Gas Chromatographic–Mass Spectrometric Determination of ^{13}C -Glucose Level for Evaluating the Effect of α -Glucosidase Inhibitor Acarbose on the Digestion of $[\text{U}-^{13}\text{C}]$ Starch in Rat

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The effect of acarbose on the digestion of starch was examined by a stable isotope tracer technique. $[\text{U}-^{13}\text{C}]$ -Starch was administered orally to rats with or without acarbose. After the addition of $[\text{H}_3]$ -D-glucose as the internal standard, the plasma samples were treated successively for defatting, deproteinizing and desalting. Glucose was converted to sorbitol by reduction with sodium borohydride. The cyclic butylboronate of sorbitol was injected into a gas chromatograph-mass spectrometer, and the concentration of labeled glucose was measured by selected monitoring of the quasi-molecular ion. The plasma concentration of labeled glucose was decreased significantly by the addition of acarbose. The effect of acarbose on the digestion of starch was clearly confirmed using $[\text{U}-^{13}\text{C}]$ starch.

Keywords ^{13}C starch; digestion; co-administration; acarbose; ^{13}C -glucose; GC-MS

Acarbose¹⁾ (*O*-4,6-dideoxy-4-[[*(1S,4R,5S,6S)*4,5,6-trihydroxy-3-(hydroxymethyl)-2-cyclohexen-1-yl]amino]- α -D-glucopyranosyl-(1 $\rightarrow$ 4)-*O*- α -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-glucopyranose) is a complex oligosaccharide which has been isolated from the culture broths of actinoplanes. Acarbose inhibits intestinal α -glucosidases and consequently delays the digestion and absorption of carbohydrates.²⁾ Therefore, acarbose can be expected to prevent postprandial hyperglycemia.

The effect of acarbose has been evaluated relative to the extent of the increase in blood glucose concentration after the administration of starch or sucrose with acarbose, as compared with that after a single administration of carbohydrates.³⁾ The ^{13}C -breath test was also utilized to evaluate acarbose.⁴⁾ However, part of the expiratory ^{13}C -carbon dioxide originated from organic acid, which was the result of unabsorbed carbohydrates by bacterial fermentation after the administration of ^{13}C -enriched carbohydrates with acarbose.

Therefore, the need for an accurate method allowing the measurement of newly absorbed glucose originating from administered carbohydrates seems to be desirable in an evaluation study of acarbose. The aim of our study was to evaluate the effect of acarbose based on mass spectrometric determination of blood ^{13}C -glucose after the administration of ^{13}C uniform-labeled starch with or without acarbose.

MATERIALS AND METHODS

Materials Acarbose was donated by Bayer Yakuin, Ltd. $[\text{U}-^{13}\text{C}]$ Starch was biosynthesized from ^{13}C -labeled carbon dioxide (99.4 atom%, MSD) using tobacco leaves, as described in our previous report.⁵⁾ The ^{13}C content in the glucose unit of labeled starch was assessed by the mass spectra of the derivative after acid hydrolysis of the starch. The labeled starch was composed of the $^{13}\text{C}_6$ -species (33.0%), $^{13}\text{C}_5$ -species (16.1%), $^{13}\text{C}_4$ -species (6.4%), $^{13}\text{C}_3$ -species (7.7%), $^{13}\text{C}_2$ -species (1.7%) and unlabeled glucose (35.0%). Unlabeled glucose originated

from the residual starch in tobacco leaves before incubation with ^{13}C -labeled carbon dioxide. The ^{13}C -abundance of newly formed glucose after incubation amounted to 84.9 atom% in labeled starch. The uniformity of labeled ^{13}C atoms in the glucose unit was confirmed by ^{13}C -NMR spectra.

$[\text{U}-^{13}\text{C}]$ -D-Glucose (98.2 atom%) and deuterated D-glucose (glucose- $[\text{5,6,6-}^2\text{H}_3]$, 98.8 atom%) were purchased from MSD.

Derivation of Glucose D-Glucose (1 mg) was added to 2 ml of aqueous sodium borohydride solution (0.2 mg/ml), and the solution was allowed to stand at room temperature for 1 h to reduce glucose to sorbitol. Acetic acid was then added to the solution for degradation of the reducing reagent, and the solution was evaporated to dryness. The residue was dissolved in methanolic hydrochloric acid solution, and the solution was evaporated to dryness. This procedure was repeated three times. The residue was dissolved in 1.5 ml of water, then 200 mg of ion exchange resin (AG 501-X8) was added to the solution, and the solution was allowed to stand for at least 5 h. The solution was then filtered and the filtrate was evaporated to dryness. The residue was dissolved in 50 μl of *n*-butylboronic acid-pyridine solution (40 mg/ml), and the solution was heated at 60 $^\circ\text{C}$ for a few minutes.

Animal Experiments Male Wistar rats weighing 230–260 g were used after being fasted for 24 h prior to the experiments. An aqueous suspension containing labeled starch (200 mg/kg) with acarbose (0, 0.75, 1.5 or 3.0 mg/kg) was orally administered to the rats. Blood samples were taken at 10, 20, 30, 45, 60, 90 and 120 min after administration, followed by the separation of the plasma. Aqueous $[\text{H}_3]$ -D-glucose (20 $\mu\text{g}/\text{ml}$) solution (1 ml) was added to 0.1 ml of plasma as the internal standard. The plasma sample was treated with 1 ml of chloroform, and the aqueous phase was separated. Then, 5 ml of ethyl alcohol was added to the aqueous solution, and the sample was shaken and centrifuged. The upper ethanolic layer was separated and evaporated. The residue containing glucose was treated using the derivation procedure.

Gas Chromatography-Mass Spectrometry (GC-MS) GC-MS analysis was performed on a JEOL D-300 gas chromatograph-mass spectrometer-computer system. The mass spectrometer was operated in the chemical ionization (CI) mode with isobutane as the reagent gas. A coiled glass column (1 m $\times$ 2 mm i.d.) packed with 1.5% OV-17 on Chromosorb W (80–100 mesh) was used. The helium gas flow rate was 20 ml/min. The temperatures of the injection port, column, separator and ion source were 250, 220, 270 and 270 $^{\circ}$ C, respectively. The ionizing energy was 200 eV, the trap current 300 μ A and the accelerating voltage 3 kV.

Correction of Ion Intensity Correction of ion intensity is required to remove the contributions of the quasi-molecular (QM)-3 ion (m/z 384) of the 13 C-glucose derivative (S-6) to the QM ion (m/z 384) of the $[^2\text{H}_3]$ -glucose derivative (S-3), and of the QM + 3 ion (m/z 387) of S-3 to the QM ion (m/z 387) of S-6 (Fig. 1). The QM + 3 ion (m/z 384) of the D-glucose derivative (S-0) did not contribute to the QM ion of S-3. The observed ratio (K) of ion intensity at m/z 387 to that at m/z 384 will be expressed as $K = (a \times A + B)/(A + b \times B)$, where A and B are the amounts of $[^2\text{H}_3]$ -D-glucose and $[\text{U-}^{13}\text{C}]$ -D-glucose in the sample, and a and b are the relative intensities of the QM + 3 ion (m/z 387) to the QM ion (m/z 384) of S-3 and of the QM – 3 ion (m/z 384) to the QM ion (m/z 387) of S-6, respectively. The ratio of $[\text{U-}^{13}\text{C}]$ -D-glucose to $[^2\text{H}_3]$ -D-glucose (B/A) was determined from the equation: $B/A = (K - a)/(1 - b \times K)$. The value of a was estimated to be 0.011 from the CI mass spectrum of the $[^2\text{H}_3]$ -D-glucose derivative (Fig. 1, S-3), and the values of b were estimated to be 0.0539 and 0.433 from those of the derivatives of $[\text{U-}^{13}\text{C}]$ -D-glucose (Fig. 1, S-6) and the hydrolyzed product of labeled starch (Fig. 1, S- $^{13}\text{C}_{0-6}$), respectively.

The $\Sigma\%$ of ion intensity at m/z 387 to total intensities of the ions at the surrounded QM ion was 46.1% in S-6, while in S- $^{13}\text{C}_{0-6}$ it was 17.1%. The same amount of $[\text{U-}^{13}\text{C}]$ -D-glucose and the labeled glucose originating from labeled starch in plasma gave the same total intensity of the ions in the range of m/z 378 to m/z 389, but the ratio of the ion intensity at m/z 387 in S- $^{13}\text{C}_{0-6}$ to that in S-6 was 0.371 (17.1/46.1). Therefore, the plasma concentration of labeled glucose was estimated from the equation:

$$B/A = (K - 0.011)/(1 - 0.433 \times K)/0.371$$

RESULTS AND DISCUSSION

Stable isotope tracers have been widely applied in the kinetic studies of endogenous materials.⁶⁾ Especially, ^{13}C -labeled compounds have been used as tracers in isotopic studies of glucose,⁷⁾ because ^{13}C -glucose has demonstrated no kinetic isotope effect in metabolic processes. Highly sensitive detection of labeled glucose with GC-MS requires the presence of a sharp peak in GC and of a high intensity ion which holds all labeled isotopes in MS.

D-Glucose has been subjected to GC analysis using various derivatives. Methylation, acetylation and tri-

methylsilylation of D-glucose are suitable methods for detection with GC, but these derivations give complex peaks in GC by anomerization. A solution to the problem of the formation of multiple peaks is to reduce D-glucose to D-sorbitol. It has been reported⁸⁾ that the butylboronate of D-sorbitol showed a sharp peak in GC. D-Glucose yielded the same alditol upon reduction, whereas D-glucose is negligible in normal plasma.

D-Glucose, $[^2\text{H}_3]$ -D-glucose and $[\text{U-}^{13}\text{C}]$ -D-glucose were reduced with sodium borohydride and then derived with butylboronic acid to cyclic butylboronic ethers of D-sorbitol (S-0), $[^2\text{H}_3]$ -D-sorbitol (S-3) and $[\text{U-}^{13}\text{C}]$ -D-sorbitol (S-6), respectively. These derivatives showed a single peak in GC. The derivative is estimated to form three five-membered rings in a molecule by electron impact (EI) mass spectrum; however, no suitable ions for a target were found in this mode.

The CI mass spectra of the derivatives are shown in Fig. 1. High intensity QM ions were observed at m/z 381 ($\Sigma\%$ 47.2) in S-0, at m/z 384 ($\Sigma\%$ 40.0) in S-3, and at m/z 387 ($\Sigma\%$ 46.1) in S-6. These QM ions were suitable as target ions for the determination of glucose. The derivative of the hydrolyzed product of labeled starch was observed as the mixture of ^{13}C -isotopomers and S-0 in S- $^{13}\text{C}_{0-6}$. The $\Sigma\%$ of the ion at m/z 387 was 17.1%.

The concentration of $[\text{U-}^{13}\text{C}]$ -D-glucose in plasma was determined by isotope dilution analysis using $[^2\text{H}_3]$ -D-glucose as the internal standard. An added amount of $[^2\text{H}_3]$ -D-glucose was fixed at 20 μg more than the content of ^{13}C -labeled glucose in a sample to decrease relatively the contribution of the ^{13}C -isotopomer to the ion at m/z 384. The calibration curve for $[\text{U-}^{13}\text{C}]$ -D-glucose (2–14 μg) and $[^2\text{H}_3]$ -D-glucose (20 μg) were derived, and the peak height of the ions at m/z 384 and 387 were measured. The corrected ratio of ion intensity (m/z 387/ m/z 384) and weight ratio ($[\text{U-}^{13}\text{C}]$ -D-glucose/ $[^2\text{H}_3]$ -D-

Fig. 1. CI Mass Spectra of Cyclic Butylboronic Ether of D-Sorbitol (S-0), $[^2\text{H}_3]$ -D-Sorbitol (S-3), $[\text{U-}^{13}\text{C}]$ -D-Sorbitol (S-6) and Labeled Sorbitol (S- $^{13}\text{C}_{0-6}$) as Reduced Product from D-Glucose, $[^2\text{H}_3]$ -D-Glucose, $[\text{U-}^{13}\text{C}]$ -D-Glucose and Labeled Glucose Obtained from Labeled Starch by Acid Hydrolysis

TABLE I. Precision for the Determination of [U-¹³C]-D-Glucose Spiked in Plasma

Sample	Added amount (μg to 100 μl)	Concentration found ($\mu\text{g/ml}$) Mean $\pm$ S.D. (n=3)	C.V. (%)
1	2.00	20.1 $\pm$ 0.1	0.5
2	4.00	39.9 $\pm$ 0.3	0.8
3	6.00	57.8 $\pm$ 3.7	6.4
4	8.00	79.1 $\pm$ 0.4	0.5
5	10.00	98.6 $\pm$ 1.1	1.1
6	12.00	118 $\pm$ 0.5	0.4
7	14.00	135 $\pm$ 2.7	2.0

Fig. 2. Plasma Concentration of the Glucose Originated from Labeled Starch after the Oral Administration of Labeled Starch (200 mg/kg) without (○) and with 0.75 mg/kg (▲), 1.5 mg/kg (●) or 3.0 mg/kg (△) of Acarbose

The concentration of the glucose was determined from the corrected ratio of ion intensity (m/z 387/ m/z 384) using 100 μl of plasma. Values are presented as an average $\pm$ S.E. of three rats for each point. a) $p < 0.01$, b) $p < 0.005$, when compared with the control. c) $p < 0.05$, d) $p < 0.02$, e) $p < 0.005$, when compared with the coadministration of acarbose (0.75 mg/kg).

glucose) indicated sufficient linearity, and the correlation coefficient obtained by regression analysis was 0.999. After the addition of known amounts of [U-¹³C]-D-glucose (2–14 μg) to 0.1 ml of rat control plasma, the plasma sample was treated successively for defatting, deproteinizing and desalting. The concentrations of [U-¹³C]-D-glucose thus found are summarized in Table I. The coefficients of variation (C.V.) in this method were within 6.4% in all samples.

The plasma concentrations of labeled glucose after the oral administration of labeled starch with or without acarbose are shown in Fig. 2. The effect of acarbose on the digestion and absorption of starch is clearly indicated. A maximum level of labeled glucose was observed within 10 min in the case of a single administration of labeled starch, while over 120 min was required in the case of the

co-administration of acarbose at a dose of 3.0 mg/kg. In all cases of dosing with acarbose, the initial increase of labeled glucose in plasma was prevented.

The area under the concentration time curve (*AUC*) was assessed from the concentration of labeled glucose in plasma after dosing of labeled starch by the trapezoidal rule. The co-administration of acarbose causes a significant decrease in the *AUC* at a high dose. The *AUC* from 0 to 120 min after administration of acarbose at doses of 1.5 and 3.0 mg/kg were 49.1 and 52.5%, respectively, compared to that of the control case. These observations indicated that the absorption amount of glucose was reduced to about one-half by acarbose during this period. The decrease in the *AUC* might be caused by the inhibition of acarbose on the digestion process of starch. Acarbose is scarcely absorbed in an unchanged form and is therefore restricted to exerting its activity within the intestinal wall.¹⁾ This fact suggests that the elimination rate of glucose is not varied by the administration of acarbose. In our radio tracer experiment, radioactivity in the small intestine after the oral administration of ¹⁴C-labeled starch was increased more than two-fold by the co-administration of acarbose at 1 h after dosing (unpublished data).

Azuma *et al.*⁹⁾ reported that the inhibitory effects of acarbose on the *AUC* of plasma glucose were dose-dependent in high correlation with the dosing amount of acarbose (50–200 mg/body) after the administration of sucrose in man. In our experiment, the *AUC* from 0 to 120 min after the co-administration of acarbose at a dose of 1.5 mg/kg was almost the same as that at a dose of 3.0 mg/kg. This difference in dose dependency may be caused by a difference in the carbohydrates used, the experimental subject or the evaluating method.

It has been reported¹⁰⁾ that isotopic randomization occurs in the liver by a gluconeogenic pathway. This glucose recycling forms the ¹³C₂-species and ¹³C₃-species of labeled glucose, and these species give the ion at m/z 384 in MS; therefore, the reliability of this method might be easily affected. The formation of ¹³C₃-species from ¹³C₆-glucose was not observed in the ratio of ion intensity (m/z 384/ m/z 387) in plasma samples after the oral administration of [U-¹³C]-D-glucose to rats. From this observation, the plasma concentration of labeled glucose which was obtained by this method was not significantly affected by the glucose recycling process.

The present study reveals that acarbose has remarkable effects on the digestion and absorption of starch in rats. Further investigation is required to reveal the effect of acarbose in man utilizing the advantages of the stable isotope tracer.

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