Determination of D-Glucosone by Colorimetry and by High-Performance Liquid Chromatography

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Methods employing colorimetry and high-performance liquid chromatography (HPLC) were developed for determination of D-glucosone. Colorimetry with phenazine methosulfate and nitro blue tetrazolium as electron acceptors made it possible to determine D-glucosone (sensitivity: 50 nmol) in the presence of excess amounts of D-glucose and D-fructose. Further, by using a high-performance liquid chromatograph equipped with a ligand-exchange mode column, we could separate D-glucosone in human and rat sera from D-glucose. The blood level of D-glucosone 5 min after intraperitoneal administration (1.68 mmol/kg) of the sugar to rats was determined by the HPLC method to be 1.3 mM in portal blood and 0.5 mM in postcaval blood. The maximum increase in the blood glucose level was observed 2 h after administration of the D-glucosone. A similar increase of blood glucose was observed after the administration of 2-deoxy-D-glucose, and seemed to continue until 5 h. The assay methods described are useful for biochemical studies on D-glucosone as an analogue of D-glucose.

Keywords D-glucosone; spectrophotometry; HPLC; D-glucosone analysis; 2-deoxy-D-glucose; carbohydrate; serum; intraperitoneal administration; blood glucose level

D-Glucosone (D-*arabino*-hexos-2-ulose), a sugar that can exist in acyclic and cyclic pyranose and furanose forms,[1] has a very similar structure to D-glucose and 2-deoxy-D-glucose. In connection with the biochemical and physiological actions of D-glucosone, some investigators have reported that D-glucosone has a high affinity for hexokinase[2] and is an inhibitor of glycolysis in yeast and rat brain.[3] Moreover, it was shown that this sugar acts as an uridylate-trapping agent and causes uridine triphosphate (UTP) depression in tumor cells.[4] Mutagenicity of this sugar has also been reported.[5]

D-Glucosone is of interest as an intermediate in a process for conversion of D-glucose to D-fructose[1] and as a radiolytic product in some foodstuffs containing D-fructose.[5,6]

In earlier work we prepared D-glucosone by using an immobilized glucose 2-oxidase–catalase gel, and reported some of its biochemical actions in rats: 1) depression of gastric motility produced by glucopenia, like D-glucose, 2) inhibition of glucose-stimulated insulin secretion from rat pancreatic islets, and 3) production of a sweetness sensation similar to that of D-glucose.[7]

For further study of the biochemical actions of D-glucosone as an analogue of D-glucose, it is necessary to develop an assay method for D-glucosone in biological samples such as serum. Colorimetry of D-glucosone with 2,3,5-triphenyltetrazolium chloride (TTC)[8,9] and the use of high-performance liquid chromatography (HPLC) have already been reported; however, these methods can not be applied directly to biological materials such as serum.

In this report, we describe a new colorimetry of D-glucosone with phenazine methosulfate (PMS)–nitro blue tetrazolium (NBT), in which excess amounts of D-glucose and D-fructose do not interfere, as well as an HPLC method for D-glucosone in human and rat sera. Furthermore, by applying these analytical methods, we compared the effect of D-glucosone on the blood glucose level in rats with that of 2-deoxy-D-glucose.

Experimental

Materials D-Glucosone was prepared from D-glucose using glucose 2-oxidase (pyranose oxidase, EC 1.1.3.10) and catalase (EC 1.11.1.6) immobilized on Affigel-10 (Bio-Rad Laboratories).[7] 2,3,5-Triphenyltetrazolium chloride (TTC), PMS, NBT, sugars, and other reagents were purchased from Nakarai Tesque, Inc. (Japan).

Colorimetric Analysis of D-Glucosone The colorimetry described here was based on the differential rate of reduction of dye (oxidized form) by reducing sugars. We chose a PMS–NBT coupling system as an electron acceptor. A 0.3 ml aliquot of Tris–HCl (50 mM, pH 7.5) containing Triton X-100 (5%), plus 0.1 ml of sample, and 0.1 ml of 0.1% NBT solution containing 0.01% of PMS (PMS–NBT solution) were placed in a 5 ml test tube. After incubation of the mixture at 50 °C for exactly 30 min, 1.0 ml of 0.5 N HCl containing 5% Triton X-100 was added to the mixture to stabilize the reduced dye. Absorbance of reduced NBT (diformazan) was measured at 525 nm using a Ubest-30 spectrophotometer (Japan Spectroscopic Co., Ltd., Japan). When human serum was used as the sample an equal volume of $ZnSO_4$ solution (2%) was added to the sample for deproteinization, and the supernatant obtained after centrifugation was utilized as the test sample.

HPLC The liquid chromatograph used in this study was a 880-PU type (Japan Spectroscopic Co., Ltd., Japan) equipped with a guard column of Shodex KS-800P (0.6 × 5 cm) and a ligand-exchange mode column of Shodex SUGAR SP-1010 (0.8 × 30 cm; Showa Denko K.K., Japan). Distilled water as the eluant for HPLC was degassed by stirring under reduced pressure. The flow rate was kept constant at 1.0 ml/min, and the column temperature was maintained at 80 °C. The column eluate was monitored with a Shodex RI SE-51 refractive index detector (Showa Denko K.K.). Areas of the sugar peaks on the chromatograms were calculated using a Hitachi D-2500 Chromato-integrator (Hitachi, Ltd., Japan). Human or rat serum (50—300 μl) containing D-glucosone was mixed with 50 μl of galactitol solution (50 mM, as an internal standard). The mixture was deproteinized by passage through a centrifugal membrane filter (Ultrafree-C3TK, Millipore Corp., U.S.A.). The filtrate was used as the sample for HPLC analyses.

Administration of D-Glucosone to Rats Male Sprague-Dawley rats weighing from 180 to 250 g were starved for 20 h. After anesthetizing the animals with sodium pentobarbital (45 mg/kg, i.p.), we injected them intraperitoneally with D-glucosone, D-glucose, and 2-deoxy-D-glucose solutions (0.3 M) at a dose of 1.68 mmol/kg. The sera of these animals were obtained from postcaval and portal blood collected under ventral celiotomy, and they were analyzed by the HPLC method described above.

Some of the animals were bilaterally adrenalectomized about 30 min before injection of the sugars to eliminate the effect of adrenalin on the blood glucose level and their blood was collected from the tail vein at regular intervals. The blood sugar level of these animals was determined using the GOD-Perid Test (Boehringer Mannheim Biochemicals, GmbH., W. Germany). The concentrations of D-glucosone in these samples were also determined by the HPLC method.

Results

Determination of D-Glucosone by Colorimetry Figure 1 shows the calibration curve of D-glucosone (in buffer)

obtained by the PMS–NBT method. The plot was linear between 50—4000 nmol of D-glucosone. The result of this colorimetry showed a good correlation with that of the previous method with TTC as an electron acceptor.[8,9] The coefficient of correlation (r value) between these two colorimetries was calculated to be 0.988.

The sensitivity of the TTC method was 18 times higher than that of the PMS–NBT method; however, the calibration curve of the TTC method did not pass through the zero point in the plot of absorbance against amount of D-glucosone.

The PMS–NBT method showed no response to D-glucose at less than 0.1 mmol (Fig. 2A), while the absorbance in the TTC method increased with increasing amount (25—100 μmol) of D-glucose added, as shown in Fig. 2A. An excess amount of D-fructose caused an increase in absorbance in both methods (Fig. 2B); however, D-fructose reduced the PMS–NBT system to a red-purple dye about 120 times more slowly than when an equimolar amount of D-glucosone was used. The reactivity of D-fructose, represented by the slope in Fig. 2B, in the TTC method was 136 times higher than that of the sugar in the PMS–NBT method.

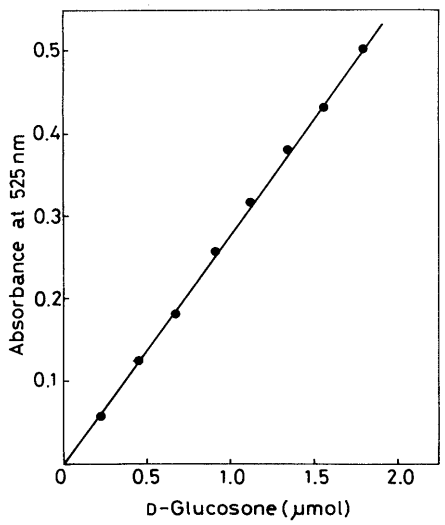

Fig. 1. Calibration Curve for D-Glucosone by Colorimetry with Phenazine Methosulfate (PMS) and Nitro Blue Tetrazolium (NBT)

D-Glucosone solutions (2.25—18.0 mM) were determined as described in Materials and Methods.

These results suggest that the PMS–NBT method is a suitable procedure to determine D-glucosone in the presence of an excess amount of D-glucose or D-fructose.

A linear calibration curve was also obtained by the PMS–NBT method when D-glucosone added to human serum was used as the sample. In this method, however, even the deproteinized serum without D-glucosone (0.1 ml) caused a slight color development (an absorbance of *ca.* 0.02 at 525 nm) due to the physiological reductants in the serum. Thus, for determination of D-glucosone in serum, the absorbance due to the reductants in serum before the administration of D-glucosone should be subtracted from the value (absorbance) obtained after the administration.

Determination of D-Glucosone by HPLC By applying the HPLC method as described above, we determined D-glucosone and D-glucose simultaneously by comparing the areas of the two peaks with the area of the galactitol (internal standard) peak. A typical chromatogram is shown in Fig. 3.

The retention times of D-glucose, D-glucosone, and galactitol were 9.8, 11.4, and 21.1 min, respectively. D-Fructose was eluted just after D-glucosone, with a retention time of 12.3 min. The peak area of D-glucosone increased linearly with increasing amount of D-glucosone dissolved in human serum (Fig. 4).

Almost the same curve was obtained from a similar experiment using an aqueous solution of D-glucosone instead of human serum containing D-glucosone. In this case, the lower limit of detection was approximately 0.5 nmol/assay.

Administration of D-Glucosone to Rats The intraperitoneal administration of D-glucosone to non-anesthetized rats (1.68 mmol/kg of body weight) produced a protuberance of the eyes, limping, sprawling, and twitching movements within 5—15 min. These symptoms were completely gone within 20—30 min after the administration. None of these symptoms was observed in rats administered 2-deoxy-D-glucose at the same dose.

By using the above HPLC method, we determined D-

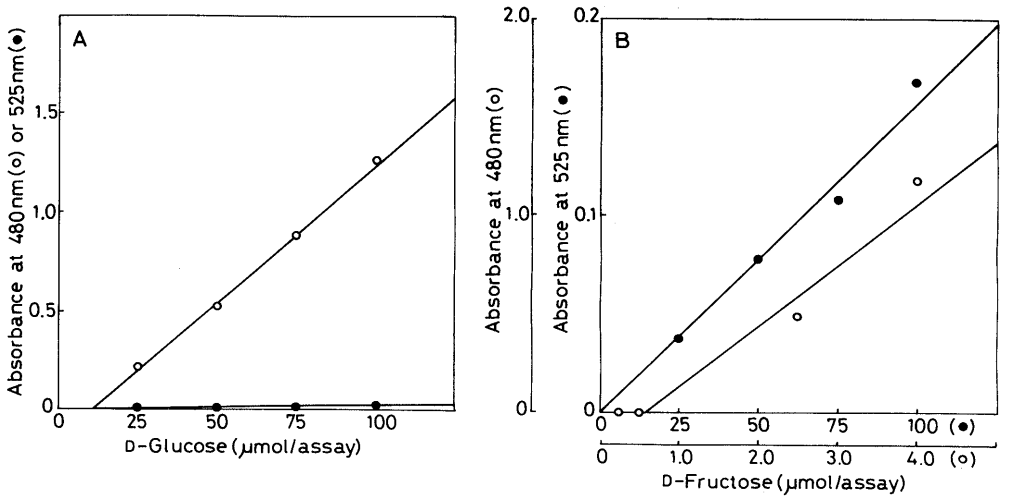

Fig. 2. Effects of Excess Amounts of D-Glucose (A) or D-Fructose (B) on the PMS-NBT Methods (●) and the TTC Method (○)

A: 0.1-ml volume of D-glucose solution (0—100 μmol) was added to the assay mixtures in the PMS–NBT and TTC methods. B: A 0.1-ml volume of D-fructose solution (0—100 μmol) was added to the assay mixture in the PMS–NBT method and the same volume (0—5 μmol) to that in the TTC method.

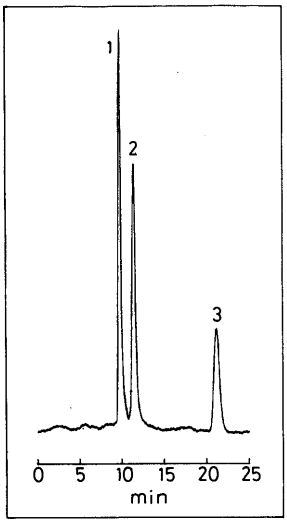

Fig. 3. HPLC Chromatogram of D-Glucose, D-Glucosone, and Galactitol (Internal Standard)

A sample (20 μl) containing 30 nmol of D-glucose, 25 nmol of D-glucosone, and 15 nmol of galactitol was injected into the HPLC system as described in Materials and Methods. Retention times of the sugars were as follows: 9.8 min for D-glucose (1), 11.4 min for D-glucosone (2), and 21.1 min for galactitol (3).

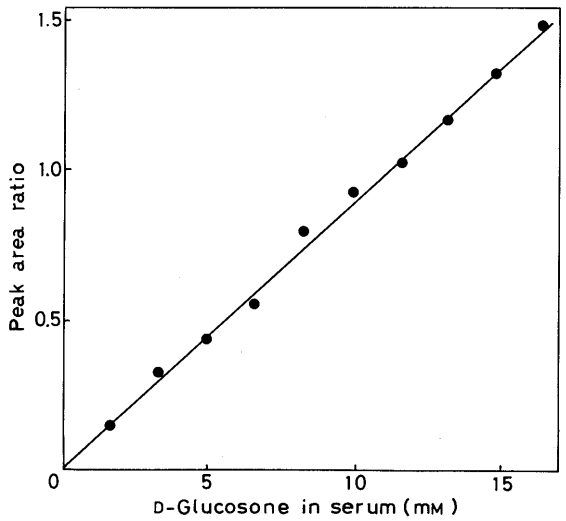

Fig. 4. Linear Relation between the Peak Area Ratio of D-Glucosone Added to Human Serum (with Respect to the Internal Standard, Galactitol) and Amount Added

Fifty microliters of galactitol (50 mM) was added to 0.3 ml of human serum containing 5—50 μmol of D-glucosone, and 20 μl of the mixture, after deproteinization, was injected into the HPLC system, as described in Materials and Methods.

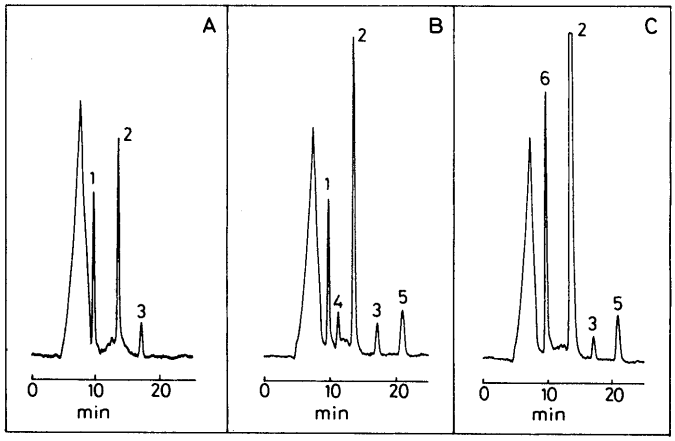

Fig. 5. Typical HPLC Chromatograms of Serum from Rat Portal Blood Obtained 5 min after the Intraperitoneal Administration of Saline (A), D-Glucosone (B), or 2-Deoxy-D-glucose (C)

The administration of these substances and the sample preparation for HPLC are described in Materials and Methods. The corresponding peaks are as follows: (1) D-glucose, (2) D-glycerol from membrane filter, (3) unknown substance in serum, (4) D-glucosone, (5) galactitol used as an internal standard, and (6) D-glucose and 2-deoxy-D-glucose.

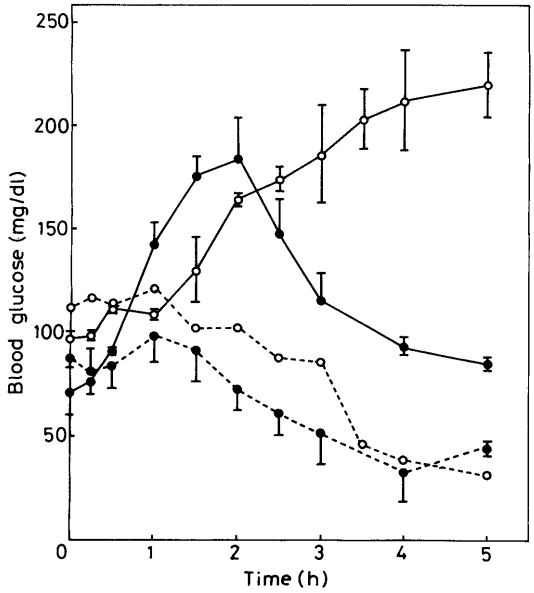

Fig. 6. Effects of D-Glucosone and 2-Deoxy-D-glucose on Blood Glucose Levels of Rats

After the intraperitoneal administration of D-glucosone (●) or 2-deoxy-D-glucose (○), the blood glucose levels were determineld as described in Materials and Methods. Dotted lines show the blood glucose levels in adrenalectomized animals. Results are given as the means (±S.D.) of 5 rats except in the case of the adrenalectomized rats loaded with 2-deoxy-D-glucose (means of 2 rats).

glucosone levels in rat serum after intraperitoneal administration of this sugar. A typical chromatogram obtained is shown in Fig. 5B. The concentrations of D-glucosone at 5 min after administration were calculated to be 1.3 mM in portal blood and 0.5 mM in postcaval blood. No peak was observed at the position of D-glucosone when saline or 2-deoxy-D-glucose was administered and the peak of 2-deoxy-D-glucose (retention time, 9.9 min) was superimposed on that of D-glucose (Fig. 5A, C).

The administration of D-glucosone (1.68 mmol/kg) to rats caused an increase in the blood glucose level to twice the original level within 2 h, and the value reverted to the initial one within the next 3 h (Fig. 6). In this experiment, the maximal concentration of D-glucosone (0.48 mM) in peripheral blood was found at 15 min after administration by an HPLC method. The concentration of D-glucosone decreased to half the maximum level within the next 30 min. The blood glucose level also increased after the administration of 2-deoxy-D-glucose; however, the level continued to increase with time (even 5 h) after administration. On the other hand, no increase in blood glucose was noted when these analogues of D-glucose were injected into adrenalectomized rats.

Discussion

D-Glucosone is reported to be the key intermediate in the conversion of D-glucose to D-fructose,[1] and pure D-

glucosone has been prepared from D-glucose enzymatically[7,9] in addition to its chemical synthesis.[10] Interestingly, this sugar appears to be one of the major radiolytic products of D-fructose in some subtropical fruits treated by irradiation,[6] and it is mutagenic toward *Salmonella* TA 100.[5]

As interest in D-glucosone has increased, it has become necessary to develop a convenient procedure for determination of D-glucosone even in samples containing excess amounts of D-glucose and D-fructose. A colorimetric method with TTC has been reported[8,9]; however, this method was affected by coexisting D-glucose and D-fructose, as shown in Fig. 4. The assay method with TTC is based on the differential rate of reduction of TTC by sugars in a strongly alkaline solution.

In the present colorimetry with PMS–NBT as an electron acceptor, the reducing power of D-glucose and D-fructose was considerably diminished by maintenance of the pH of the reaction mixture at 7.5. At this pH, D-glucosone still showed reducing activity and caused color development. It seems to be possible to determine D-glucosone in biological samples such as serum by comparison of the absorbance values before and after administration of D-glucosone. Because of its low sensitivity to D-glucose and D-fructose, the PMS–NBT method seems to be suitable for the assay of D-glucosone in the presence of excess amounts of these sugars, such as in irradiated foodstuffs or in the conversion reaction of D-glucose to D-fructose.

The determination of D-glucosone by HPLC has been reported by some investigators.[6,11-13] In these reports, an amino-bound silica column or a strongly basic anion-exchange column was used for the separation of D-glucosone from other substances. We chose a ligand-exchange mode column for determination of D-glucosone in which distilled water could be used as the HPLC eluant. In our method, the peak of D-glucose was completely separated from that of D-glucosone. Though the peak of D-fructose was close to that of D-glucosone, these peaks were separated sufficiently to differentiate the two. In the determination by this HPLC method of D-glucosone added to serum, the serum was deproteinized by membrane filtration, which removed large molecules (above M.W. 30000) in the serum. Among many procedures available for deproteinization, we chose this membrane filter approach because it offered good recovery of D-glucosone in the serum. It is not recommended to apply this deproteinizing method in the PMS–NBT method, because low-molecular reductants in the serum cannot be removed.

The administration of D-glucosone to rats elicited similar symptoms to those reported by Herring *et al.*[14] The present HPLC procedure makes it possible to determine D-glucosone appearing in rat serum after intraperitoneal administration of the sugar. Under the conditions used, D-glucosone appeared in the portal blood within 5 min after administration. The drastic increase in the blood glucose level caused by D-glucosone or 2-deoxy-D-glucose started 1 h after the administration. As reported previously,[7] D-glucosone can pass through the blood-brain-barrier and causes a considerable response of the central glucose-recognizing system. It is well known that 2-deoxy-D-glucose disturbs the response to D-glucose by glucose-recognizing systems located in the liver and central nervous system.[15] A recognition of sham glucopenia may produce an increase in blood glucose over the normal level.

In the case of adrenalectomized rats, no effects of these analogues of D-glucose on the blood glucose level were observed. These results suggest that the increase in blood glucose is due to the release of adrenic hormones by efferent signals from the central nervous system, in which one of the glucose-recognizing systems is located. By a similar mechanism, D-glucosone and 2-deoxy-D-glucose may increase the blood glucose level. As shown in Fig. 6, the D-glucosone-increased level of blood glucose returned to the normal level within 5 h after the administration; however, that by 2-deoxy-D-glucose continued for 5 h after the administration. Though the reason for this is not clear at present, the difference may reflect differential metabolic behavior between the two analogues of D-glucose with respect to the glucose-recognizing systems.

A study on the metabolism of D-glucosone by glycolytic enzymes of mammals is in progress in our laboratory. Details will be reported elsewhere.

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