Determination of Glucose in Plasma Using Immobilized Enzymes in a Flow System

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Glucose was determined using an apparatus containing an enzyme reactor in a flow line. The enzymes used for glucose assay were hexokinase and glucose-6-phosphate dehydrogenase. NADH formed by enzymatic reactions was fluorometrically detected. The calibration curve for glucose was linear within the range of 0.2—50 μM (r=0.9996), and the detection limit was 0.1 μM (S/N=3). This method was applied to the analysis of glucose in plasma. No influence of sodium fluoride on the activities of immobilized enzymes was observed. Recovery of glucose added to plasma or serum was found to be in the range of 98 to 106%. We investigated the correlation between the glucose content determined by other methods and that determined by the present method. A linear relationship was observed between that determined by the present method and that by both the glucose oxidase-electrode method (y=0.986x−4.6, r=0.9993) and the soluble enzyme method (y=1.004x+1.3, r=0.9982).

Key words —— glucose, immobilized enzyme, flow injection analysis, plasma

INTRODUCTION

Glucose content in blood has typically been determined using chemical reactions such as the Somogyi-Nelson method,[1] the Hagedorn-Jensen method[2] and the *ortho*-toluidine boric acid(OTB) method.[3] For the purpose of glucose analysis, enzymatic reactions catalyzed by glucose oxidase(GOD), glucose dehydrogenase, or hexokinase(HK)-glucose-6-phosphate dehydrogenase(G6PD) have been applied. Enzymes can be utilized in both immobilized and soluble forms. In particular, GOD has frequently been utilized in the form of an immobilized enzyme, such as an enzyme reactor[4,5] in a flow system or as an enzyme electrode.[6,7] HK-G6PD has also been used as an enzyme reactor.[8,9] In certain cases[4-7] these reactions have been applied to the analysis of glucose in blood.

We previously described[10] the determination of glucose using immobilized HK-G6PD in a flow system, and reported its application to determination of the glucose content in beverages. In the present study, this method was applied to the determination of glucose in plasma, and the correlation between the contents determined by other methods and those found by the present method were compared. NADH formed by the enzymatic reactions shown below was detected fluorometrically.

$$\text{glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{glucose-6-phosphate} + \text{ADP}$$

$$\text{glucose-6-phosphate} + \text{NAD}^+ \xrightarrow{\text{G6PD}} \text{6-phosphogluconolactone} + \text{NADH}$$

MATERIALS AND METHODS

Materials —— G6PD from *Leuconostoc mesenteroides*, HK from bakers yeast, NAD^+, 25% aqueous solution of glutaraldehyde and aminopropyl glass (500 Å pore size, 200—400 mesh) were purchased from Sigma Chemical Co. (U.S.A.).

Enzyme Immobilization —— Enzymes were immobilized as described below. Bovine serum albumin(BSA) was added to the reaction mixture of enzyme immobilization at the final stage of the reaction to stabilize the immobilized enzymes.[10] To aminopropyl glass beads (0.5 g) in 4.5 ml of 0.1 M phosphate buffer (pH 10.0) was added 0.5 ml of a 25% aqueous solution of glutaraldehyde. The mixture was bubbled with N_2 gas for 1 h at room temperature. After the activated aminopropyl glass was filtered and washed with 100 ml water, 4.0 ml of 0.05 M phosphate buffer (pH 6.0), 100 units of G6PD and 100 units of HK were added. One unit of G6PD or HK was defined as the amount of enzyme catalyzing the production of 1 μmol of 6-phosphogluconolactone or

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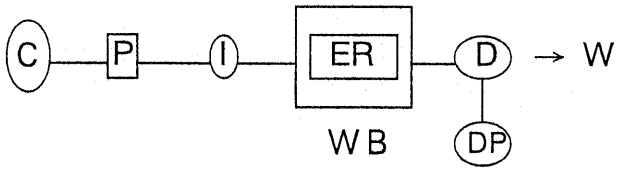

Fig. 1. Apparatus for Determination of Glucose

C, carrier reservoir; P, pump; I, sample injector; ER, enzyme reactor; D, spectrofluorometer; DP, data processor; WB, water bath; W, waste.

glucose-6-phosphate per min, respectively. The mixture was stirred with a shaker for 20 h at 4℃, then 48.3 mg of BSA(1 w/v%) was added and the mixture was stirred for 4 h. Enzyme-immobilized glass beads were filtered and washed first with 100 ml of 0.05 M phosphate buffer (pH 6.0) and then with 100 ml of water. They were stored in 4.0 ml of 0.05 M phosphate buffer (pH 6.0). The percent activities of the immobilized enzymes were 14% and 5% of the initially applied enzymes for G6PD and HK, respectively. The enzyme-immobilized glass beads thus prepared were packed in a stainless steel column (4 cm, i.d. 2 mm).

Apparatus —— A schematic diagram of the apparatus used in the study is shown in Fig. 1. The enzyme reactor (ER) containing immobilized HK and G6PD was immersed in a water bath (WB) thermoregulated at 30℃. The carrier used was 0.1 M triethanolamine buffer(pH 7.0) containing 2 mM ATP, 1.5 mM NAD^+ and 10 mM $MgCl_2$. The carrier was delivered at a rate of 0.4 ml/min by a Shimadzu LC-10AD pump (P). NADH formed by the enzymatic reactions was fluorometrically detected (λ_{ex}, 340 nm; λ_{em}, 460 nm) by a Hitachi F-1050 spectrofluorometer (D) with a 12 μl flow cell. This instrument is considerably more sensitive than that used in the previous study (Hitachi F-1000).[10] The peak area was obtained by a Hitachi D-2500 data processor (DP). The sample injection volume was 50 μl.

The plasma or serum samples for analysis were prepared by 500 fold dilution of the original plasma with a carrier.

RESULTS AND DISCUSSION

The carrier used in the present study was one which yielded the largest peak area due to NADH of all carriers investigated earlier.[10] The peak due to NADH was observed at approximately 1.4 min. The calibration curve formed between glucose concentration and peak area was linear($r=0.9996$) within the glucose concentration range of 0.2 to 50 μM. The detection limit ($S/N=3$) was 0.1 μM, and the relative standard deviations of the peak area at 1 μM and 10 μM were 0.7% ($n=7$) and 1.0% ($n=7$), respectively. The detection limit of glucose in the present study was considerably lower than that previously reported[10] likely due to the high sensitivity of the spectrofluorometer used. When a plasma sample for injection was prepared by 500-fold dilution of an original sample, a 0.2 μM of diluted sample corresponded to 1.8 mg/dl of the original sample.

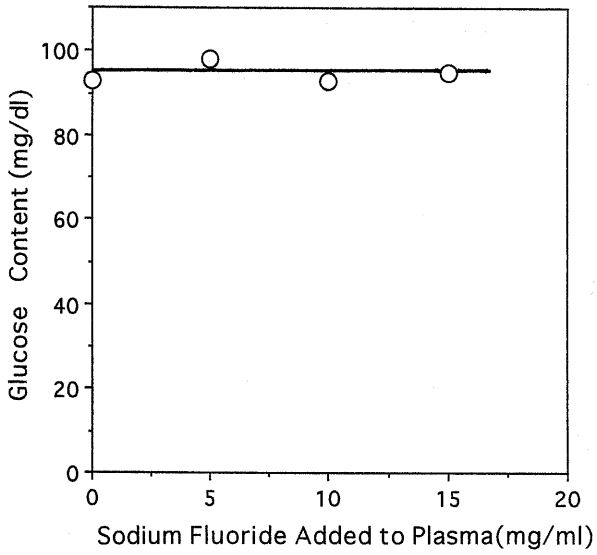

Fig. 2. Influence of Sodium Fluoride on the Glucose Content in Plasma Measured by the Present Method

Plasma used was prepared by treating blood with heparin. Values were averages of three separate determinations.

The plasma used for analysis was prepared by treating blood with sodium fluoride, which is an anticoagulant that inhibits glycolysis. Thus, the influence of sodium fluoride on the activities of the immobilized enzymes was evaluated. As shown in Fig. 2, which illustrates the effect of sodium fluoride on the glucose content in plasma determined by the present method, it was demonstrated that sodium fluoride did not influence the activities of immobilized enzymes. Table 1 shows the results of the recovery test of the glucose added to plasma or serum. Observed recoveries were in the range of 98 to 106%, indicating the reliability of the present method in determining glucose content. Furthermore, these results show that the present method is applicable to the determination of glucose in serum as well as that in plasma.

As shown in Fig. 3, a linear relationship was

Table 1. Recovery of Glucose Added to Plasma or Serum

	Added glucose (mg/dl)	Observed glucose (mg/dl)	Recovery (%)
Plasma	0	98	—
Plasma	50	147	98
Plasma	100	199	101
Plasma	200	297	100
Plasma	401	495	99
Serum	0	99	—
Serum	50	152	106
Serum	100	202	103
Serum	200	300	101
Serum	401	497	99

Values are averages of two separate determinations. Plasma was prepared by treating blood with sodium fluoride.

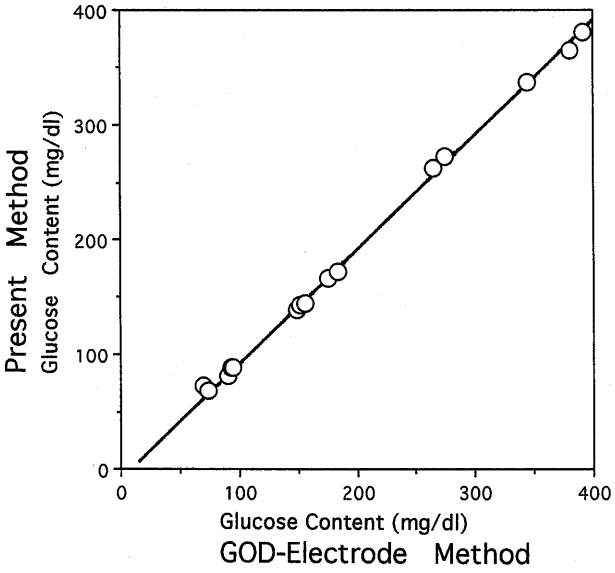

Fig. 3. Correlation between the Glucose Contents in Plasma Determined by the GOD-Electrode Method and That Determined by the Present Method

observed: between the glucose content in plasma as determined by the GOD-electrode method (Kyoto Daiichikagaku GA-1160) and that determined by the present method ($y=0.986x-4.7$, and $r=0.9993$, $n=15$). When the plasma samples were analyzed using a carrier containing no NAD^+, and thus the enzymatic reaction catalyzed by G6PD did not occur, a small peak was detected at approximately 1.2 min for respective samples. This peak overlapped with the peak due to NADH formed in the glucose analysis. The areas of these peaks corresponded to glucose contents in the range from 0.8 to 1.9 mg/dl. After these values were subtracted from the glucose contents obtained by the present method, a linear relationship was also obtained: $y=0.986x-5.8$, and $r=0.9993$. The correlation (Fig. 4) between the glucose content in plasma obtained by the present method and that by a commercially available test kit(Boehringer Mannheim F-kit) in which HK-G6PD is present in the form of soluble enzyme showed a linear relationship with a slope approaching unity: ($y=1.004x+1.3$, and $r=0.9982$, $n=15$). After calibrating the glucose content obtained from the peak areas observed using a carrier containing no NAD^+, a linear relationship was also obtained ($y=1.004x+0.2$, and $r=0.9983$). Thus, the glucose content in plasma determined here correlated closely with that determined by other methods.

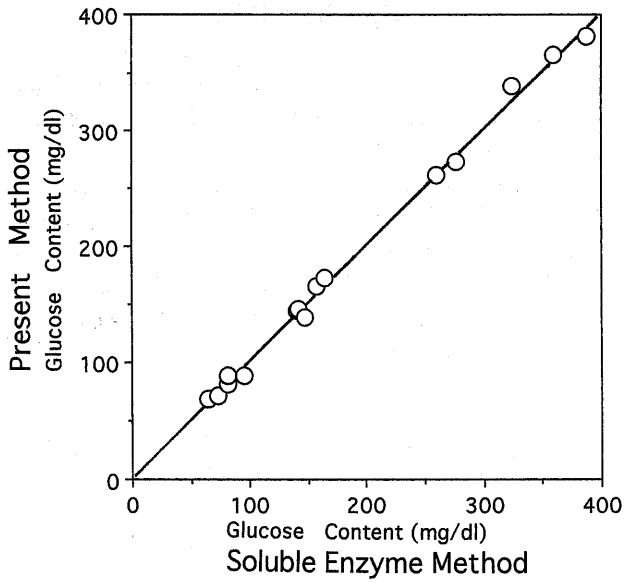

Fig. 4. Correlation between the Glucose Contents in Plasma Determined by the Soluble Enzyme Method and That Determined by the Present Method

The peak area for 10 μM(0.180 mg/dl) glucose was almost equivalent for all 300 samples, including standard samples for calibration curves, whereas in the previous study[10] this value was constant across the analysis of 50 samples. The lifetime of the immobilized enzymes was thus prolonged, perhaps due to the BSA which was added at the final stage of enzyme immobilization reaction. The sampling rate calculated from the peak width at baseline was 30 samples/h. In contrast to exclusive and expensive commercially available instruments, the apparatus used in the present study can be easily assembled by substituting the separation column in an HPLC system with the enzyme reactor.

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