JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of 1-Deoxynojirimycin in Mulberry Leaves Using Hydrophilic Interaction Chromatography with Evaporative Light Scattering Detection

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A simple and rapid method for determining 1-deoxynojirimycin (DNJ), a potent glucosidase inihibitor present in mulberry leaves (*Morus alba* and *Morus bombysis*), by high-performance liquid chromatography coupled to an evaporative light scattering detector (ELSD) has been developed. DNJ was separated from an extract of mulberry leaves on a TSKgel Amide-80 column, which is a representative column for hydrophilic interaction chromatography. During postcolumn detection, DNJ was detected by ELSD and concurrently identified by mass spectrometry. The detection limit was 100 ng. This method is sufficiently sensitive for determining DNJ in mulberry leaves and other related products.

KEYWORDS: 1-Deoxynojirimycin; evaporative light scattering detector; mulberry leaves; *Morus alba*

INTRODUCTION

In the past three decades, there has been a continued interest in aza-sugars (also termed imino sugars) because of their higher potency as glycosidase inhibitors (*1*−*4*). Many of them have great potential as drugs in the treatment of a variety of carbohydrate-mediated disorders such as diabetes, cancer, viral diseases (i.e., HIV, HBV, and influenza virus), and glycosphingolipid storage diseases (for reviews, see references *2*−*7*).

Among aza-sugars, 1-deoxynojirimycin (DNJ) is a typical naturally occurring alkaloid (*8*−*10*), with promising biological activity in vivo (i.e., inhibition of intestinal α-glucosidase and α-amylase) (*1*, *11*, *12*). The compound is a D-glucose analogue with an NH group substituting for the oxygen atom of the pyranose ring (**Figure 1**). In 1976, DNJ was isolated from the root bark of the mulberry tree and named moranoline by Yagi et al. (*13*). This was the initial isolation of DNJ from nature, which prompted the concept that dietary mulberry intake might be beneficial for the suppression of blood glucose levels, thereby preventing diabetes. To date, several animal studies supporting this concept have been published, and particularly interesting are the reports by Nojima and his co-workers (*11*, *14*), who clearly showed that the administration of mulberry leaf extract repaired glucose metabolism and hyperglycemic conditions in streptozotocin-induced diabetic mice.

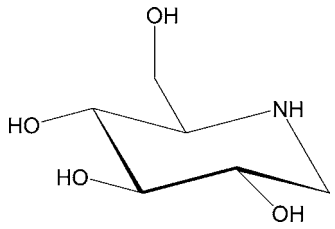

Figure 1. Chemical strucuture of 1-deoxynojirimycin.

Against this background, various food products containing mulberry leaves have recently been manufactured and commercially supplied as functional foods in Japan and other countries (*15*). They have shown the bioavailability of DNJ (*15*); nevertheless, we could not find any products that display the DNJ amount, which is presumably due, in part, to the lack of a suitable method for determining DNJ. Therefore, in this study, we focused on establishing a rapid and convenient quantitative method for determining the DNJ present in mulberry leaves and other related products and discussed the efficient separation of DNJ with hydrophilic interaction chromatography (HILIC).

MATERIALS AND METHODS

Chemicals and Solvents. DNJ was purchased from Wako (Osaka, Japan) and used as a reference standard in determination experiments. Acetonitrile and distilled water were obtained from Kanto (Tokyo,

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10.1021/jf0306901 CCC: $27.50
Published on Web 02/19/2004

Japan). Ammonium acetate was from Sigma (St. Louis, MO). All other reagents used were of analytical grade.

Plant Materials. Mulberry leaves (*Morus alba* Kinuyutaka and Kairyonezumigaeshi and *Morus bombysis* Shinkenmochi) were harvested from field trees grown at the Fukushima agricultural experiment station (Fukushima, Japan) in June 2002. The leaves were cleaned with water, frozen at −40 °C, and lyophilized for 48 h. Dried leaves were disintegrated and then stored at −4 °C until DNJ analysis.

Instrumentation and Chromatography. The HPLC system consisted of a Shimadzu LC-10AD pump (Tokyo, Japan), a Shimadzu DGU-14A degasser, a Shimadzu CTO-10A column oven, and a Reodyne 7125 injector (Cotati, CA) having a 100 μL sample loop. A TSKgel Amide-80 column (4.6 × 250 mm; Tosoh, Tokyo, Japan) was used. The separation was performed using a mixture of acetonitrile and distilled water (81:19, v/v; containing 6.5 mM ammonium acetate; pH 5.5). The flow rate was adjusted to 1 mL/min, and the column temperature was maintained at 40 °C. The eluent was split at the postcolumn. One of the split eluents (flow rate = 0.95 mL/min) was sent to a Sedex 55 evaporative light scattering detector (ELSD) (Sedere, Alfortville, France). ELSD conditions were optimized in order to achieve maximum sensitivity; the temperature of the drift tube was set at 55 °C, nitrogen gas was used as the nebulizing gas at a pressure of 1.2 bar, and the gain was usually set at 8. The peak areas were registered using a Shimadzu C-R6A Chromatopac integrator. The other split column eluent (flow rate = 0.05 mL/min) was sent to a Mariner electrospray ionization time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA). MS was carried out in the positive ion measurement mode with a spray voltage of 2900 V, a nozzle potential of 220 V, and a nozzle temperature of 140 °C. The flow rate of the nebulizer gas was 0.3 mL/min. Full-scan spectra were obtained by scanning masses between *m*/*z* 100 and 1000 amu at 3 s/scan.

Extraction and Quantification of DNJ. The DNJ standard was weighed accurately and dissolved in a mixture of acetonitrile and water (50:50; containing 6.5 mM ammonium acetate; pH 5.5), then prepared for a stock solution containing 5 mg/mL of DNJ, and stored at −4 °C until analysis. We have verified that the stock solution is stable for up to 3 months under these conditions. Before determining DNJ in mulberry leaves, we prepared dilutions containing 0.010, 0.032, 0.10, 0.32, 1.0, and 3.2 mg/mL of standard DNJ according to its expected levels in leaves. A 10 μL sample of these solutions (containing 100−3200 ng of DNJ) was subjected to HPLC-ELSD, and a calibration curve was made.

Mulberry DNJ was extracted from sample lyophilized leaves (0.1 g) with 1 mL of a mixture of acetonitrile and water (50:50; containing 6.5 mM ammonium acetate; pH 5.5) in a microtube by sonication for 1 min. The resulting suspension was centrifuged at 15000*g* for 5 min. The supernatant was filtered through a PTFE filter (0.45 μm pore size; Advantec, Tokyo, Japan), and a 10 μL aliquot was applied to the HPLC-ELSD/MS system. The DNJ concentration in leaves was calculated using the equation of the calibration curve. Determinations were performed within 3 months of leaf sampling. In contrast, mulberry leaf related products (i.e., tea leaves, tablets, and leaf powder) were purchased from the general market in Japan and subjected to DNJ determination. Leaf powder was used intact, and tea leaves and tablets were ground to powder and then used for analysis. The procedure of sample treatment and measurement was the same as that of the mulberry leaves described above.

RESULTS AND DISCUSSION

To determine DNJ by HPLC, we first attempted to seek the most appropriate column that would enable DNJ, which is a highly polar compound (**Figure 1**), to be retained. Ligand exchange and aminopropyl columns are commonly applied for HPLC determination of relatively polar compounds such as carbohydrates and water soluble vitamins (*16*). However, even though these classical columns were tested under various conditions, reasonable retention was not obtained for standard DNJ. This indicates that interactions between the stationary phase of these columns and DNJ were extensively weak. The same result was obtained when a reversed-phase column was used. Finally, assays were tried using HILIC, which has been developed as an efficient tool to analyze highly polar compounds, and its analytical application for carbohydrates (*17*) and peptide (*18*) has been reported. As Tolstikov and Fiehn described (*19*), the retention ability of this column basically depends on the hydrophilicity of analytes, and if the compound has amino groups, its retention time is prone to elongation. Hence, it seemed logical to anticipate that a TSKgel Amide-80 column might be a desirable column for efficient separation of DNJ. When standard DNJ (1000 ng) was subjected to HPLC coupled to MS using a TSKgel Amide-80 column, a well-defined peak ascribed to DNJ was detected at a suitable retention time (24.5 min) in the total ion current chromatogram. This peak component gave a molecular ion $[M + H]^+$ at *m*/*z* 164.1 amu, which corresponds to DNJ.

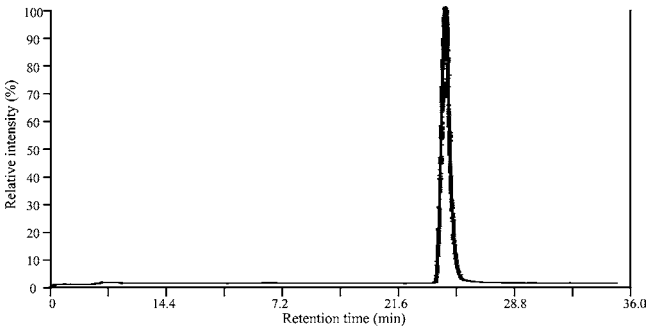

Figure 2. HPLC-ELSD chromatogram of standard 1-deoxynojirimycin (DNJ).

As mentioned above, we succeeded in analyzing DNJ by using an amide column equipped with a MS detector. However, the MS instrument tends to be rather complicated, expensive, time-consuming, and occasionally less reproducible, and it is therefore difficult to use for routine analysis. DNJ has no chromophore in its molecule, and this causes difficulties with UV and fluorescence detection. As an alternative, we decided to use ELSD, a semiuniversal detector that can detect any nonvolatile analyte. During the past decade, the HPLC-ELSD method has proven to be advantageous for determining several lipids (*20*), carbohydrates (*21*), and amino acids (*22*) bearing weak or no chromophores. However, to our knowledge, alkaloids such as DNJ have never been analyzed by HPLC-ELSD. As shown in **Figure 2**, ELSD enabled the detection of standard DNJ as a distinct single peak with a retention time of 24.5 min.

Generally, DNJ has been extracted from plant sources with hot water (*8*, *9*), but such a method requires quite a long extraction time and extra cleanup procedures. In this study, lyophilized leaves were put in organic solvent, sonicated, and centrifuged, and the supernatant was injected directly into an HPLC column, giving clear chromatograms (**Figure 3A**,**B**) through HPLC-ELSD/MS analysis. Mulberry DNJ eluted as a defined peak at a retention time of 24.5 min with the baseline separated from the other peak components (**Figure 3A**). This peak (24.5 min) was identified as DNJ on the basis of the MS profile (**Figure 3B**,**C**). On the other hand, because a slight tailing was observed for the mulberry DNJ peak (**Figure 3A**,**B**), its purity was checked by HPLC/MS. MS spectra in a region close to mulberry DNJ peak (22.0−26.0 min) were accumulated (**Figure 3C**). The accumulated spectrum contained only one DNJ peak ($[M + H]^+$ at *m*/*z* 164.1 amu), and no apparent peaks for the impurities. This indicated that no impurities coelute with the mulberry DNJ peak. Like the mulberry DNJ peak, some

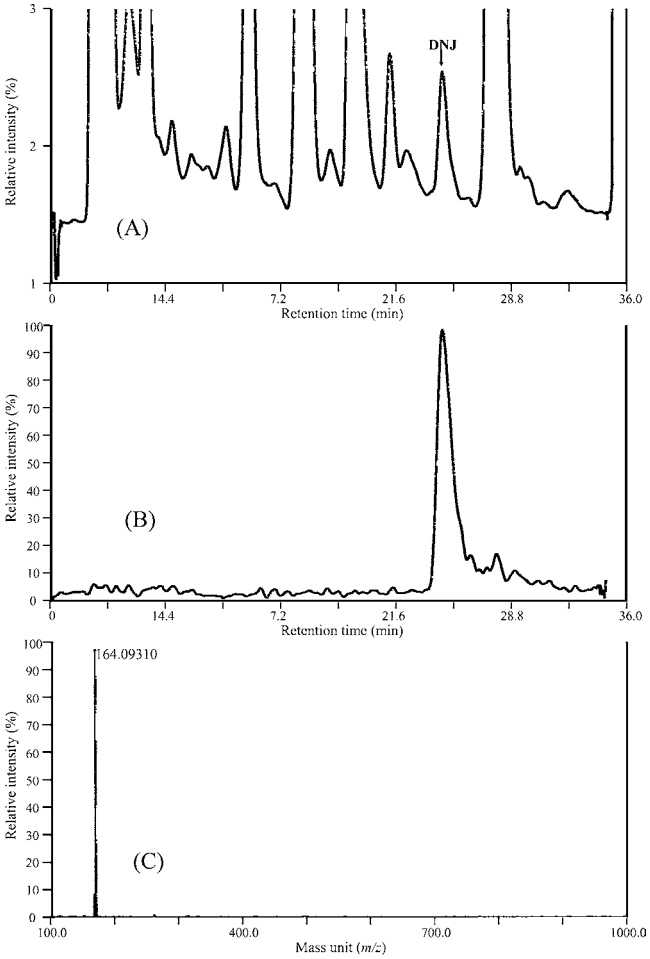

Figure 3. HPLC-ELSD/MS analysis of 1-deoxynojirimycin (DNJ) present in mulberry leaves: (**A**) ELSD chromatogram; (**B**) single-ion plot of the mass corresponding to the $[M + H]^+$ ion of DNJ (*m/z* 164.1); (**C**) accumulated MS spectrum of the peak detected between 22.0 and 26.0 min in chromatogram **B**.

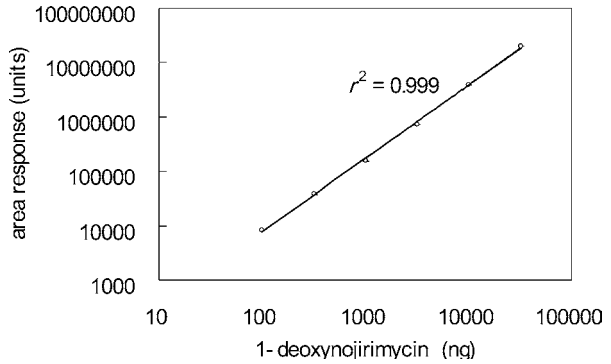

Figure 4. Standard curve for 1-deoxynojirimycin using ELSD detector.

peak tailing was also observed for standard DNJ. Therefore, such tailing might depend on the nature of the TSKgel Amide-80 column.

To apply the present method for determining mulberry DNJ, its standard curve was made. As shown in **Figure 4**, the standard curve of DNJ using ELSD was not linear, which is a common feature of this detector (*22*−*24*). The correlation between dose and response is best described by the following equation: injected amount = 0.1295 × area units$^{0.7423}$; r^2 = 0.999. The detection limit was 100 ng (0.613 nmol) for DNJ at a signal-to-noise ratio of 5. Generally, the HPLC-ELSD detection limit of carbohydrates is >100 ng (*21*). Hence, we can see that the sensitivity of the present ELSD method for DNJ is relatively high. By using this standard curve, the concentration of DNJ in three cultivars of mulberry leaves and its related products were defined as 100−480 mg/100 g (0.10−0.48%) lyophilized leaves (**Tables 1** and **2**). Without recourse to cultivars and product forms, the present method showed enough sensitivity to determine DNJ concentration. Finally, to ascertain the recovery of DNJ from mulberry leaves, we spiked standard DNJ (50−200 μg) to 100 mg of mulberry leaf powder (Kinuyutaka). After that, DNJ was extracted and quantified according to the present method. The recovery of DNJ was calculated as >90%.

Table 1. 1-Deoxynojirimycin (DNJ) Concentration of Mulberry Leaves in Three Cultivars

cultivar	DNJ concn[a] (%)
Kinuyutaka (*Morus alba*)	0.14 ± 0.0029
Kairyonezumikaeshi (*Morus alba*)	0.13 ± 0.0026
Shinkenmochi (*Morus bombysis*)	0.10 ± 0.0020

[a] Values are means ± SD, $n = 3$.

Table 2. 1-Deoxynojirimycin (DNJ) Concentration of Mulberry Leaf Products on the Market

product (form)	DNJ concn[a] (%)	product (form)	DNJ concn[a] (%)
A (tea leaf)	0.23 ± 0.0036	G (tea leaf)	<0.05
B (tea leaf)	0.23 ± 0.0019	H (powder)	0.20 ± 0.0087
C (tea leaf)	0.21 ± 0.0039	I (powder)	0.20 ± 0.0013
D (tea leaf)	0.17 ± 0.0028	J (tablet)	0.48 ± 0.0062
E (tea leaf)	0.17 ± 0.0017	K (tablet)	0.28 ± 0.0068
F (tea leaf)	0.13 ± 0.0019		

[a] Values are means ± SD, $n = 3$.

Most recently, Kim et al. (*25*) has described the determination procedures of DNJ by its derivatization with 9-fluorenylmethyl chloroformate, followed by reversed-phase HPLC. These authors mentioned that the concentration of DNJ in mulberry leaves harvested in Korea is ∼0.4%, which is almost consistent with our determined values (**Tables 1** and **2**) (*25*). In the method used by Kim et al. (*25*), the sensitivity toward DNJ is high (at least 1 ng), but the determination procedures are relatively complicated due to the necessity of the derivatization. In contrast, our HPLC-ELSD method enables the simple and convenient determination of DNJ without any labeling procedures. This is a great advantage when multiple samples are analyzed.

In conclusion, we established a simple quantitative determination method for DNJ present in mulberry leaves. Currently, various supplements that boast the bioavailability of DNJ are available to consumers in Japan. Nevertheless, their DNJ contents are not ordinarily specified. As there is no official method to measure DNJ, the present method may be applicable to the assay of DNJ present in various supplements, tablets, and other related products as well as in multiple plant sources.

LITERATURE CITED

(1) Niwa, T.; Inoue, S.; Tsuruoka, T.; Koaze, Y.; Niida, T. "Nojirimycin" as a potent inhibitor of glucosidase. *Agric. Biol. Chem.* **1970**, *34*, 966−968.

(2) Fellows, L. E.; Kite, G. E.; Nash, R. J.; Simmonds, M. S. J.; Scofield, A. M. Castanospermine, swainsonine and related polyhydroxy alkaloids: structure, distribution and biological activity. In *Plant Nitrogen Metabolism*; Poulton, J. E., Romero, J. T., Conn, E. E., Eds.; Plenum Publishers: New York, 1989; pp 395−427.

(3) Fellows, L. E.; Kite, G. C.; Nash, R. J.; Simmonds, M. S. J.; Scofield, A. M. Distribution and biological activity of alkaloidal glycosidase inhibitors from plants. In *Nitrogen Metabolism of Plants*; Mengel, K., Pibeam, D. J., Eds.; Clarendon Press: Oxford, U.K., 1922; pp 271−284.

(4) Asano, N.; Nash, R. J.; Molyneux, R. J.; Fleet, G. W. J. Sugar-mimic glycosidase inhibitors: natural occurrence, biological activity and prospects for therapeutic application. *Tetrahedron Asymmetry* **2000**, *11*, 1645−1680.

(5) Leglar, G. Glycosidase hydrolases: mechanistic information from studies with reversible and irreversible inhibitors. *Adv. Carbohydr. Chem. Biochem.* **1990**, *48*, 319−384.

(6) Elbein, A. D. Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains. *Annu. Rev. Biochem.* **1987**, *56*, 497−534.

(7) Asano, N.; Kizu, H.; Oseki, K.; Tomioka, E.; Matsui, K.; Okamoto, M.; Baba, M. *N*-Alkylated nitrogen in the ring sugars: conformational basis of inhibition of glycosidases and HIV-1 replication. *J. Med. Chem.* **1995**, *38*, 2349−2356.

(8) Asano, N.; Tomioka, E.; Kizu, H.; Matsui, K. Sugar with nitrogen in the ring isolated from the leaves of *Morus bombycis*. *Carbohydr. Res.* **1994**, *253*, 235−245.

(9) Asano, N.; Oseki, K.; Tomioka, E.; Kizu, H.; Matsui, K. *N*-Containing sugars from *Morus alba* and their glycosidase inhibitory activities. *Carbohydr. Res.* **1994**, *259*, 243−255.

(10) Asano, N.; Yamashita, T.; Yasuda, K.; Ikeda, K.; Kizu, H.; Kameda, Y.; Kato, A.; Nash, R. J.; Lee, H. S.; Ryu, K. S. Polyhydroxylated alkaloids isolated from mulberry trees (*Morus alba* L.) and silkworms (*Bombyx mori* L.). *J. Agric. Food Chem.* **2001**, *49*, 4208−4213.

(11) Nojima, H.; Kimura, I.; Chen, F.-J.; Sugihara, Y.; Haruno, M.; Kato, A.; Asano, N. Antihyperglycemic effects of N-containing sugars from *Xanthocercis zambesiaca*, *Morus bombycis*, *Aglaonema treubii*, and *Castanospermum australe* in streptozotocin-diabetic mice. *J. Nat. Prod.* **1998**, *61*, 397−400.

(12) Yoshikuni, Y. Inhibition of intestinal α-glucosidase activity and postprandial hyperglycemia by moranoline and its N-alkyl derivatives. *Agric. Biol. Chem.* **1988**, *52*, 121−128.

(13) Yagi, M.; Kouno, T.; Aoyagi, Y.; Murai, H. The structure of Moraoline, a piperidine alkaloid from *Morus* species. *Nippon Nougei Kagaku Kaishi* **1976**, *50*, 571−572 (in Japanese).

(14) Kimura, M.; Chen, F.-J.; Nakashima, N.; Kimura, I.; Asano, N.; Koya, S. Antihyperglycemic effects of N-containing sugars derived from mulberry leaves in streptozotocin-induced diabetic mice. *J. Trad. Med.* **1995**, *12*, 214−219.

(15) Lee, H. S.; Chung, K. S.; Kim, S. Y.; Ryu, K. S.; Lee, W. C. Effect of several sericultural products on blood glucose lowering for Alloxan-induced hyperglycemic mice. *Korean J. Seric. Sci.* **1998**, *40*, 38−42.

(16) Sharpless, K. E.; Margolis, S.; Thomas, J. B. Determination of vitamins in food-matrix standard reference materials. *J. Chromatogr. A* **2000**, *881*, 171−181.

(17) Alpert, A. J.; Shukla, M.; Shukla, A. K.; Zieske, L. R.; Yuen, S. W.; Ferguson, M. A. J.; Mehlert, A.; Pauly, M.; Orlando, R. Hydrophilic-interaction chromatography of complex carbohydrates. *J. Chromatogr. A* **1994**, *676*, 191−202.

(18) Alpert, A. J. Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. *J. Chromatogr.* **1990**, *499*, 177−196.

(19) Tolstikov, V. V.; Fiehn, O. Analysis of highly polar compounds of plant origin: combination of hydrophilic interaction chromatography and electrospray ion trap mass spectrometry. *Anal. Biochem.* **2002**, *301*, 298−307.

(20) Sugawara, T.; Miyazawa, T. Separation and determination of glycolipids from edible plant sources by high-performance liquid chromatography and evaporative light-scattering detection. *Lipids* **1999**, *34*, 1231−1237.

(21) Wei, Y.; Ding, M.-Y. Analysis of carbohydrates in drinks by high-performance liquid chromatography with a dynamically modified amino column and evaporative light scattering detection. *J. Chromatogr. A* **2000**, *904*, 113−117.

(22) Petritis, K.; Elfakir, C.; Dreux, M. A comparative study of commercial liquid chromatographic detectors for the analysis of underivatized amino acids. *J. Chromatogr. A* **2002**, *961*, 9−21.

(23) Christie, W. W. Rapid separation and quantification of lipid classes by high performance liquid chromatography and mass (light-scattering) detection. *J. Lipid Res.* **1985**, *26*, 507−512.

(24) Christie, W. W. Separation of lipid classes by high-performance liquid chromatography with the "mass detector". *J. Chromatogr.* **1986**, *361*, 396−399.

(25) Kim, J. W.; Kim, S.-U.; Lee, H. S.; Kim, I.; Ahn, M. Y.; Ryu, K. S. Determination of 1-deoxynojirimycin in *Morus alba* L. leaves by derivatization with 9-fluorenylmethyl chloroformate followed by reversed-phase high-performance liquid chromatography. *J. Chromatogr. A* **2003**, *1002*, 93−99.

Received for review October 2, 2003. Revised manuscript received December 12, 2003. Accepted December 15, 2003.

JF0306901