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누에에 함유된 1-Deoxynojirimycin의 분석을 위한 HPLC-ELSD 분석법 밸리데이션

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Development and Validation of a Unique HPLC-ELSD Method for Analysis of 1-Deoxynojirimycin Derived from Silkworms

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Abstract – A simple and accurate assay was developed for the quantitative analysis of 1-deoxynojirimycin (1-DNJ) derived from the silkworm (*Bombyx mori*). Normal-phase high-performance liquid chromatography coupled with an evaporative light scattering detector (HPLC-ELSD) and a hydrophilic interaction liquid chromatography column was used. Various parameters were applied to optimize the analysis method. The limits of detection and quantification of 1-DNJ were 2.97×10^{-3} and 9.00×10^{-3} mg/mL, respectively. The calibration curve showed good linearity results. The concentration range and the r^2 value were 0.0625–1.0 mg/mL and 0.9997, respectively. The accuracy test demonstrated a significantly high recovery rate (89.95–103.22%). The relative standard deviation was ≤ 1.00%. Thus, a method for the accurate identification and quantitative analysis of 1-DNJ in silkworms was developed. Moreover, in this procedure, the process of derivatization of 1-DNJ, which was required in previous experiments, could be eliminated. This technique may be actively utilized for the development of pharmaceuticals and health functional foods using 1-DNJ.

Keywords – Silkworm, 1-Deoxynojirimycin, HPLC-ELSD, Method validation

The importance of insects as a future food source has been highlighted. They produce lesser amounts of greenhouse gases than the livestock industry, respond to climate change, and are a valuable alternative food.[1-3] The silkworm, an eco-friendly resource, has been recognized for its nutritional value, and its value is gradually rising. However, research regarding on the effects of the functional ingredients that can be obtained directly or indirectly after ingestion on the human body is insufficient.[4,5]

The silkworm is the larva of the silkworm moth belonging to the family Bombycidae[6] and grows by feeding mainly on mulberry leaves. Its cylindrical body can be distinguished into the head, thorax, and ventral segments.[7] Before larvae develop into a moth, they have hatched several times and spun a cocoon and the silk filament which is used to make silk fabric is extracted by brushing the cocoon.[8-10] Among the industrial insects, the silkworm, which has been traditionally used as an edible resource, is known to have various physiological activities such as antidiabetic and antioxidant effects.[11-14]

1-deoxynojirimycin (1-DNJ), a natural substance found in silkworms, has been isolated in large quantities; it lowers blood sugar and has the potential to be developed as a functional

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food and specialty drug effective in the treatment of diabetes.[15-17] Structurally, the 1-DNJ lacks a characteristic functional group.[18] Furthermore, analysis of this molecule using high-performance liquid chromatography (HPLC), which is the common method, is difficult because it cannot be detected by an ultraviolet (UV) detector.[19,20] An evaporative light scattering detector (ELSD) is recommended for substances, such as 1-DNJ, which lack without an ultraviolet chromophore.[21,22] Usually, an ELSD is used during HPLC analysis that includes the following three steps: nebulization, evaporation of the eluent, and measurement of the scattered light produced by the particles.[23] The relationship between the signal and concentration of the sample it produces does not obey the Beer–Lambert law but rather an exponential equation of Rayleigh's law, $y = ax^b$, whose linear mathematical expression form is as follows: $\log y = \log a + b \log x$.[24] Currently, the experimental process is complicated because 1-DNJ must be analyzed through the well-known 9-fluorenylmethyloxycarbonyl chloroformate derivatization process.[25,26]

Therefore, a simpler, faster, and more accurate analysis method is required to compensate for the error caused by the loss of 1-DNJ that may occur due to the additional material pretreatment process. In this study, we present a novel method that has industrial and medical value to analyze 1-DNJ extracted from silkworms.

Materials and Methods

Materials – Freeze-dried powder of 3rd-day 5th instar silkworm (Baekokjam) was purchased from a sericulture farmhouse in Geongbok Yechoen, Korea (May 2021) and stored at −80 ℃ for later use.

Extraction Procedure – The dried silkworm powder (15 g) was extracted for 3 h twice using a reflux extractor with 30% ethanol (EtOH) in water (300 mL) as the extraction solvent. Subsequently, it was strained through a filter paper (pore size, 5–8 μm), and the ethanol in the extraction solvent was evaporated using a vacuum rotary evaporator. The remaining water was evaporated using a freeze dryer to obtain the silkworm extract.

Instrumentation, Chemicals, and Reagents – Analyses were performed using an HPLC system (Waters 1525 Binary pump, USA) that comprised an auto-sampler, pump, and ELSD (Waters 2424 ELS detector, USA). The HPLC-grade solvents, including water, EtOH, trifluoroacetic acid (TFA), and acetonitrile (ACN), were purchased from J. T. Baker (Phillipsburg, PA, USA). 1-DNJ was commercially acquired from the Natural Product Institute of Science and Technology (www.nist.re.kr), Anseong, Korea (Fig. 1).

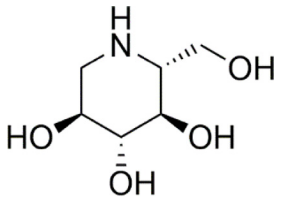

Fig. 1. Chemical structure of 1-DNJ.

Preparation of Standard and Sample Solutions for HPLC – The 1-DNJ standard was precisely weighed and dissolved in water to prepare a standard stock solution of 1 mg/mL concentration and filtered through a polytetrafluoroethylene (PTFE) membrane syringe filter (pore size, 0.45 μm). After diluting the standard stock solution to the required concentration (0.0625-1.0 mg/mL), it was analyzed to obtain a calibration curve. 1-DNJ was extracted from the sample silkworm extract powder (500 mg) with 5 mL water by sonication for 20 min. The extracted suspension was centrifuged at 10,000 rpm for 10 min. The supernatant was filtered through a PTFE membrane syringe filter (pore size, 0.45 μm) to prepare a sample stock solution, which was diluted to the required concentrations (25.0, 50.0, 100.0 mg/mL) for analyses.

HPLC-ELSD Conditions – The analysis was performed using a normal-phase HPLC system with a TSKgel Amide-80 hydrophilic interaction liquid chromatography (HILIC) column (4.6 mm × 25 cm, 5 μm; Tosoh, Tokyo, Japan) attached to an ELSD the injection volume was 5.0 μL. The column was maintained at a temperature of 40 ℃ and the drift tube of the detector was maintained at 60 ℃. The flow rate was adjusted to 0.7 mL/min. Nitrogen gas was used as the spraying gas at 40.0 psi pressure, and the gain was set at 8. The separation was analyzed under isocratic conditions with a mixture of 0.1% TFA in 70% ACN (TFA/water/ACN, 0.1:29.97:69.93; v/v) for 40 min.

Method Validation – The method was validated by testing the specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, and precision according to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) criteria (Guideline IHT, 2015). Specificity was analyzed to identify the presence of the same compound in tests for retention time and spike test of silkworm extract and 1-DNJ. Accuracy was confirmed by performing a recovery rate test

in which the sample extract was spiked with three standards of different concentrations, and the percentage of the standard recovered from the sample extract was calculated. To further verify the accuracy, three different concentrations of silkworm sample extracts were used for intra-day (repeatability) analysis over one day. Inter-day (intermediate precision) analysis was performed by different researchers using the same equipment on various days under the same conditions with only one concentration of the sample extract. The intra- and inter-day precision of the standards were investigated by evaluating the relative standard deviation (RSD) percentage that was calculated by dividing the standard deviation by the mean value. Linearity was evaluated by injecting five different concentrations of standard solutions (0.0625–1.0 mg/mL) three times each. A calibration curve was created in proportion to the concentration of the measured standard solution based on the peak area of the chromatogram result. In ELSD, the peak signal value for concentration is in the following exponential function form: $y = ax^b$. Therefore, it is converted to the common logarithm form: $\log y = \log a + b \log x$ to generate a linear calibration curve.[27] Linearity was verified by applying the correlation coefficient (r^2) of this formula. LOD and LOQ values were derived using the standard deviation values of the slope (S) and intercept (σ) of the calibration curve of the standard material. The LOD refers to the minimum amount or concentration of an analyte that can be detected separately from the baseline, and the LOQ denotes the minimum amount that can be quantified with precision and repeatability. The LOD and LOQ were derived by the following formula: LOD = 3.3 (σ/S) and LOQ = 10 (σ/S).

Calibration Curve – A calibration curve was prepared based on the peak area of each standard solution concentration. The linearity was determined by deriving the correlation coefficient (r^2) value of the calibration curve from the common logarithm form. The 1-DNJ concentration in the sample was calculated using the prepared calibration curve. The calibration function was set using the mean ± standard deviation (n = 3) values of concentration (X-axis, mg/mL) and peak area (Y-axis).

Results and Discussion

A HPLC method for quantifying 1-DNJ from silkworms was developed and validated. A HILIC column capable of separating 1-DNJ, which is a highly polar compound (Fig. 1), was selected and analyzed using an ELSD. The results are shown in Fig. 2. 1-DNJ was defined as the peak detected with a retention time of 8.1 min. There were no concurrently eluted impurity peaks, indicating that the analytical method

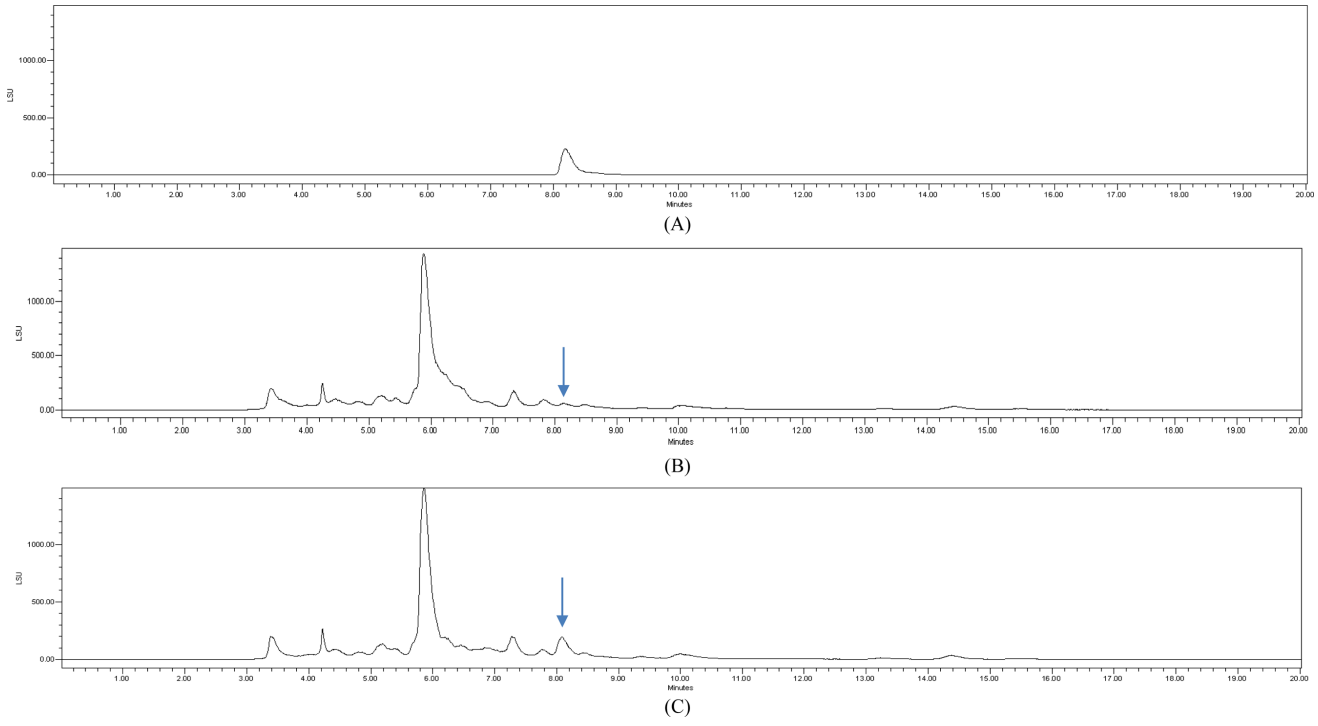

Fig. 2. HPLC results and specificity of 1-DNJ (A), silkworm (B), and 1-DNJ spiking (C).

is suitable and has high accuracy. The specificity of the analysis method was evaluated using the spike test in which the standard material was spiked with the sample solution. The presence the standard material in the silkworm was confirmed through the retention time and the 1-DNJ peak of the chromatogram spiked in the sample (Fig. 2). The HPLC method exhibited specificity by demonstrating good resolution for the chromatogram peak of 1-DNJ.

The quantitative parameters of 1-DNJ were obtained under the previously set HPLC analysis conditions. A calibration curve was generated based on the peak area obtained using HPLC analysis by dilution with various concentrations of isolate and 1-DNJ. The linearity was evaluated with five standard solutions of 1-DNJ at concentrations in the 0.0625–1.0 mg/mL range (n = 3). The correlation coefficient (r^2) and range presented in Table I show good linear regression (r^2 = 0.9997) in the range. The peak area (Y-axis) was plotted against the 1-DNJ concentrations (X-axis) in mg/mL to prepare a regression equation. The LOD and LOQ values were 2.97×10^{-3} and 9.00×10^{-3} mg/mL, respectively. These data showed that the ELSD assay method established in this study was accurate and sensitive for quantifying 1-DNJ detected in silkworm extracts.

The standard 1-DNJ solution (0.125–0.5 mg/mL) of known concentration was spiked with silkworm extract powder (25.0 mg/mL) to measure the accuracy of the analysis method. In order to calculate the recovery rate of each compound, the amount detected in the analysis result was compared with the amount of 1-DNJ spiked for each concentration. The recovery rates were in the 92.09–102.53% range (Table II), which were acceptable, thus demonstrating that the analytical method was precise.

To measure the precision of the analysis method, intra- and inter-day precision values were compared (Tables III, IV). The RSDs of the precision values obtained from the intra- and inter-day precision test were in the ranges of 0.43 to 1.00% and 0.36 to 0.96%, respectively. The inter-day RSD values are lower than the intra-day ones suggesting that 1-DNJ remains relatively stable over time after dissolution. Furthermore, these results are lower than the upper limit value recommended by the ICH (2%). Thus, the analytical method is reliable for quantifying 1-DNJ in silkworms.

The 1-DNJ content in the silkworm extract measured in this study was 5.72 mg/g, which is higher than the amount determined in other studies.[28-30] Thus, our method is effective and advantageous analytical method has been estab-

Table I. Linearity, LOD, and LOQ of 1-DNJ

Compound	t_R	Range (mg/mL)	Calibration equation[a]	r^2[b]	LOD (mg/mL)	LOQ (mg/mL)
1-DNJ	8.1	0.0625–1.0	Y = 1.5098X + 4.5921	0.9997	2.97×10^{-3}	9.00×10^{-3}

t_R: retention time
[a]Y: peak area, X: concentration of standards (mg/mL).
[b]r^2: correlation coefficient for five calibration data points (n = 3).
1-DNJ: 1-deoxynojirimycin; LOD: limit of detection; LOQ: limit of quantification

Table II. Accuracy of 1-DNJ content determination

Compound	Spiked amount (mg)	Recovery (%)					Average (%)	RSD (%)
		1st	2nd	3rd	4th	5th		
1-DNJ	0.125	89.95	93.02	91.46	92.65	93.39	92.09	1.52
	0.25	102.59	102.86	103.22	103.01	100.98	102.53	0.87
	0.5	95.12	93.90	96.61	94.83	96.98	95.49	1.34

1-DNJ: 1-deoxynojirimycin; RSD: relative standard deviation

Table III. Intra-day precision of 1-DNJ content determination

Compound	Concentration (mg/mL)	Intra-day (n = 3)	
		Measured content (mg/g)	RSD (%)
1-DNJ	25.0	5.87	0.73
	50.0	5.78	0.43
	100.0	5.23	1.00

1-DNJ: 1-deoxynojirimycin; RSD: relative standard deviation

Table IV. Inter-day precision of 1-DNJ content determinations

Compound	Concentration (mg/mL)	Inter-day (n = 5)	
		Measured content (mg/g)	RSD (%)
1-DNJ	50.0	5.76	0.36
		5.72	0.96
		5.73	0.80

1-DNJ: 1-deoxynojirimycin; RSD: relative standard deviation

lished. Several studies on the 1-DNJ content not only in silkworms but also in mulberry plants, which is the staple food of silkworms, have been conducted. Mulberry leaves contain various physiologically active substances, such as chlorogenic acid, flavonoid, γ-aminobutyric acid (GABA), and 1-DNJ, which have anticancer, antidiabetic, antihypertensive, and antioxidant properties. Thus, silkworms that ingest mulberry leaves may have several applications.[31-33)]

In this paper, as results of the development and validation of an analysis method for quantifying 1-DNJ in silkworm, a simpler and superior method was able to be derived than the previous analytical method.[19)] Mobile phase conditions are simple, diluent is water, extraction is simple, room temperature is possible, and retention time is short. Therefore, the analysis method in this paper can be said to be a superior analysis method when developing functional food using materials such as mulberry leaves and silkworms. In this study, HPLC analysis using an ELSD was optimized to quantify 1-DNJ in silkworms. The purpose of this verification study was to develop a method to analyze 1-DNJ, whose characteristics cannot be determined using a UV detector unless it undergoes a special pretreatment process, such as derivatization, since it has no chromophore. Our results showed that this method may have been useful in agricultural technology research involving many insects, including silkworms, which have high industrial value. Additionally, new analysis methods that can provide big data for silkworm research databases may be developed. In particular, this analytical method could serve as the basis for standardization in future studies involving the development of health functional foods and companion animals using silkworms.

Conclusions

An ELSD rather than the previously known UV detector and C18 reversed-phase column utilization method was used. This unique method, wherein a HILIC column was utilized, was less time-consuming than that employed in previous studies, thereby increasing the possibility of applying it in the analyses of various health functional foods that can be obtained from silkworms in the future.

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