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Occurrence of Orally Administered Mulberry 1-Deoxynojirimycin in Rat Plasma

KIYOTAKA NAKAGAWA,[†] HIROYUKI KUBOTA,[†] TOSHIYUKI KIMURA,[†,‡] SHINJI YAMASHITA,[†] TSUYOSHI TSUZUKI,[†,§] SHINICHI OIKAWA,[||] AND TERUO MIYAZAWA*,[†]

Food and Biodynamic Chemistry Laboratory, Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan, National Agricultural Research Center for the Tohoku Region, Fukushima 960-2156, Japan, Department of Food Management, School of Food, Agricultural and Environmental Sciences, Miyagi University, Sendai 982-0215, Japan, and Department of Medicine, Nippon Medical School, Tokyo 113-8603, Japan

1-Deoxynojirimycin (DNJ), a potent glucosidase inhibitor, is a characteristic constituent of the mulberry leaf. Dietary mulberry DNJ may be beneficial for the suppression of abnormally high blood glucose levels, thereby preventing diabetes mellitus. Although there is considerable interest in the effects of mulberry DNJ, the intestinal absorption and pharmacokinetic profile of orally administered mulberry DNJ have never been characterized. In this study, we developed a method for determining the level of plasma DNJ by hydrophilic interaction chromatography coupled to a mass spectrometric detector (HILIC–MS) to investigate the absorption and metabolism of orally administered mulberry DNJ in rats. DNJ was separated from plasma extract on a TSK gel Amide-80 column, a representative column for HILIC. At postcolumn, DNJ was concurrently detected and identified by MS. The plasma DNJ concentration in fasted rats was below the detection limit [<1 μg (6 nmol)/mL]; however, the concentration reached a maximum [15 μg (92 nmol)/mL] 30 min after the administration of mulberry DNJ (110 mg/kg of body weight), and the DNJ concentration decreased rapidly thereafter. When the rats received different amounts of mulberry DNJ (1.1, 11, and 110 mg/kg of body weight), dose-dependent incorporation of DNJ into the plasma was confirmed. We did not detect any DNJ metabolites in the plasma. These findings indicate that orally administered mulberry DNJ is absorbed as an intact form from the alimentary tract and then is quickly excreted from the body. The developed HILIC–MS method could be applied in determining levels of DNJ in urine and tissues, and therefore, the method would be a powerful tool for studying the metabolic fate of mulberry DNJ as well as its bioavailability.

KEYWORDS: 1-Deoxynojirimycin; HILIC–MS; mulberry; absorption; rat plasma

INTRODUCTION

There has been continued interest in aza sugars (also termed imino sugars) over the past three decades because of their high potency as glycosidase inhibitors (*1*). Many of them have great potential as drugs in the treatment of a variety of carbohydrate-mediated disorders such as diabetes and HIV/AIDS (*2*, *3*).

Among aza sugars, 1-deoxynojirimycin (DNJ) is a typical naturally occurring alkaloid with potent biological activity (i.e., the inhibition of intestinal α-glucosidase) (*2*, *3*). DNJ is a D-glucose analogue with an NH group substituting for the oxygen atom of the pyranose ring (**Figure 1**). Since DNJ is a characteristic constituent of mulberry (Moraceae) leaves that have been consumed as tea for more than 750 years in Japan, dietary mulberry DNJ has been hypothesized to be beneficial for the suppression of an abnormally high blood glucose level (*4*–*6*). To evaluate the hypothesis, we recently conducted a human study and confirmed that the administration of mulberry DNJ to humans suppressed the increase in the level of postprandial blood glucose (*7*). Therefore, mulberry DNJ is feasible for therapeutic use in oral treatment of non-insulin-dependent diabetes mellitus (type 2 diabetes).

Although there is considerable interest in the beneficial effects of mulberry DNJ, the absorption and metabolism of DNJ have never been characterized. Faber et al. (*8*) studied the pharmacokinetics of 1-deoxymannojirimycin (DMJ; structurally related to DNJ) after intravenous administration of radiolabeled DMJ to rats. These authors found that 120 min after administration, 52% of the DMJ dose was detected in unchanged form in urine,

* To whom correspondence should be addressed. Telephone: +81-22-717-8904. Fax: +81-22-717-8905. E-mail: miyazawa@biochem.tohoku.ac.jp.
[†] Tohoku University.
[‡] National Agricultural Research Center for the Tohoku Region.
[§] Miyagi University.
[||] Nippon Medical School.

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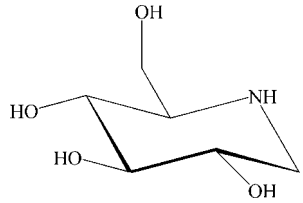

Figure 1. Chemical structure of 1-deoxynojirimycin (DNJ).

whereas only 4.9% of the dose was excreted into bile. At that time, a small amount of DMJ was detected in the liver (2.1%), kidney (1.1%), small intestine (0.9%), stomach (0.6%), and heart (0.1%). These results indicate that aza sugars may be rapidly eliminated from the body in an intact form by renal excretion. However, experiments focusing on the metabolic fate of DNJ have never been carried out. The absorption rate of DNJ in animals and humans is therefore yet to be determined.

The main goal of this study was to evaluate the absorption and metabolic fate of orally ingested mulberry DNJ. To determine the pharmacokinetic profile of mulberry DNJ orally administered to rats, we developed a method for investigating plasma DNJ by hydrophilic interaction chromatography coupled to a mass spectrometric detector (HILIC–MS). In addition, we applied the developed HILIC–MS method for DNJ analysis in urine and tissues.

MATERIALS AND METHODS

Chemicals. Standard DNJ, acetonitrile, distilled water, and ammonium formate were purchased from Wako (Osaka, Japan). All other reagents used were analytical grade.

HILIC–MS. The HILIC–MS system consisted of a Jasco (Tokyo, Japan) PU980 intelligent HPLC pump, a Jasco CO-965 column oven, and a Reodyne (Cotati, CA) 7125 injector. A TSK gel Amide-80 column (4.6 mm × 250 mm; Tosoh, Tokyo, Japan) was used as a HILIC column (*9*). The mobile phase was a mixture of acetonitrile and distilled water (72:28, v/v) containing 6.5 mM ammonium formate (pH 5.5). The flow rate was adjusted to 1.0 mL/min, and the column temperature was maintained at 40 °C. The eluent was split at postcolumn. One of the split eluents (flow rate of 0.01 mL/min) was sent to a Mariner electrospray ionization time-of-flight mass spectrometer (MS) (Applied Biosystems, Foster City, CA). MS was carried out in a positive ion measurement mode with a spray voltage of 3500 V, a nozzle potential of 100 V, and a nozzle temperature of 140 °C. The flow rate of the nebulizer gas was 0.3 mL/min. Full-scan spectra were recorded by scanning masses between *m*/*z* 100 and 400 at a rate of 3 s/scan. The other split eluent (flow rate of 0.99 mL/min) was discarded.

Experimental Design for Oral Administration. DNJ was extracted from mulberry leaves (*Morus alba*) and purified using ion-exchange chromatography followed by recrystallization (*10*). The purity of the obtained mulberry DNJ was >95%.

This study was conducted in conformity with policies and procedures detailed in the Animal Experiment Guidelines of Tohoku University. Male Sprague-Dawley rats (8 weeks old; SLC, Hamamatsu, Japan) weighing 260–290 g were housed in a room with a controlled temperature (23 ± 1 °C) and light (from 08:00 to 20:00), with free access to distilled water and MF Standard Rodent Chow (Oriental Yeast, Tokyo, Japan). After 3 days of feeding, food was withheld for 12 h, and mulberry DNJ (110 mg/kg of body weight) dissolved in water was orally administered by direct stomach intubation. Before (control) and 0.5, 1, 2, 4, and 8 h after the administration, blood was collected by decapitation, with each blood sample collected in a tube containing EDTA as an anticoagulant. Plasma was immediately prepared by centrifugation at 1500*g* for 15 min at 4 °C and was stored at −30 °C until use. Similarly, different amounts of mulberry DNJ (1.1, 11, and 110 mg/kg of body weight) were orally administered to evaluate the dose-dependent incorporation of mulberry DNJ into rats. Plasma was prepared from the blood obtained 0.5 h after DNJ administration and was then stored at −30 °C.

To evaluate DNJ concentration in urine and tissues, mulberry DNJ (110 mg/kg of body weight) was orally administered. Twenty-four hours after DNJ administration, tissues (liver, kidney, pancreas, and spleen), small intestine contents, and large intestine contents were corrected. Urine was also collected during time periods 0–24 h after administration.

DNJ Analysis. The plasma (500 μL) was lyophilized for 24 h. DNJ was extracted from the lyophilized plasma with 1 mL of mobile phase [a mixture of acetonitrile and distilled water (72:28, v/v) containing 6.5 mM ammonium formate (pH 5.5)] in a test tube by vortexing for 1 min and sonicating for 5 min. The mixture was centrifuged at 1500*g* for 10 min at 4 °C. The supernatant was filtered through a PTFE filter (0.45 μm pore size; Sartorius), and the resulting extract was dried by a nitrogen stream. The extract was redissolved in 120 μL of mobile phase, and a portion (100 μL) of the aliquot was then injected into the HILIC–MS system to determine the plasma DNJ level. Similarly, tissue (500 mg) or urine (1 mL) was lyophilized and mixed with 1 mL of mobile phase, and the supernatant was then analyzed by HILIC–MS.

RESULTS

HILIC–MS Analysis of Standard DNJ and Rat Plasma Extracts. Typical HILIC–MS chromatograms of standard DNJ are presented in **Figure 2**. A defined peak ascribed to DNJ was detected at a retention time of 13 min in both the total ion current (TIC) chromatogram (**Figure 2A**) and the single-ion monitoring (SIM) of the mass corresponding to the $[M + H]^+$ ion of DNJ (**Figure 2B**). This peak component (13 min) was identical to DNJ on the basis of its MS spectrum (**Figure 2C**). The SIM detection limit of DNJ was 500 pg (3.1 pmol) at a signal-to-noise ratio of 3.

Typical SIM chromatograms of rat plasma extracts are presented in **Figure 3**. Although there were several small peaks ascribed to background contaminants in the plasma extract taken before administration of mulberry DNJ, DNJ itself was not detected (**Figure 3A**). Thirty minutes after oral DNJ administration (110 mg/kg of body weight), a clear peak ascribed to DNJ (13 min) was observed in the plasma extract (**Figure 3B**). This peak was identified as DNJ on the basis of the MS profile (**Figure 3C**). We next searched for plausible DNJ metabolites (e.g., dehydrated, nitrogen-oxidized, carbon-oxidized, alkylated, and acetylated DNJ) in the plasma. On the basis of the molecular weights of each plausible metabolite, HLICL–MS with SIM analysis was performed. However, we did not detect any DNJ metabolites in the plasma (data not shown). These findings indicate that orally administered mulberry DNJ is absorbed as an intact form from the alimentary tract of a rat.

Determination of the Plasma DNJ Level. Before making the determination, we checked the recovery of DNJ from rat plasma during the extraction process. In the recovery rate test, standard DNJ (1–11 μg) was spiked into the control plasma (500 μL of DNJ-noningested rat plasma). The DNJ-spiked plasma was lyophilized and mixed with a mobile phase [a mixture of acetonitrile and distilled water (72:28, v/v) containing 6.5 mM ammonium formate (pH 5.5)]. The supernatant was then analyzed using HILIC–MS (see DNJ Analysis). However, the recovery rate of DNJ was found to be less than 50%. Therefore, to obtain a better quantitative result, plasma DNJ level determination was carried out using the following method instead of the absolute-calibration method. We divided the plasma sample into seven portions (each 500 μL), and known amounts (1, 3, 5, 7, 9, and 11 μg) of standard DNJ were added into six of the portions. The seven samples (six DNJ-spiked plasmas and one original plasma not treated with DNJ) were then analyzed using HILIC–MS. A calibration curve for DNJ was made on the basis of the difference between the peak areas of DNJ-spiked

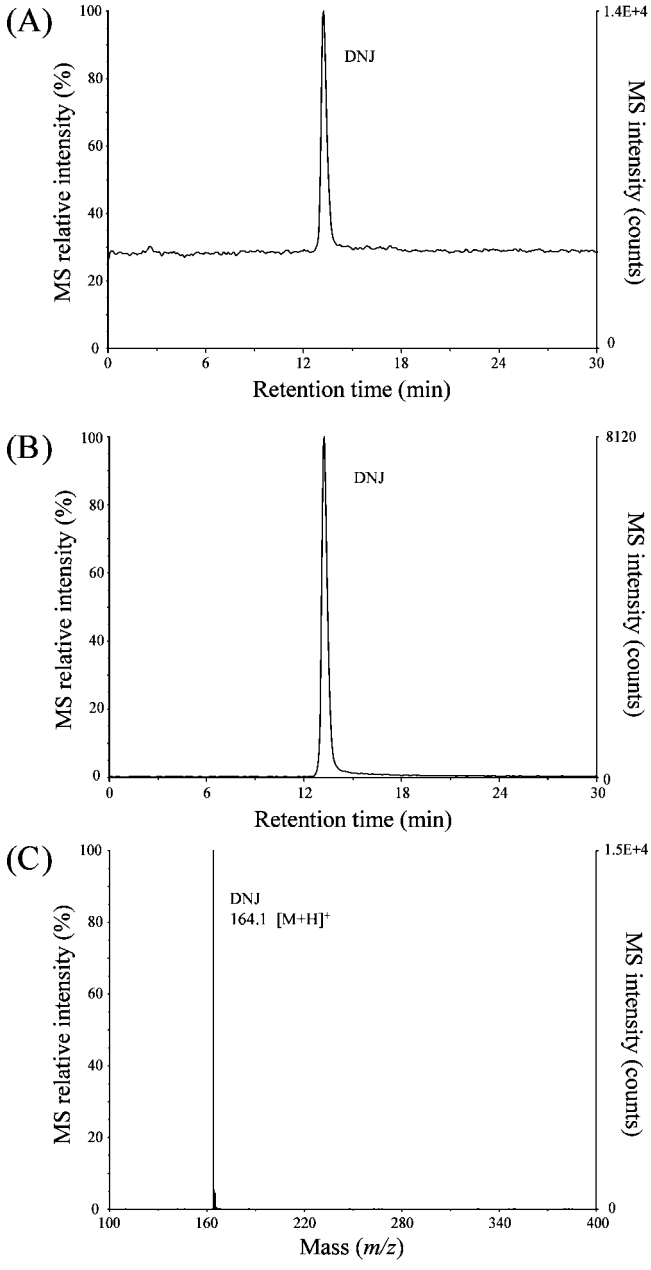

Figure 2. HILIC–MS analysis of standard 1-deoxynojirimycin (DNJ). (**A**) Total ion current (TIC) chromatogram. (**B**) Single-ion monitoring (SIM) of the mass corresponding to the $[M + H]^+$ ion of DNJ (*m/z* 164.1). (**C**) MS spectrum of the peak detected at 13 min in chromatogram B. Standard DNJ (100 ng) was analyzed using HILIC–MS.

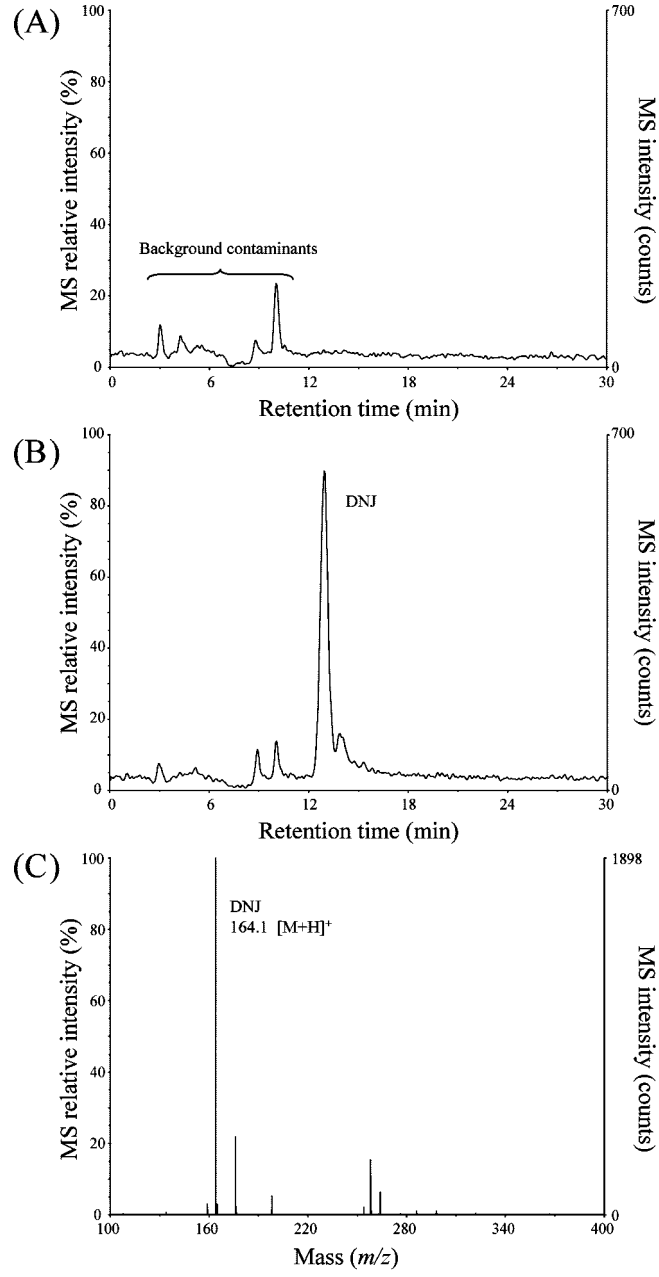

Figure 3. HILIC–MS analysis of rat plasma extracts before (**A**) and after (**B**) oral administration of mulberry 1-deoxynojirimycin (DNJ). Rat plasma extract before and 30 min after oral administration of mulberry DNJ (110 mg/kg of body weight) was analyzed using HILIC–MS with single-ion monitoring (SIM) detection (*m/z* 164.1). (**C**) MS spectrum of a peak detected at 13 min in chromatogram B.

plasma and original plasma not treated with DNJ (**Figure 4**). The DNJ concentration in the original plasma sample was calculated using this calibration curve.

Metabolic Fate of Mulberry DNJ. Figure 5A plots the time course of DNJ concentration in rat plasma after the oral administration of mulberry DNJ. The concentration of DNJ in the plasma increased to its highest level [15 μg (92 nmol)/mL] 30 min after the administration (110 mg/kg of body weight) and decreased rapidly thereafter. Plasma DNJ concentrations 4–8 h after administration were below the detection limit [<1 μg (6 nmol)/mL]. When rats received different amounts of mulberry DNJ (1.1, 11, and 110 mg/kg of body weight), dose-dependent incorporation of DNJ into plasma was confirmed (**Figure 5B**). On the other hand, the high reproducibility of the HILIC–MS method for determining the plasma DNJ concentration (coefficient of variability of <5%) was confirmed and was not altered by storage of plasma samples at −80 °C for 1–4 weeks.

Finally, we investigated DNJ in urine and tissues and found that 24 h after DNJ administration (110 mg/kg of body weight), DNJ was detected in unchanged form in urine (2% of the ingested DNJ), as well as large intestine contents (7%) and small intestine contents (1%). In contrast, DNJ concentrations in liver, kidney, pancreas, and spleen were trace levels [<1 μg (6 nmol)/g]. The results suggested that a small amount of orally administered mulberry DNJ is absorbed and then rapidly excreted into urine.

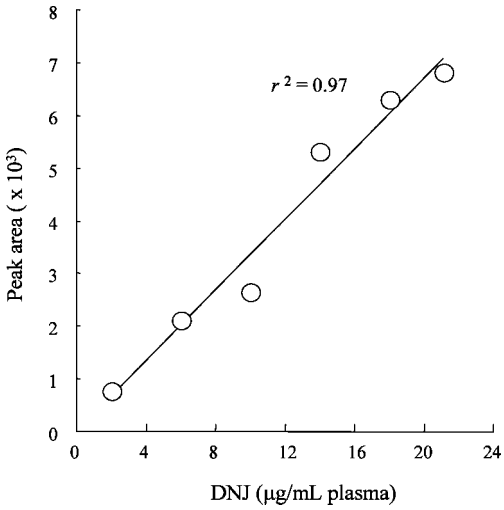

Figure 4. Calibration curve of 1-deoxynojirimycin (DNJ) for determination of its plasma concentration.

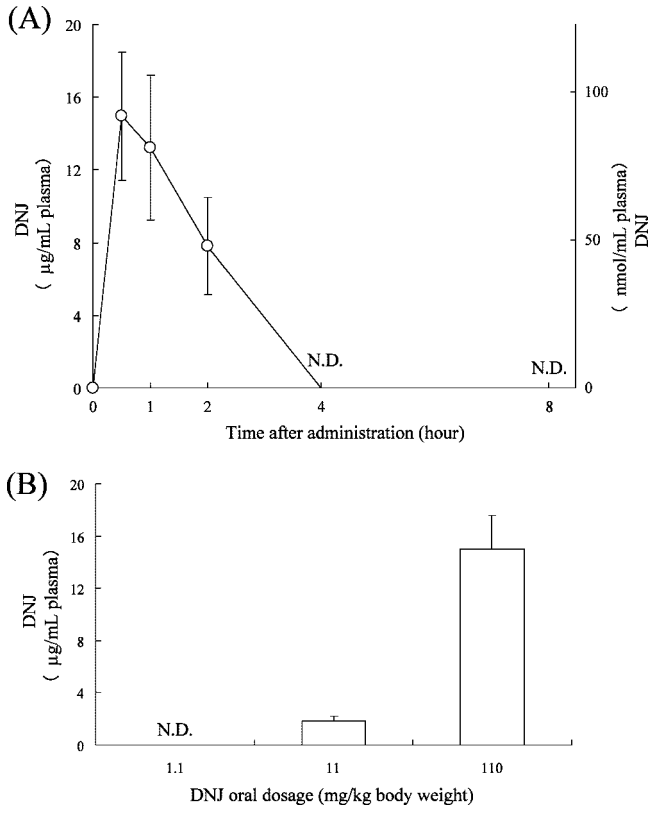

Figure 5. Concentration of 1-deoxynojirimycin (DNJ) in rat plasma after a single oral administration of mulberry DNJ. (**A**) Time course changes in the DNJ concentration in the plasma of rats receiving mulberry DNJ (110 mg/kg of body weight). (**B**) Dose-dependent incorporation of DNJ into the plasma 30 min after the administration of mulberry DNJ (1.1, 11, and 110 mg/kg of body weight). Values are means ± the standard deviation (n = 3–4). N.D., below the detection limit [<1 μg (6 nmol)/mL].

DISCUSSION

To evaluate the metabolic fate of mulberry DNJ, it is important to develop a sensitive and selective method. In particular, since DNJ is a highly polar compound, the selection of the analytical column significantly impacts the DNJ analysis (*9*). Recently, HILIC has been developed as an efficient tool for analyzing highly polar compounds, and its analytical application to carbohydrates (*11*) and peptides (*12*) has been reported. As Tolstikov and Fiehn described (*13*), the retention ability of the HILIC column basically depends on the hydrophilicity of the analytes, and if the compound has amino groups, its retention time is prone to elongation. Therefore, a HILIC column (TSK gel Amide-80) might be a preferable column for the efficient separation of DNJ. As we had expected, when standard DNJ was subjected to HILIC coupled to MS, DNJ was detected at a suitable retention time (13 min) with high sensitivity (**Figure 2**). The characteristics and advantages of our HILIC–MS method can be explained as follows. The method was satisfactorily selective and sensitive for measuring the level of DNJ present in rat plasma (**Figure 3**). Also, the method was sufficiently simple and convenient to be applied to a large number of samples. Therefore, the HILIC–MS method would be a useful tool for studying the metabolic fate of mulberry DNJ as well as its bioavailability.

We also developed a simplified method for extracting DNJ from plasma. Lyophilized plasma was mixed with a mobile phase, and the supernatant was then analyzed using HILIC–MS. However, the recovery rate of DNJ was found to be less than 50%. In this study, plasma DNJ concentrations were therefore determined using the standard DNJ addition method (see Determination of the Plasma DNJ Level) (**Figure 4**). This method enabled a quantitative analysis of plasma DNJ, but a more effective extraction procedure using an internal standard needs to be developed in the future. On the other hand, as seen in **Figures 3** and **5**, HILIC–MS with SIM is highly useful for determining plasma DNJ levels, even though the detailed structural information about the DNJ (i.e., NH group and hydroxyl moiety) was not obtained. To overcome this problem, we are currently improving our DNJ analyses by using HILIC coupled to tandem mass spectrometry (HILIC–MS/MS).

In this study, the level of DNJ was shown to be below the detection limit [<1 μg (6 nmol)/mL] in plasma samples from fasted rats. This is due to the absence of DNJ in the rat diet. After oral administration of mulberry DNJ (1.1, 11, and 110 mg/kg of body weight), DNJ was dose-dependently absorbed from the digestive tract into rat plasma (**Figures 3** and **5**). Plasma DNJ levels reached a maximum 30 min after oral intake and then rapidly decreased (**Figure 5**). The results suggest that in the pharmacokinetics of absorption, DNJ has a short half-life. As calculated from the total area under the concentration–time curve (AUC) of DNJ, 1% of the ingested DNJ was incorporated into the rat plasma. When compared other those of aza sugars with DNJ, the plasma concentration of DNJ (**Figure 5**) was slightly lower than that of miglitol (*14*). In recent years, a number of pharmacokinetic studies of plant phenolic compounds, mainly flavonoids, have been conducted to investigate their numerous physiological functions. The maximum concentration of DNJ (**Figure 5**) is higher than that of anthocyanin (*15*), catechin (*16, 17*), luteolin (*18*), genistein (*19*), or curcumin (*20*). The bioactivity of DNJ in the digestive tract (α-glucosidase inhibition) has been a focus and has been investigated thoroughly, but little attention has been paid to the effects of DNJ within the body. Iizuka et al. (*21*) studied the antidiabetic effect of mulberry DNJ using GK rat (model of non-insulin-dependent diabetes mellitus) and revealed its unique properties, increasing insulin sensitivity and improving insulin resistance. Considering the results of the animal study (*21*) as well as the plasma concentration of DNJ (**Figure 5**), it is likely that DNJ may act as a bioactive compound within the body (e.g., affecting secretion of insulin from pancreatic β-cells).

As for the metabolism of mulberry DNJ, there has been speculation that bacterial digestion of DNJ by the gastrointestinal system may occur before absorption. Also, it is likely that DNJ

is metabolized by drug-metabolizing enzymes in the liver, such as cytochrome P450s. For this study, DNJ would be considered a metabolically stable compound since the degradation products derived from DNJ as well as its metabolites (e.g., oxidized and alkylated products) were not detected in plasma. However, there is the possibility that a small portion of the ingested DNJ could be degraded or metabolized in an amount too small to be identified with the present HILIC–MS analysis [e.g., a small unknown peak appeared after DNJ at 13.3 min, (**Figure 3B**)]. Further investigation will be required to assess this possibility.

The HILIC–MS method presented here was applicable for determination of the level of DNJ in urine and tissues. On the basis of these findings, we speculate about one possible metabolic fate of mulberry DNJ. A small amount of orally administered mulberry DNJ is rapidly absorbed from the intestine into the portal vein. Since DNJ is structurally similar to glucose, the intestinal absorption of DNJ may be regulated via a glucose transporter (*22*). The DNJ incorporated into the bloodstream is quickly excreted into the urine. Because DNJ has a relatively short half-life (**Figure 5**) and we could not detect a substantial amount of DNJ in tissues, the tissue distribution and accumulation of DNJ may not be particularly high.

As for the bioactivity of aza sugars, including DNJ, these compounds are an important class of glycosidase inhibitors and are receiving considerable attention as potential therapeutic agents (i.e., as antidiabetics, antiobesities, and antivirals) (*1–3*). Among imino sugars, miglitol (glyset) has been approved as a drug (a second-generation α-glucosidase inhibitor) for treating type 2 diabetes (*23*). *N*-Butyl-DNJ (zavesca) has also been used as a drug for patients with type 1 Gaucher disease (*24*). Despite DNJ's excellent α-glucosidase inhibitory activity in vitro, its efficacy in vivo was reported to be rather moderate (*25*). We therefore consider DNJ suitable for use as a "functional food" instead of as a drug. For this reason, we recently produced a food-grade mulberry powder enriched with DNJ (*7*). We then conducted a human study, confirming that administration of the DNJ-enriched powder to humans suppressed the rise of postprandial blood glucose (*7*). Considering the result presented here (**Figure 5**), the ingested DNJ would be rapidly eliminated from the body in an intact form and therefore may lack side effects. Hence, DNJ-enriched powder might be therapeutically used as an effective safety food in the treatment of non-insulin-dependent diabetes mellitus (type 2 diabetes). This possibility is currently being investigated in clinical studies, to obtain its approval for "Food for Specified Health Use (FOSHU)" status in Japan.

In conclusion, although there have been several reports about the absorption and metabolic fate of aza sugars in animals (*8, 14*), they have not provided any information about the intestinal absorption, metabolites, and pharmacokinetic profiles of orally administered mulberry DNJ. In this study, we used the developed HILIC–MS system to demonstrate that orally administered mulberry DNJ is absorbed as an intact form from the alimentary tract of a rat and then rapidly excreted from the body.

ABBREVIATIONS USED

AUC, total area under the concentration–time curve; DMJ, 1-deoxymannojirimycin; DNJ, 1-deoxynojirymycin; EDTA, ethylenediaminetetraacetic acid; HILIC, hydrophilic interaction chromatography; MS, mass spectrometer; MS/MS, tandem mass spectrometry; PTFE, polytetrafluoroethylene; SIM, single-ion monitoring; TIC, total ion current chromatogram.

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