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Note

Gas chromatographic determination of glucose in serum with glucose oxidase—catalase system

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Glucose in serum has been determined with the help of enzymes. There are several enzymatic methods such as the glucose oxidase—peroxidase method [1—3], the glucose dehydrogenase—NAD^+ method [4, 5] and the hexokinase—glucose-6-phosphate dehydrogenase method [6]. Among these, the hexokinase—glucose-6-phosphate dehydrogenase method reacts with both glucose and glucose-6-phosphate, and insufficient purity of the enzymes results in false values [6]. Glucose dehydrogenase is difficult to obtain in high purity and is very expensive. On the other hand, glucose oxidase is available in high purity, inexpensive and specifically oxidizes β-D-glucose. However, the glucose oxidase—peroxidase method has a disadvantage that peroxidase is unspecific in activity for indicator substrate, so it is susceptible to interference of reducing compounds. In a previous paper [7], a new method for the gas chromatographic (GC) determination of hydrogen peroxide using a methanol—catalase system was reported, which involved the enzymatic conversion of hydrogen peroxide into formaldehyde and derivatization of the formaldehyde with pentafluorobenzyloxylamine (PFBOA). The purpose of this present paper is to develop a new GC method for the assay of glucose in serum with the glucose oxidase—catalase system as an extension of our previous work on the GC determination of hydrogen peroxide using the methanol—catalase system.

EXPERIMENTAL

Reagents

PFBOA hydrochloride (melting point 115°C) was synthesized from pentafluorobenzyl bromide (Aldrich, Milwaukee, WI, U.S.A.) and N-hydroxyphthalimide (Tokyo Kasei, Tokyo, Japan) [8]. Iodobenzene was used as an

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internal standard. An aqueous solution of glucose (60 μg/ml) was prepared by dissolving glucose in water.

Enzymes

Catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6) (270,000 U/ml) was obtained from Boehringer (Mannheim, G.F.R.). A stock solution (20,000 U/ml) was prepared by diluting it with distilled water.

Glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) (17.8 U/mg) was purchased from Sigma (St. Louis, MO, U.S.A.). A stock solution (5 U/ml) was prepared by dissolving it in distilled water.

Apparatus and conditions

A Shimadzu GC-4CPF gas chromatograph equipped with a hydrogen flame ionization detector (FID) was used. A 2-m glass column packed with 3% XE-60 on 80—100 mesh Celite 545 AW DMCS was used, with a column temperature of 100°C, a detector temperature of 150°C and a chart speed of 0.25 cm/min.

Standard procedure

To the mixture of 1.25 ml of 0.1 *M* phosphate buffer, pH 5.6, 0.15 ml of methanol, 0.1 ml of catalase solution and 0.5 ml of glucose oxidase, were added 0.5 ml of sample solution containing glucose (or 0.5 ml of ten-fold diluted serum with distilled water) and 0.5 ml of aqueous PFBOA solution (1 mg/ml as the hydrochloride) in a 10-ml test-tube. After mixing, the tube was incubated in a water-bath at 37°C for 90 min. After saturation with sodium chloride and acidification with one drop of 18 *N* sulfuric acid, the PFBOA derivative of formaldehyde was extracted with 0.3 ml of *n*-hexane containing iodobenzene (200 μg/ml) as internal standard. Excess sodium chloride and the aqueous layer were removed with the aid of a syringe with a long needle. An aliquot of the extract was applied to the GC column. Quantitation was carried out using calibration graphs obtained from known amounts of glucose. Blank tests were performed using water instead of sample solution.

RESULTS AND DISCUSSION

The factors affecting the reaction in the methanol—catalase system were investigated in detail in the previous paper [7]. In the present paper, the following factors affecting the overall reaction in the glucose oxidase—catalase system were investigated in further detail.

pH of the reaction solution

Using 30 μg of glucose, the optimal pH in the reaction solution was examined in the range 4—8. The pH was adjusted with 0.1 *M* acetate buffer or 0.1 *M* phosphate buffer. The results in Fig. 1 show the overall effects on the glucose oxidase reaction, on the catalase reaction and on the condensation reaction of formaldehyde produced with PFBOA. The optimal pH was between 5 and 6, which agreed with the fact that glucose oxidase is most active at pH 5.6.

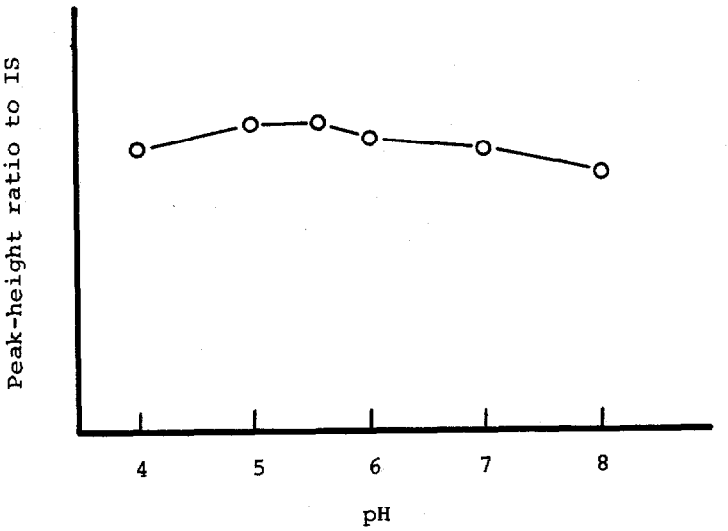

Fig. 1. Effect of pH on the overall reaction in glucose oxidase—catalase system.

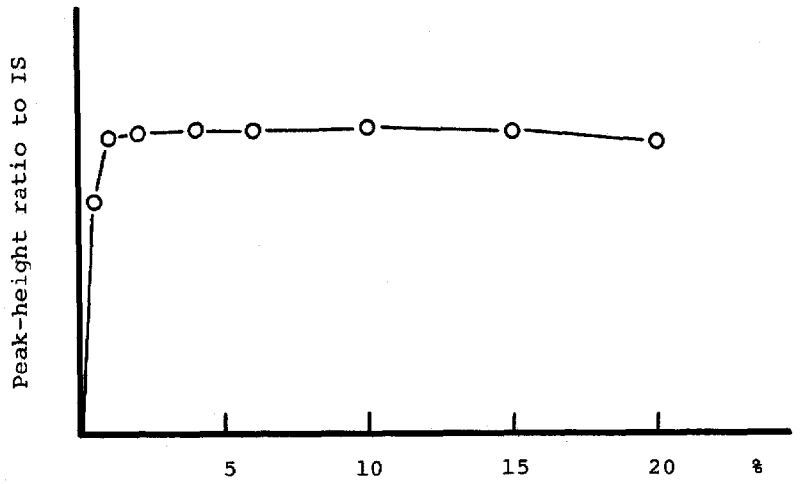

Fig. 2. Effect of methanol concentration.

Methanol concentration

Using 30 μg of glucose, the appropriate concentration of methanol for the glucose oxidase—catalase reaction was examined in the range 1—20% and was found to be between 2 and 10%, as shown in Fig. 2. In the previous paper [7] the suitable concentration of methanol for the determination of hydrogen peroxide using the catalase reaction was found to be between 8.0 and 30%. However, glucose oxidase activity seems to be inhibited in the presence of high concentrations of methanol. Therefore, the measurements in this method were made in the presence of 5% of methanol.

Glucose oxidase concentration

The necessary concentration of glucose oxidase for the determination of

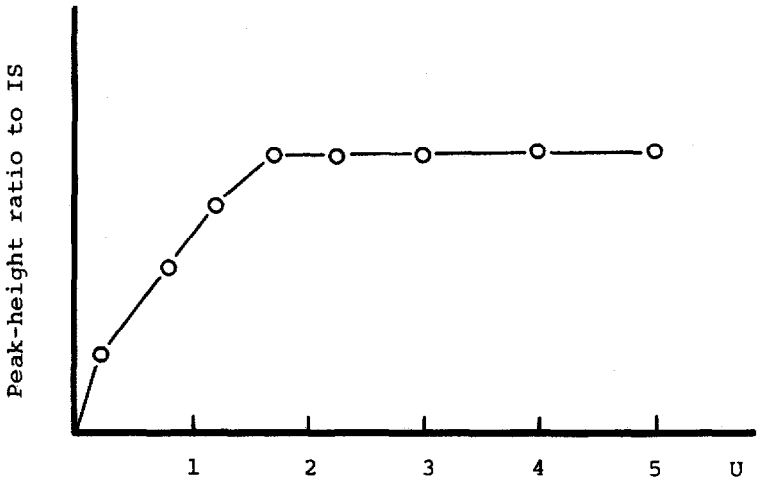

Fig. 3. Effect of glucose oxidase concentration.

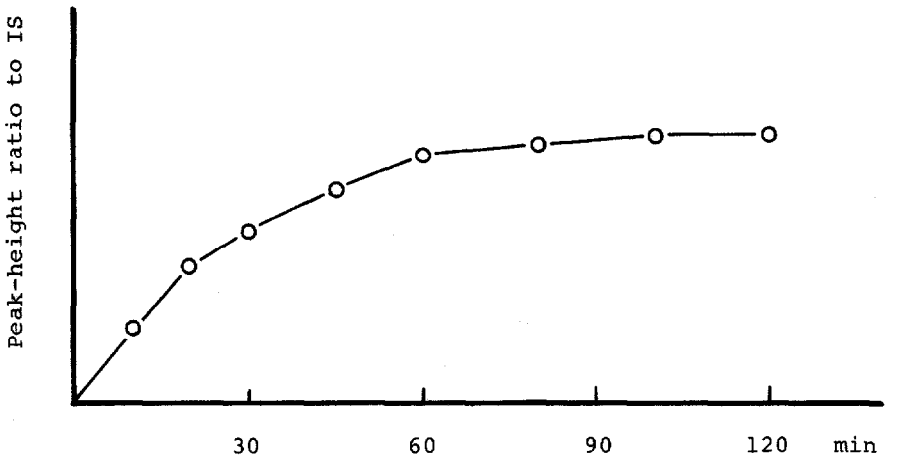

Fig. 4. Effect of reaction period.

glucose was examined. As shown in Fig. 3, it was found that the concentration of glucose oxidase sufficient to obtain a constant value was 2 U for 30 μg of glucose in the reaction system.

Reaction period

Using 30 μg of glucose, the effect of the reaction period was investigated through the entire procedure. It can be seen that the reaction was sufficient in 90 min to obtain a constant glucose value, although after this time the measured values increased slowly with reaction time as shown in Fig. 4.

Influence of reducing compounds

L-Ascorbic acid and uric acid were examined as examples of reducing agents. L-Ascorbic acid (5—20 μg) or uric acid (5—40 μg) was added to 3 ml of the reaction solution with 30 μg of glucose. The results are shown in Fig. 5. Within the range of reducing agent concentrations tested, the measured values were

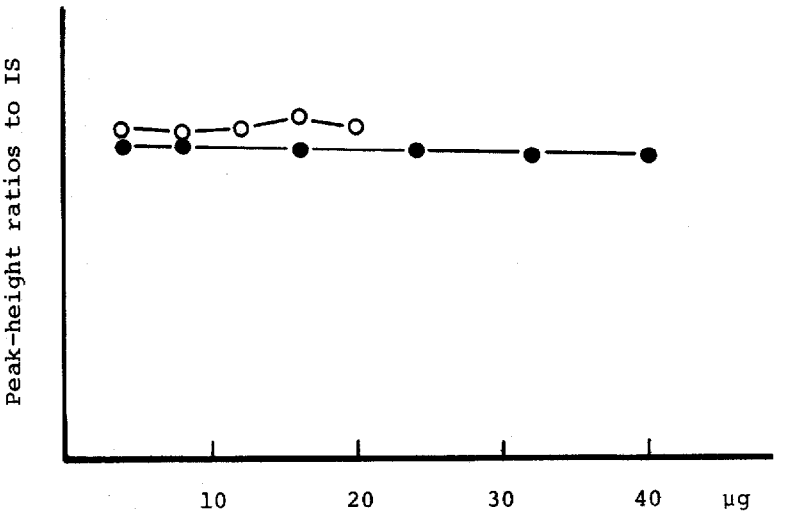

Fig. 5. Effect of coexistence of reducing compounds: ascorbic acid (○) and uric acid (●).

constant. From these results, it can be concluded that the methanol—catalase system is nearly specific for the conversion of hydrogen peroxide into formaldehyde, as expected in the previous paper.

Application

A sample solution containing an individual amount of glucose was measured according to the standard procedure described under Experimental and peak height ratios of formaldehyde formed enzymatically to that of the internal standard were proportional in the range 20—100 μg of glucose in 0.5 ml of sample solution. The coefficient of correlation for the calibration graphs was 0.9991. The reproducibilities of the method were examined with an identical sample solution containing 30 μg of glucose and the coefficient of variation obtained was 2.55% (n = 5).

A typical GC separation of glucose in serum is illustrated in Fig. 6. Iodobenzene was used as internal standard. The peak height ratio of formaldehyde to the internal standard on the chromatogram corresponded to 84 mg/dl glucose in serum. The blank values were found to be negligible towards the FID. A recovery test was carried out on five 0.5-ml portions of an identical ten-fold diluted serum sample spiked with 30 μg of glucose, and the values obtained were calculated to be 102 ± 2.7% (n = 5).

Glucose oxidase is very specific for glucose. However, the glucose oxidase peroxidase method which has widely been used has a disadvantage that it is susceptible to interference from the presence of reducing compounds commonly existing in serum such as ascorbic acid. This important subject remains open. So it is still required to establish a specific method for the assay of glucose in serum.

In this respect, catalase converts methanol specifically to formaldehyde through the action of hydrogen peroxide formed in the glucose oxidation reaction and the catalase reaction is not subject to interference from coexisting reducing compounds. On the other hand, GC is an excellent technique for the

Fig. 6. Gas chromatogram of formaldehyde produced from glucose in serum in glucose oxidase catalase system on a 2.0 m 3% XE-60 column at 100°C, with FID. Peaks: 1 = formaldehyde PFBOA; 2 = iodobenzene.

determination of formaldehyde. We are not aware of any report dealing with catalase reaction combined with GC for serum glucose determination. The present paper describes a reliable and sensitive method for the determination of glucose by connecting the catalase reaction and the derivatization of formaldehyde with PFBOA.

The proposed method appears to be a little more time-consuming compared with non-chromatographic methods, but a one-step procedure was established to permit the three reactions, glucose oxidase reaction, catalase reaction and condensation reaction with PFBOA, simultaneously. All these things are put together during the incubation; thus, the derivative is formed "on-line", i.e. as the enzyme reacts. In case we need a reliable value of glucose in serum, the method would be useful and helpful.

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