorometer equipped with a flow cell (15 μl). The spectral bandwidths for both excitation and emission were 20 nm. Peak heights were used for quantification.

Administration of 2-Deoxy-D-glucose, and Serum Sample Preparation Rats (total 54, Sprague-Dawley, female, five weeks old, weight 104—152 g, fasted for 24 h) were divided into 9 groups (6 in each group) and given 0.25 ml of saline (control group) or 0.25 ml of 2-deoxy-D-glucose (100 mg/kg rat) in saline for the other 8 groups by single injection into the tail vein. Two additional rats in each group except for the control group (total 16 rats) were given saline as a control. After the administration, the rats were allowed only water. Blood samples were drawn from the hearts of rats in each group at 0, 5, 10, 30, 60, 90, 120, 200 and 300 min after administration. Serum samples obtained in the usual manner were stored at −20 °C.

Deproteinization of Serum Samples To 100 μl of serum, 10 μl of 1.0 μmol/ml L-fucose (internal standard) and 400 μl of 0.5 M trichloroacetic acid (the preferred reagent) or 0.5 M perchloric acid or acetonitrile were successively added, and the mixture was centrifuged at 1500 *g* for 5 min at 4 °C. Then 100 μl of 1.0 M borate buffer (pH 11.0 for the acids, pH 9.0 for acetonitrile) was added to 100 μl of the supernatant. A 100-μl aliquot of the mixture was injected into the chromatograph.

Results and Discussion

Separation and Fluorescence Derivatization of the Sugars The conditions of HPLC separation and subsequent post-column fluorescence derivatization were investigated using 2-deoxy-D-glucose, D-glucose and L-fucose (internal standard) together with other deoxy sugars, 2-deoxy-D-ribose and L-rhamnose. The conditions of the separation and derivatization were essentially the same as those of the previous HPLC method for the quantification of typical reducing sugars,[10] but some modifications were made to optimize the conditions for the deoxy sugars as follows. Higher concentration and pH of borate buffer as the mobile phase allowed the sugars to be eluted more rapidly; 1.0 M borate buffer of pH 9.0 afforded the fastest and most satisfactory separation. Sodium hydroxide for the derivatization reaction yielded almost maximum peak heights at 0—0.4 M for D-glucose and L-rhamnose, and at 0.6—0.8 M for the other deoxy sugars (Fig. 2); 0.6 M was adopted.

Figure 3A shows a chromatogram obtained with a standard mixture of the sugars [retention times (min): 2-deoxy-D-ribose, 11.2; 2-deoxy-D-glucose, 12.4; L-rhamnose, 15.2; L-fucose, 22.2; D-glucose, 28.0]. The fluorescence excitation and emission spectra of the reaction mixture for all these sugars were almost identical in shape and maxima (excitation, 330 nm; emission, 460 nm) with those obtained with D-glucose in the manual spectrofluorometric method.[11]

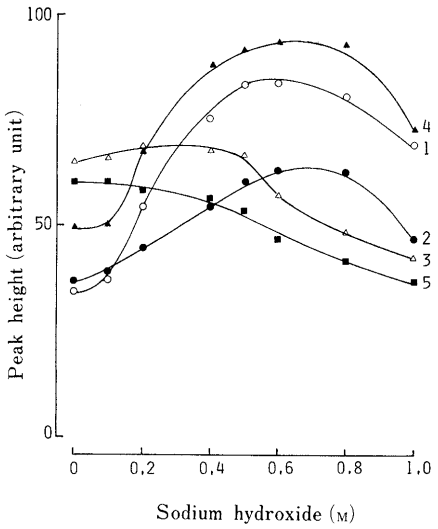

Fig. 2. Effect of Sodium Hydroxide Concentration on the Derivatization of Four Deoxy Sugars and D-Glucose

Concentrations: 2 nmol/injection volume each. Curves: 1, 2-deoxy-D-ribose; 2, 2-deoxy-D-glucose; 3, L-rhamnose; 4, L-fucose; 5, D-glucose.

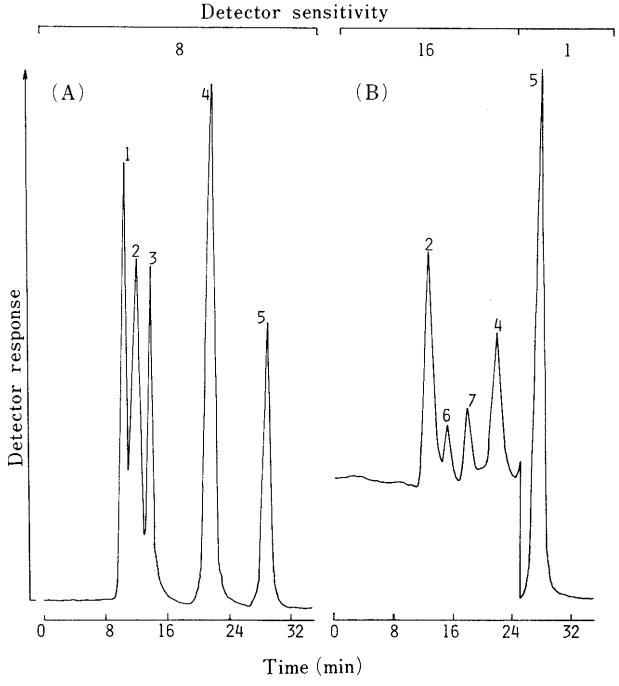

Fig. 3. Chromatograms Obtained with (A) a Standard Mixture of Four Deoxy Sugars and D-Glucose and (B) Rat Serum 60 min after Administration of 2-Deoxy-D-glucose

Peaks: 1, 2-deoxy-D-ribose; 2, 2-deoxy-D-glucose; 3, L-rhamnose; 4, L-fucose; 5, D-glucose; 6, unidentified (probably reducing sugar); 7, D-mannose. Concentrations: (A), 2 nmol/injection volume each; (B, μmol/ml serum): 2, 0.2; 4, 0.1 (internal standard); 5, 10.2.

The calibration graphs for the sugars were linear up to at least 100 nmol/injection volume of 100 μl, and passed through the origin. The detection limits (pmol/injection volume of 100 μl; $S/N=3$) were 21 (2-deoxy-D-ribose), 26 (2-deoxy-D-glucose), 27 (L-rhamnose), 18 (L-fucose) and 28 (D-glucose). The precision was established by repeated determinations ($n=10$) of a standard mixture of the sugars (10 nmol/ml each). The relative standard variations (%) were 1.7 (2-deoxy-D-ribose), 1.5 (2-deoxy-D-glucose), 2.1 (L-rhamnose), 1.5 (L-fucose) and 1.9 (D-glucose).

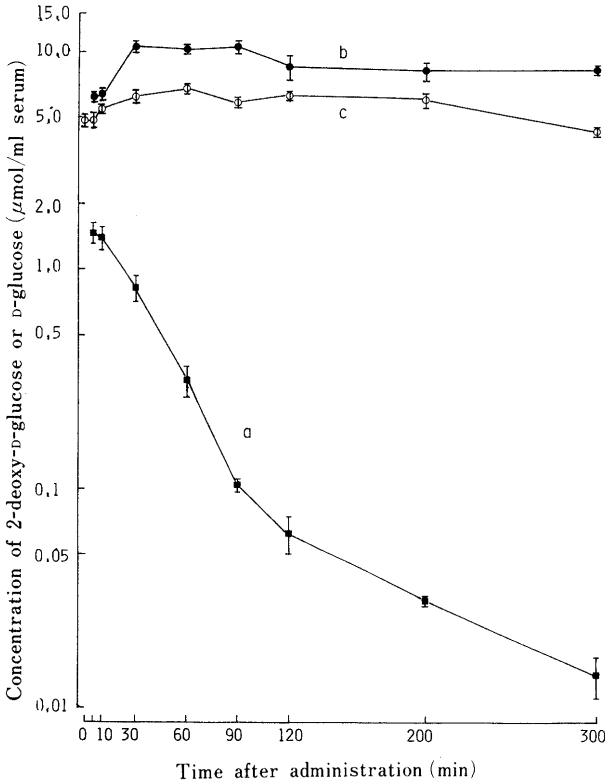

Fig. 4. 2-Deoxy-D-glucose and D-Glucose Concentrations in Rat Serum after 2-Deoxy-D-glucose Injection into the Tail Vein

Curves: a, 2-deoxy-D-glucose; b, D-glucose after 2-deoxy-D-glucose administration; c, D-glucose in control rat. Each point and vertical line represent the mean and standard deviation, respectively.

Determination of 2-Deoxy-D-glucose and D-Glucose in Rat Serum L-Fucose does not occur in rat serum. Therefore it was used as an internal standard. Three deproteinization procedures with trichloroacetic acid, perchloric acid and acetonitrile were compared. The recommended procedure with trichloroacetic acid provided complete and consistent recoveries (99%) of 2-deoxy-D-glucose, D-glucose and L-fucose added to serum samples. The other procedures gave somewhat lower and less consistent recoveries (perchloric acid, 97—101%; acetonitrile, 74—81%).

Figure 3B depicts a typical chromatogram obtained with rat serum 60 min after single intravenous administration of 2-deoxy-D-glucose (100 mg/kg). The peak components were identified on the basis of the retention times in comparison with standard solutions and also by co-chromatography of the standards and the samples. The peak for 2-deoxy-D-glucose (peak 2) partially overlapped with an unidentified peak (peak 6), but 2-deoxy-D-glucose could be determined precisely by the baseline method; the recoveries of 5 nmol of 2-deoxy-D-glucose added to 100 μl of control serum were $99 \pm 2\%$ [mean $\pm$ relative standard deviations (R.S.D.), $n=10$] though the R.S.D. for the height of peak 6 in the control serum was 25%. Peak 6 appeared to be due to a reducing sugar for the following reasons. The fluorescence excitation and emission maxima of the eluate were at 330 and 460 nm, respectively, which are characteristic of reducing sugars,[11] and the peak disappeared when *p*-MOED was omitted in the HPLC system. The limits of detection ($S/N=3$) for 2-deoxy-D-glucose and D-glucose in rat serum were 0.52 and 0.56 nmol/ml (corresponding to 26 and 28 pmol/injection volume of 100 μl), respectively. The R.S.D. in multiple determinations ($n=10$) of 2-deoxy-D-glucose and D-glucose in serum were 1.5 and 1.6% at mean concentrations of 0.3 and 9.9 μmol/ml, respectively.

The concentration–time plots for 2-deoxy-D-glucose and D-glucose in sera of rats dosed intravenously with 2-deoxy-D-glucose are shown in Fig. 4. The serum concentrations in rats after injection of 2-deoxy-D-glucose (100 mg/kg) showed a biphasic decrease; the first half-life was 0.7 h and the second was 2.9 h. Blood D-glucose level increased rapidly to approximately 1.6 times the control values and remained elevated for at least 5 h. The pattern of the curves was almost identical with that reported by other workers.[13-15]

This study provides the first HPLC method for the simultaneous determination of 2-deoxy-D-glucose and D-glucose. The method is simple and sensitive enough to measure deoxy sugars in only small amounts of biological samples, and it may be useful in biological and biomedical studies of 2-deoxy-D-glucose and other deoxy sugars.

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