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Simultaneous determination of glucose and 1-deoxyglucose in serum by anion-exchange chromatography with an immobilized pyranose oxidase reactor

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ABSTRACT

A liquid chromatographic system for the determination of glucose and 1-deoxyglucose in serum using an immobilized pyranose oxidase reactor and chemiluminescence detection is described. Separation was achieved on a TSK gel SAX column (2 cm × 4 mm I.D.) with an eluent of 0.1 *M* sodium hydroxide solution. Serum was diluted 30-fold with 0.1 *M* NaOH. Sample solution (50 μl) was injected into the system. The hydrogen peroxide produced was detected by measuring the chemiluminescence emitted on admixing with luminol and potassium hexacyanoferrate (III). The calibration graphs were linear from 1 to 600 μ*M* glucose and from 0.6 to 400 μ*M* 1-deoxyglucose; the detection limits for glucose and 1-deoxyglucose were 0.5 and 0.3 μ*M*, respectively. The sample throughput was 10/h. The immobilized enzyme reactor was stable for at least 2 months.

INTRODUCTION

It is important to diagnose diabetes mellitus at an early stage. The oral glucose tolerance test (OGTT) has been accepted as the standard diagnostic test [1]. However, the OGTT is not an appropriate test for mass screening. Fasting blood glucose (FBG) has been assessed for usefulness as a screening test for diabetes mellitus as an alternative to the OGTT [2]. Rapid methods for determining glucose in a number of clinical samples have been reported [3–7].

Recently, it has been shown that the serum 1-deoxyglucose level is substantially reduced in patients with diabetes mellitus [8] and provides analytical information on glycaemic control in patients [9,10]. A flow-injection method for the rapid assay of serum 1-deoxyglucose has been proposed [11].

FBG is difficult to standardize and is influenced by unexpected dietary deviation. On the other hand, since a decrease in serum 1-deoxyglucose level is also observed in other diseases [12,13], it is dangerous to use a single random estimation of the 1-deoxyglucose level as an alternative to FBG. Simultaneous determination of glucose and 1-deoxyglucose in serum provides a reliable test for diagnosis of diabetes mellitus.

In this work, immobilized pyranose oxidase was used as a column reactor (4 cm × 4 mm I.D.) in a chromatographic system for the specific detection of glucose and 1-deoxyglucose. Glucose and 1-deoxyglucose were separated on an anion-exchange resin column (2 cm × 4 mm I.D.) with 0.1 *M* sodium hydroxide solution as eluent. The

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hydrogen peroxide produced in the reactor was detected chemiluminometrically by a luminol–ferricyanide reaction. Sample throughput was 10/h.

EXPERIMENTAL

Materials

Pyranose oxidase (EC 1.1.3.10, from *Polyporus obtusus*, 10 U/mg) was obtained from Takara Shuzo (Kyoto, Japan). Aminopropyl-CPG (mean pore diameter 59 nm, amount of amine 76 μmol/g, particle size 200–400 mesh) and anion-exchange resin [TSK gel SAX (5 μm), Cl type] were purchased from CPG (Fairfield, NJ, USA) and Tosoh (Tokyo, Japan), respectively. D-Glucose and 1-deoxyglucose were obtained from Sigma (St. Louis, MO, USA). All other reagents were commercially available and were of analytical reagent grade.

Stock solutions of D-glucose (10 m*M*) and 1-deoxyglucose (1 m*M*) were prepared in water and stored in a refrigerator. McIlvaine buffer (pH 3.5) was prepared from 0.1 *M* sodium monohydrogen phosphate and 0.05 *M* citric acid. Luminol solution [1 m*M* luminol in 0.4 *M* carbonate buffer (pH 10.5)] was prepared daily. A potassium hexacyanoferrate(III) solution (20 m*M*) was prepared every week. Eluent (0.1 *M* NaOH) was prepared from carbonate-free 50% NaOH solution and protected from carbon dioxide contamination with a soda lime trap.

The anion-exchange resin was packed into a stainless-steel column (2 cm × 4 mm I.D.). The packed column was washed with 0.5 *M* NaOH at a flow-rate of 0.1 ml/min for 1 h and then with 0.1 *M* NaOH at a flow-rate of 0.4 ml/min for 20 min.

The enzyme was immobilized onto the aminopropyl-CPG, which was activated by glutaraldehyde. The details for the preparation of the immobilized enzyme have been reported previously [7,11]. The enzyme was packed into a stainless-steel column (4 cm × 4 mm I.D.).

Flow system and procedure

A schematic diagram of the flow system is shown in Fig. 1. The system consisted of two LC pumps (Hitachi L-6000) and a reagent-delivery pump (Kyowa Seimitsu KHU-W-52), an injector (Sanuki SV1-6M2) equipped with a 50-μl loop and a luminometer (Niti-On LF-800). The samples were injected into the separation column at a flow-rate of 0.4 ml/min in 0.1 *M* NaOH. The eluate from the column was neutralized to pH 6.2 by mixing with McIlvaine buffer (pH 3.5) prior to elution through the enzyme reactor. The total flow-rate through the enzyme reactor was 1.0 ml/min. The eluate from the enzyme reactor was combined with the luminol solution and the potassium hexacyanoferrate solution at a four-way valve and then mixed with a mixing tube placed before the detector flow cell. The total flow-rate through the flow cell was 2.0 ml/min. The peak heights were measured by a data processor (Chromatocorder II). The immobilized enzyme reactor was thermostated at 45°C.

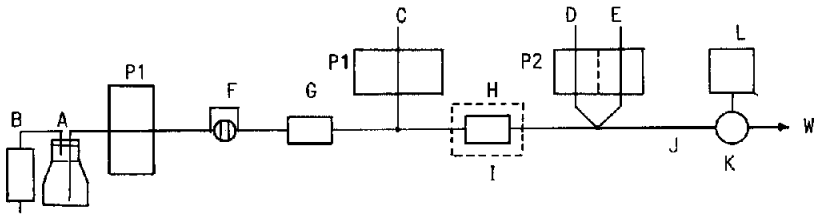

Fig. 1. Flow diagram of the liquid chromatographic system for chemiluminometric detection of glucose and 1-deoxyglucose with an immobilized enzyme column reactor. A = Eluent (0.1 *M* NaOH, 0.4 ml/min); B = soda lime tube (10 cm × 10 mm I.D.); C = MacIlvaine buffer solution (pH 3.5, 0.6 ml/min); D = luminol solution (0.5 ml/min); E = potassium ferricyanide solution (20 m*M*, 0.5 ml/min); F = injector; G = separation column (TSK gel SAX); H = immobilized pyranose oxidase reactor (4 cm × 4 mm I.D.); I = water bath (45°C); J = mixing tube (50 cm × 0.5 mm I.D.); K = chemiluminometer; L = data processor; P1 = LC pump; P2 = reagent pump; W = waste.

Separation of glucose and 1-deoxyglucose was investigated with refractive index (RI) detection using a differential refractometer (Waters R401).

Serum samples (3 μl) were diluted 30-fold with 0.1 *M* NaOH and then filtered through a membrane filter (Millipore PTGC, cut-off at relative molecular mass 10 000). An aliquot (50 μl) of the filtrate was injected into the system.

RESULTS AND DISCUSSION

Separation of glucose and 1-deoxyglucose

Separation of glucose and 1-deoxyglucose was studied using anion-exchange chromatography, with borate buffer or sodium hydroxide solution as eluent. Since borate ion interfered with the enzymatic reaction owing to the formation of complexes with the compounds, sodium hydroxide solution was selected as eluent. The separation was effected by a TSK gel SAX (5 μm) column (2 cm × 4 mm I.D.) with 0.1 *M* NaOH as eluent at a flow-rate of 0.4 ml/min at ambient temperature. Fig. 2 shows an example of a chromatogram demonstrating the separation of glucose and 1-deoxyglucose. Under these conditions the retention times (min) for glucose, 1-deoxyglucose and related compounds were as follows: glucose, 4.6 min; 1-deoxyglucose, 1.4; D-galactose, 4.6; L-sorbose, 4.8; D-xylose, 5.0; and maltose, 20.6. At NaOH concentrations of less than 0.05 *M*, retention of glucose decreased gradually because the affinity of chloride ion for the resin is greater than that of OH^- and chloride ion in serum samples accumulates on the resin. Column efficiency increased as the NaOH concentration was increased. Since it was difficult to neutralize the more concentrated NaOH solutions with one step in the flow system, 0.1 *M* NaOH was selected as eluent. Retention decreased as the eluent was contaminated by carbon dioxide. Proper precautionary measures included the use of a soda lime tube on the eluent bottle and high-purity deionized water when making up eluent. When the column temperature exceeded 40 °C, tailing and a secondary peak for glucose were observed.

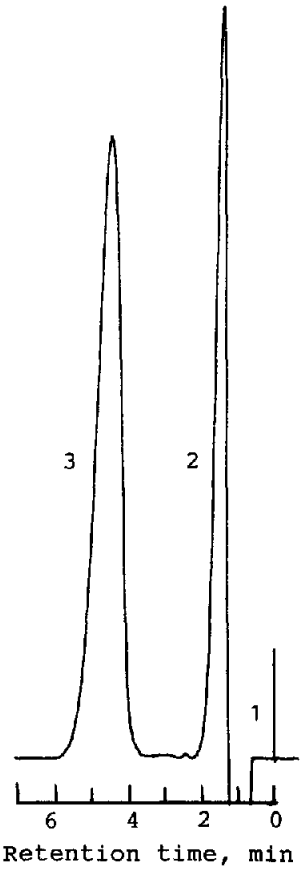

Fig. 2. Chromatogram of a standard mixture of glucose and 1-deoxyglucose (each 2 g/l). 1 = Water; 2 = 1-deoxyglucose; 3 = glucose. Detection: refractive index (att. ×32). A TSK gel SAX column (2 cm × 4 mm I.D.) was used at room temperature with 0.1 *M* NaOH as the eluent at a flow-rate of 0.4 ml/min.

Reactor performance

To test the activity of the enzyme reactor, the system shown in Fig. 1 was used in a flow-injection mode by omitting the separation column. Measurements were established optimum pH with 0.05 *M* phosphate buffer between pH 5.0 and 7.5. Fig. 3 shows the effects of pH on the peak height. Maximum responses for glucose and 1-deoxyglucose were obtained at pH 6.0 and 6.5, respectively. pH 6.2 was selected for further work.

The effect of temperature on the activity was examined over the range 30–55°C. The reactor exhibited the highest activity at 55°C, as shown in

Fig. 3. To prolong the lifetime of the enzyme, 45°C was selected. At this temperature, the conversion efficiency to H_2O_2 was 69%.

The reactor was used for 10 h (about 100 injections) per day and stored at 4°C in 0.1 *M* phosphate buffer (pH 7.0) when not in use. The activity remained at 90% of the initial value for 2 months.

Using 0.1 *M* phosphate buffer (pH 6.2) at a flow-rate of 1.0 ml/min at 45°C, the relative activities of the reactor for glucose, 1-dehydroglucose, D-galactose, L-sorbose, D-xylose and maltose were 100, 94, 94, 89, 121 and 124, respectively.

Linearity of the standard curve

Under the conditions shown in Fig. 1, the plot of peak height against the concentration was linear from $1 \cdot 10^{-6}$ to $6 \cdot 10^{-4}$ *M* for glucose and from $6 \cdot 10^{-7}$ to $4 \cdot 10^{-4}$ *M* for 1-deoxyglucose. The least-squares calibration equation for glucose was $y = 0.525x + 3.789$, where y is the common logarithm of peak height (cm) and x is the common logarithm of glucose concentration (*M*), with a linear correlation coefficient of $r = 0.998$ (eleven data points). The calibration equation for 1-deoxyglucose was $y = 0.613x + 4.344$, with $r = 0.991$ (eleven points). The relative standard deviation (R.S.D.) for ten injections of standard sample was 0.7% at 150 μM glucose and 1.1% at 5 μM 1-deoxyglucose. The detection limits (signal-to-noise ratio = 3) for a standard mixture were 0.5 μM for glucose (4 ng) and 0.3 μM for 1-deoxyglucose (2 ng in a 50-μl injection). The detection limits for serum samples were 3 mg/l for glucose and 2 mg/l for 1-deoxyglucose.

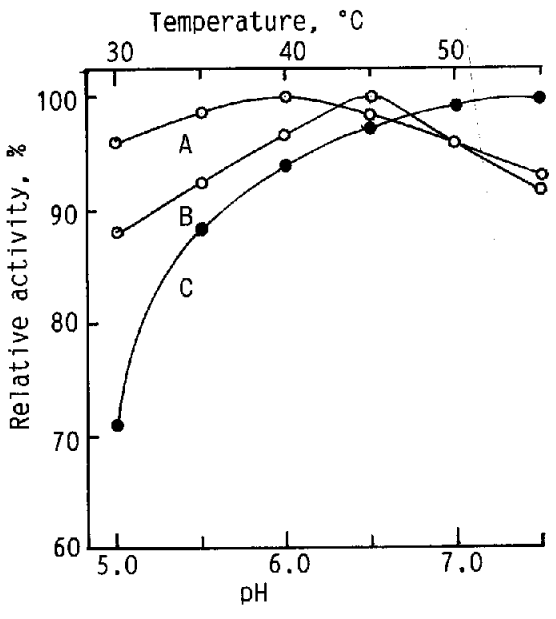

Fig. 3. Effects of pH (○) and temperature (●) on the activity of the immobilized enzyme. A = Glucose; B = 1-deoxyglucose; C = 1-deoxyglucose.

Precision and reproducibility

Pooled human serum was repeatedly analysed over 15 days. Fig. 4 shows a chromatogram obtained from the serum sample. The system was

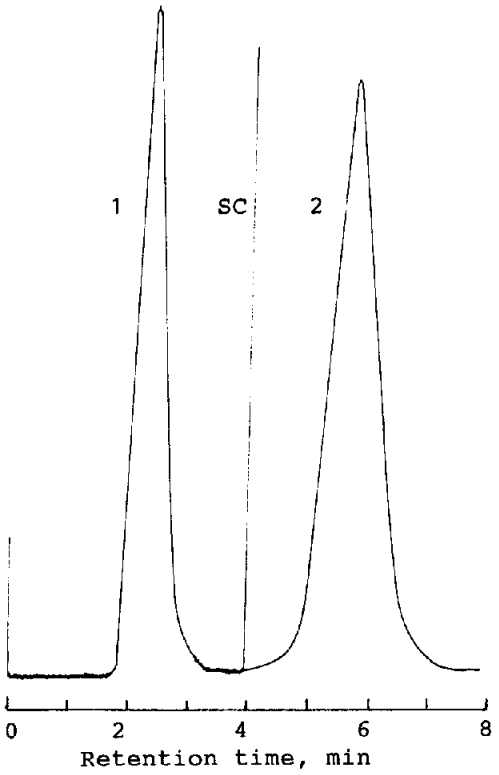

Fig. 4. Chromatogram of serum sample from normal subject. 1 = 1-Deoxyglucose; SC = sensitivity change noise (the sensitivity for glucose peak was reduced to 1/40 that for 1-deoxyglucose), 2 = glucose. Serum (3 μl) was diluted 30-fold with 0.1 *M* NaOH and filtered with a membrane filter. The filtrate (50 μl) was injected into the system. The conditions are the same as in Fig. 1.

used for the assay of 100 samples in a day and the reactor was stored in a refrigerator when not in use. In order to correct the variation of the conversion efficiency, standards were measured at 100-sample intervals. The anion-exchange resin column was renewed every 400 determinations. This system gave satisfactorily precise and reproducible results; for serum containing 5.47 m*M* (985 mg/l) glucose and 132 μM (21.6 mg/l) 1-deoxyglucose, the within-day R.S.D. for glucose and 1-deoxyglucose was 1.3% and 1.8%, respectively, and day-to-day R.S.D. for glucose and 1-deoxyglucose was 1.7% and 2.4%, respectively.

Comparison

Results for serum glucose [$n = 25$, range 3.92–10.1 m*M* (705–1823 mg/l)] and 1-deoxyglucose [$n = 25$, range 37.8–205 μM (6.2–33.7 mg/l)] obtained using this system compared well with results obtained using a Hitachi 736 automatic analyser with the hexokinase method for glucose and using a batchwise method [9] with soluble pyranose oxidase–peroxidase for 1-deoxyglucose. The calculated linear regressions and correlation coefficients for glucose and 1-deoxyglucose were $y = 0.992x + 0.006$ and $r = 0.993$, and $y = 1.03x + 0.03$ and $r = 0.989$, respectively, indicating good agreement between the results.

CONCLUSION

Serum glucose and 1-deoxyglucose, as well as glycosylated haemoglobin and fructosamine, are clinical markers of glycaemic control in diabetes mellitus, but they differ from one another in clinical significance. Simultaneous and rapid measurements of the marker level provide a reliable screening test for diabetes. We attempted to determine simultaneously glucose and 1-deoxyglucose in serum in the chromatographic system. We succeeded in determining glucose and 1-deoxyglucose in serum at a rate of 10/h. The present system gave precise and reproducible results that show an excellent correlation with results obtained by enzymatic methods.

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