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Short communication

Post-column enzyme reactors for chemiluminometric detection of glucose, 1,5-anhydroglucitol and 3-hydroxybutyrate in an anion-exchange chromatographic system

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Abstract

A liquid chromatographic system consisting of a co-immobilized 3-hydroxybutyrate dehydrogenase–NADH oxidase reactor and an immobilized pyranose oxidase reactor in series and a chemiluminometer was developed for the simultaneous determination of glucose, 1,5-anhydroglucitol and 3-hydroxybutyrate in plasma. The enzymes were immobilized on toresylated poly(vinyl alcohol) beads. Separation was achieved on a TSK gel SAX column (40×4 mm I.D.) with an eluent of 50 m*M* NaOH containing 30 m*M* sodium butyrate. The hydrogen peroxide produced was detected by measuring the chemiluminescence emitted on admixing with luminol and potassium hexacyanoferrate(III). The calibration curves were linear from 0.8 to 500 μ*M* (7 ng–4 μg) for glucose, from 0.8 to 400 μ*M* (7 ng–3 μg) for 1,5-anhydroglucitol and from 1 to 700 μ*M* (5 ng–4 μg in a 50-μl injection) for 3-hydroxybutyrate. The sample throughput was four per hour. The reactors were stable for at least ten days.

Keywords: Glucose; 1,5-Anhydroglucitol; 3-Hydroxybutyrate

1. Introduction

The measurement of glucose and 3-hydroxybutyrate (HB) is of clinical importance in diagnosis and management of metabolic derangement of carbohydrate metabolism; both the plasma glucose and HB concentrations increase in diabetes mellitus (DM). Though fasting blood glucose has been assessed for usefulness as a test for DM, it is influenced by unexpected dietary variation. Simultaneous determination of these substances in plasma provides a reliable test for diagnosis of DM. A flow-injection (FI) system with a co-immobilized glucose dehydrogenase (GDH)/NADH oxidase (NAOD) reactor and a 3-hydroxybutyrate dehydrogenase (HBDH)/NAOD reactor was used for rapid determination of glucose and HB in plasma [1]. In the method, the immobilization process was time-consuming because glutaraldehyde-activated beads were used for the coupling of the enzymes. The method is not very sensitive because the coupling yields were not high; the lower detection limits for glucose and HB were 2 and 0.8 μ*M*, respectively. This method permits sensitive detection without the necessity for tedious sample treatment.

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DM has been found and has recently been proposed as a novel marker of glycaemic control in DM [2–5]. An anion-exchange chromatographic system with an immobilized pyranose oxidase (EC 1.1.3.10, PyOD) has been developed for the selective determination of AG in serum [6,7]. This method did not give good precision because it was difficult to reproducibly neutralize the eluent (0.2 *M* NaOH) with one step in the flow system; the precision was about 7%.

In the present study, we have tried to determine glucose, AG and HB simultaneously, by combining the anion-exchange chromatographic system with a co-immobilized HBDH/NAOD reactor and an immobilized PyOD. The separation was effected with 50 m*M* NaOH containing 30 m*M* sodium butyrate as eluent. Tresylated poly(vinyl alcohol) beads were used for the rapid preparation of the reactors with high activities. In the HBDH/NAOD reactor, HBDH catalyzes the oxidation of HB in the presence of β-nicotinamide adenine dinucleotide (NAD^+) and the resulting reduced form of NAD^+ (NADH) produced is removed by the NAOD with concomitant formation of hydrogen peroxide. In the PyOD reactor, hydrogen peroxide is produced by the oxidation of glucose and AG in the presence of molecular oxygen. The hydrogen peroxide formed is detected by measuring the chemiluminescence emitted when mixed with luminol and potassium hexacyanoferrate(III). The method was applied to the determination of glucose, HB and AG in plasma.

2. Experimental

2.1. Reagents

PyOD (from *Polyporus obtusus*, 10 U mg^{-1}) was purchased from Takara Shuzo (Kyoto, Japan). HBDH (EC 1.1.1.30, from *Pseudomonas* sp., grade III, 120 U mg^{-1}) and NAOD (EC number not assigned, from *Bacillus megaterium*, 50 U mg^{-1}) were obtained from Toyobo (Osaka, Japan) and Asahi Kasei (Tokyo, Japan), respectively. NAD^+ (free acid, 96%) was purchased from Kohjin (Tokyo). Poly(vinyl alcohol) beads (GS 520, particle size 13 μm) and anion-exchange resin [T SKgel SAX (5 μm), Cl type] were from Showa Denko (Tokyo) and Tososh (Tokyo), respectively. All other reagents were of analytical-reagent grade.

Luminol solution [2 m*M* luminol in carbonate buffer (pH 10.0) consisting of 0.4 *M* sodium carbonate–0.4 *M* sodium hydrogen carbonate] was prepared and stored for one day in a refrigerator before use [8,9]. A potassium hexacyanoferrate(III) stock solution (0.1 *M*) was prepared and diluted ten-fold with water before use. NAD^+ solution [3.5 m*M* in 0.1 *M* acetate buffer (pH 5.0)] was prepared daily. Stock solutions of D-glucose (10 m*M*) and AG (1 m*M*) were prepared in water and stored in a refrigerator. Stock solution of HB (10 m*M*) was made by dissolving D,L-3-hydroxybutyrate sodium salt (D,L-HB salt) in water. D-Isomer concentration was assumed to be half of the D,L-HB salt concentration. The eluent was 50 m*M* NaOH containing 30 m*M* sodium butyrate; NaOH solution (100 m*M*) was prepared by dilution of carbonate-free 50% NaOH solution with water and diluted with the same volume of sodium butyrate solution (60 m*M*). The eluent was protected from carbon dioxide contamination with a soda lime trap. The anion-exchange resin was packed into a stainless-steel column (40×4 mm I.D.). The column was washed with 0.5 *M* NaOH at a flow-rate of 0.1 ml min^{-1} for 1 h, and afterwards washed with the eluent at a flow-rate of 0.4 ml min^{-1} for 20 min.

2.2. Preparation of enzyme reactors

A co-immobilized HBDH/NAOD reactor and an immobilized PyOD reactor were prepared.

The method for the preparation of the tresylated poly(vinyl alcohol) beads was similar to that described previously [10]. The beads were packed into two stainless-steel columns (40×4 mm I.D. each). Enzyme solution [5 mg of HBDH (600 U) and 5 mg of NAOD (250 U) or 10 mg of PyOD (100 U) in 10 ml of 0.05 *M* phosphate buffer (pH 7.0)] was circulated through the column at 0.2 ml min^{-1} for 4 h at room temperature. NAOD and PyOD were immobilized with 89 and 75% yields, with precisions of 2 and 1% ($n=5$), respectively. The time taken to prepare each reactor was about 4.5 h.

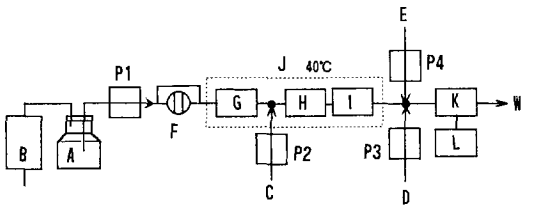

Fig. 1. Schematic diagram of the liquid chromatographic system for chemiluminometric detection of glucose, 1,5-anhydroglucitol and 3-hydroxybutyrate with immobilized pyranose oxidase reactor and co-immobilized 3-hydroxybutyrate dehydrogenase/NADH oxidase reactor. A=eluent (0.70 ml min^{-1}), B=soda lime tube, C=NAD^+ solution (0.28 ml min^{-1}), D=luminol solution (0.5 ml min^{-1}), E=potassium hexacyanoferrate(III) solution (0.5 ml min^{-1}), F=injector with a 50-μl loop, G=analytical column, H=immobilized pyranose oxidase reactor, I=co-immobilized 3-hydroxybutyrate dehydrogenase/NADH oxidase reactor, J=water bath, K=luminometer with a flow-through cell, L=data processor, P1, P2, P3 and P4=pumps, W=waste.

2.3. System

The liquid chromatographic system is shown in Fig. 1. It consisted of four piston pumps (Hitachi L-6000), an injector (Sanuki SVI-6M2) with a 50-μl loop, a separation column, two reactors and a luminometer (Soma S-3400) with a flow-through cell (100 μl) connected to a data processor (Chromatocorder II). The reactors were maintained at 40°C.

The separation of glucose, AG and HD was optimised using a differential refractometer (Shodex SE-51) as detector. Venous blood was treated to obtain plasma in the same manner as that described previously [10]. Plasma (10 μl) was diluted 20-fold with 50 m*M* NaOH and filtered through an ultrafiltration membrane (Advantec Q0100, nominal molecular mass cut-off 10 000). The filtrate (50 μl) was injected via an injector.

The results obtained by the present method were compared with those obtained by a Hitachi 736 automatic analyzer with soluble hexokinase-glucose-6-phosphate dehydrogenase as detector for glucose, by a batchwise method [11] with soluble PyOD peroxidase for AG, and by a manual method [12] with soluble HBDH for HB.

Prior to the first run in each day, the separation column was washed with 0.5 *M* NaOH at a flow-rate of 0.5 ml min^{-1} for 20 min and then with the eluent at a flow-rate of 0.7 ml min^{-1} for 10 min.

3. Results and discussion

3.1. Separation

Separation of glucose, AG and HB was studied using anion-exchange chromatography, with 50 m*M* NaOH containing 1.0–150 m*M* sodium butyrate as a modifier to elute glucose and HB. In the system, a differential refractometer was used as detector because of the convenience. Though column efficiency increased as the NaOH concentration was increased, it was difficult to neutralize the more concentrated NaOH solutions with one step in the flow system. The NaOH solution of 50 m*M* was selected. Sodium acetate and sodium butyrate were examined as modifier. The elution strength of sodium acetate was weaker than that of sodium butyrate. The effect of sodium butyrate concentration on the capacity factors for glucose, AG and HB is shown in Fig. 2. Changes in sodium butyrate concentration did not affect the mutual elution order of these substances. The capacity factors of glucose and HB decreased with an increase in the sodium butyrate concentration, while AG was not retained above 30 m*M* sodium butyrate. The most optimal separation was effected by a TSK gel SAX (5 μm) column (40×4 mm I.D.) with 50 m*M* NaOH containing 30 m*M* sodium butyrate at a flow-rate of 0.7 ml min^{-1} at ambient temperature. Fig. 3 shows an example of a chromatogram demonstrating the separation of glucose, AG and HB.

3.2. Reactor performance

To test the activity of the reactors, the system shown in Fig. 1 was used in a flow-injection mode by omitting the separation column. Measurements were made to study the effect of pH using borate buffer consisting of 0.1 *M* boric acid–0.1 *M* KCl–0.1 *M* sodium carbonate between pH 7.5 and 9.5. Maximum responses for glucose, AG and HB were obtained at pH 9.0, 9.0 and 9.2, respectively. pH 9.2 was selected for further work.

The effect of temperature on the activity was

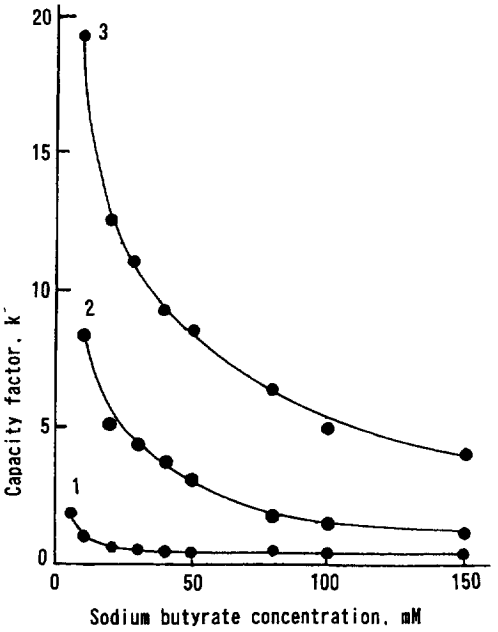

Fig. 2. Relationship between sodium butyrate concentration in the eluent and the capacity factors of glucose, 1,5-anhydroglucitol and 3-hydroxybutyrate. Chromatographic conditions are given in Section 2.

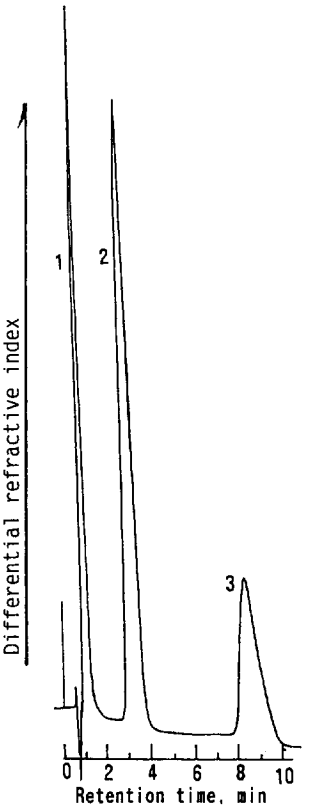

Fig. 3. Chromatogram of a standard mixture of glucose, 1,5-anhydroglucitol and 3-hydroxybutyrate (each 2g/l). 1=1,5-anhydroglucitol, 2=glucose, 3=3-hydroxybutyrate. Detection: refractive index (att.×32). Chromatographic conditions are given in Section 2.

examined over the range 30–50°C. The reactors exhibited the highest activity at 50°C. To prolong the lifetime of the reactors, 40°C was selected.

The effect of NAD^+ concentration on the peak height for HB, glucose and AG was examined over the range 0.2–4 m*M*. The optimum NAD^+ concentration for HB was 2 m*M*; at this concentration, the NAD^+ concentration in the reactor was 1 m*M*. The peak heights for glucose and AG were not affected when the concentration of NAD^+ varied from 0.2 to 4 m*M*.

The reactors were used for 10 h (about 80 injections) per day and stored at 4°C in 0.1 *M* phosphate buffer (pH 7.0) when not in use. The activities of the HBDH/NAOD reactor and PyOD reactor decreased to 84 and 88% of the initial values, respectively, after 3 weeks.

Under the conditions of pH 9.2 and 2 m*M* NAD^+ at 40°C at a flow-rate of 1.0 ml min^{-1}, standard solution (10 μ*M*) was injected into the system and the peak heights were compared with the peak height for H_2O_2. The conversion efficiencies for glucose, AG and HB to H_2O_2 were 82, 69 and 62%, respectively.

3.3. Calibration

Under the conditions in Fig. 1, the plot of peak area against the concentration was linear from 0.8 to 500 μ*M* (7 ng–4 μg) for glucose, from 0.8 to 400 μ*M* (7 ng–3 μg) for AG and from 1 to 700 μ*M* (5 ng–4 μg in a 50-μl injection) for HB. The relative standard deviation (R.S.D.) for five injections of standard sample was 0.8% at 200 μ*M* glucose, 1.8% at 5 μ*M* AG and 2.5% at 5 μ*M* HB. The detection limits (signal-to-noise ratio=3) were 0.5 μ*M* for glucose (5 ng), 0.4 μ*M* for AG (4 ng) and 0.8 μ*M* HB (5 ng in a 50-μl injection). The detection limits

for plasma samples were 2 mg l^{-1} for glucose, 2 mg l^{-1} for AG and 2 mg l^{-1} for HB.

The contribution of the post-column system to band broadening was about 80% of the peak width.

3.4. Application

This system was used to determine the amount of glucose, AG and HB in plasma. A typical chromatogram is shown in Fig. 4 for plasma sample, in which the term SC describes sensitivity change noise.

3.4.1. Precision

The plasma sample was repeatedly analyzed over 30 days. The system was used for analyses of 30 samples per day and standards were measured at 15 sample intervals, in order to check the variation of the conversion efficiencies. The retention of glucose and HB decreased gradually because the affinity of chloride ion for the resin is greater than those of OH^- and butyrate ion, and chloride ion in samples accumulates on the resin. The analytical column and reactors were renewed every 10 days. The system gave satisfactorily precise and reproducible results; for a plasma sample containing 5.99 m*M* glucose, 135 μ*M* AG and 65.7 μ*M* HB, the within-day R.S.D. values for glucose, AG and HB were 1.1, 2.5 and 3.3%, respectively, and day-to-day R.S.D. for glucose, AG and HB were 1.5, 2.9 and 3.8%, respectively.

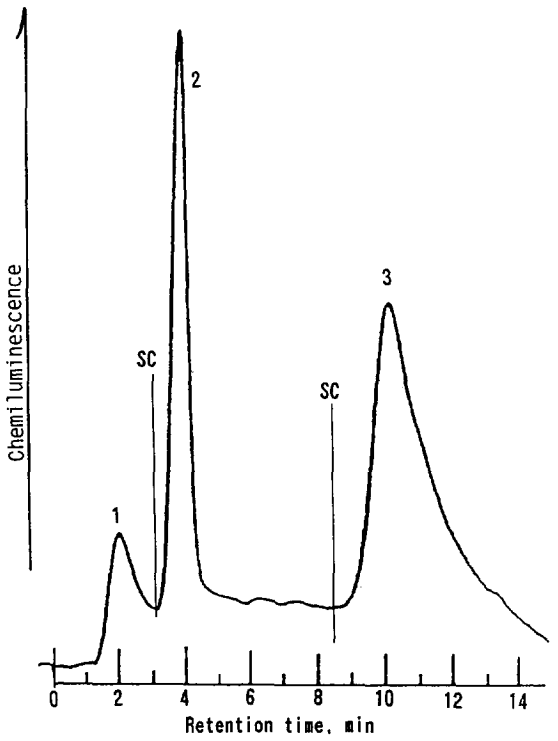

Fig. 4. Chromatogram of plasma sample from normal subject. 1=AG, 2=glucose, 3=HB, SC=sensitivity change noise (the sensitivity for glucose peaks was reduced to one tenth of that for AG and HB). Detection: chemiluminescence. The conditions are the same as in Fig. 1.

3.4.2. Comparison

Results for plasma glucose [n=20, range 4.10–9.78 m*M* (738–1760 mg l^{-1})], AG [n=20, range 40.5–1.88 μ*M* (6.64–30.8 mg l^{-1})] and HB [n=20, range 37.2–1840 μ*M* (3.87–295 mg l^{-1})] obtained using this system compared well with the results obtained using a Hitachi 736 automatic analyzer with the hexokinase method for glucose, using a method [11] with soluble PyOD-peroxidase for AG and a method [12] with soluble HBDH for HB. The calculated linear regression equations and correlation coefficients for glucose, AG and HB were y=1.017x+0.201 and r=0.999, y=1.010x+0.201 and r=0.993 and y=0.989x−0.008 and r=0.995, respectively.

4. Conclusion

Plasma glucose, AG and HB, as well as glycosylated haemoglobin and fructosamine, are clinical markers of glycaemic control in DM, but they differ from one another in clinical significance. Simultaneous and simple measurements of the marker levels provide a reliable test for diabetes. We attempted to determine simultaneously glucose, AG and HB in plasma in the chromatographic system without tedious pretreatment, except for ultrafiltration. It was proved that the immobilized PyOD reactor and co-immobilized HBDH/NAOD reactor are useful for the sensitive detection of glucose, AG and HB using anion-exchange chromatography and chemiluminometric detection. Compared with the FI method utilizing a co-immobilized GDH/NAOD reactor

and HBDH/NAOD reactor [1] and anion-exchange chromatographic methods with an immobilized PyOD [6,7], the sensitivity and precision are about two times higher. The reactors in the post-column system were stable enough to permit the measurement of more than 300 samples for 10 days. The present system gave precise and reproducible results that show a good correlation with results obtained by enzymatic methods.

References

[1] N. Kiba, H. Koemado and M. Furusawa, Anal. Sci., 11 (1995) 605.
[2] H. Akamuna, K. Ogawa, Y. Lee and Y. Akanuma, J. Biochem., 90 (1981) 157.
[3] T. Yamanouchi, H. Akanuma, F. Takaku and Y. Akanuma, Diabetes, 35 (1986) 204.
[4] T. Yamanouchi, S. Minoda, M. Yabuuchi, Y. Akanuma, H. Akanuma, H. Miyashita and I. Akaoka, Diabetes, 38 (1989) 723.
[5] A.L. Frattali and B.A. Wolf, Clin. Chem., 40 (1994) 1991.
[6] N. Kiba, Y. Goto and M. Furusawa, J. Chromatogr., 620 (1993) 9.
[7] S. Tajima, M. Hashiba, T. Suzuki, H. Akanuma and M. Yabuuchi, Biomed. Chromatogr., 7 (1993) 41.
[8] M. Tabata, C. Fukunaga, M. Ohyabu and T. Murachi, J. Appl. Biochem., 6 (1984) 251.
[9] C.A. Koerner and T.A. Nieman, Anal. Chem., 58 (1986) 116.
[10] N. Kiba, Y. Oyama, A. Kato and M. Furusawa, J. Chromatogr. A, 724 (1996) 354.
[11] M. Yabuuchi, M. Masuda, K. Katoh, T. Nakamura and H. Akamuma, Clin. Chem., 35 (1989) 2039.
[12] P.A. Ruell and G.C. Gass, Ann. Clin. Biochem., 28 (1991) 183.