Short Communication

Determination of Asperulosidic Acid and Deacetylasperulosidic Acid in Rat Plasma After Administration of *Morinda citrifolia* Juice

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Abstract

Determination of asperulosidic acid (AA) and deacetylasperulosidic acid (DAA) in rat plasma after administration of *Morinda citrifolia* juice by HPLC-UV detection was described. Deproteinized rat plasma (200 μ L) with MeOH for the HPLC analysis was injected. AA and DAA was separated on ZIC[®]-HILIC (250 $\times$ 4.6 mm, i.d., 5 μ m) with a mixture of CH₃CN/MeOH/0.1% formic acid aqueous solution (=70:25:5, v/v/v) at a flow rate of 0.75 mL/min. The absorbance of eluate was monitored at 235 nm. Under this condition, the separation of AA and DAA was achieved within 25 min. The calibration curves using plasma spiked with standards indicated good linearity ($r \geq 0.996$) in the range of 0.8-20 μ g/mL for AA and 4-100 μ g/mL for DAA, respectively. The limits of quantitation for AA and DAA at a signal-to-noise ratio of 10 were 0.3 and 1.1 μ g/mL, respectively. The validation parameters of the method such as recovery (94.9-101.7 %), precisions (less than 7.1 % for intra-day and less than 9.8 % for inter-day) and accuracy (95.0-101.7 %) were acceptable. The analytes in rat plasma were stable after three freeze-thaw cycles or storage at room temperature for up to 6 h. Furthermore, monitoring of AA and DAA after administration of the *Morinda citrifolia* juice in which AA and DAA were determined, was successfully demonstrated. This is the first report to determine AA and DAA in rat plasma after administration of *Morinda citrifolia* juice.

Keywords: Asperulosidic acid; Deacetylasperulosidic acid; *Morinda citrifolia* (Noni)

1. Introduction

Morinda citrifolia (call as Noni) is native from Southeast Asia to Australia and has been used for centuries as a food or medicinal plant [1]. Most *Morinda citrifolia* fruit is consumed as juice and its tea and supplement are also commercially available. The main proven functionalities of *Morinda citrifolia* are related to the control of several diseases. Basic research and limited experiments with animals have shown that *Morinda citrifolia* has anti-microbial [2], anti-cancer [3-6], antioxidant [7-9], anti-inflammatory [10] and anti-cardiovascular activities

[11]. Ingredients such as amino acids, anthraquinones, coumarins, fatty acids, flavonoids and iridoids which might contribute to the bioactivities of *Morinda citrifolia*, have been identified [1,12]. However, a few determination methods for these in *Morinda citrifolia* product has been established [7,12].

Among them, iridoid derivatives focus on due to their beneficial effects (anti-microbial or anti-inflammatory activities) [10,13]. Major species of the iridoid derivatives in *Morinda citrifolia* identified were asperulosidic acid (AA) and deacetylasperulosidic acid (DAA) (Fig. 1)

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[10,13,14]. Deng *et al.*, developed an HPLC-photodiode array (HPLC-PDA) method for the determination of AA and DAA in *Morinda citrifolia* [14]. However, no method for determination of them in biological sample after administration of *Morinda citrifolia* product has been reported.

In this study, an HPLC method for determination of AA and DAA in rat plasma was established. Method validation of the proposed method and stability of analytes in plasma was also evaluated. Furthermore, monitoring of AA and DAA after administration of the *Morinda citrifolia* juice in which AA and DAA was determined, was demonstrated. This is the first report to determine AA and DAA in rat plasma after administration of *Morinda citrifolia* product.

Fig. 1. Chemical structures of AA and DAA.

2. Experiments

2.1. Chemicals

AA and DAA were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). CH₃CN, MeOH, formic acid and ethyl carbamate from Wako Pure Chem. Co. (Osaka, Japan) were used. *Morinda citrifolia* juices were kindly gifted from Dr. Nishigaki (M&K Laboratories, Nagano, Japan). Distilled water was passed through a Pure Line WL21P system (Yamato Scientific Co., Tokyo, Japan). All other chemicals were of analytical grade. A standard stock solutions (10 mg/mL) of AA and DAA in MeOH was prepared and storing at 4°C until analysis. For the evaluation of method validation, the stock solution was appropriately diluted with MeOH.

2.2. Sample preparations

Two-hundred micro-liter of rat plasma were added to 400 µL of MeOH for deproteinization, and then centrifuged (2000 g) at 4°C for 15 min. After filtration with the membrane filter (0.45 µm), the sample supernatant was injected into the HPLC system.

For determination of AA and DAA in *Morinda citrifolia* juices, the sample diluted 10 times with MeOH was vortex-mixed for 1 min and centrifuged (2000 g) at 10°C for 10 min. The filtered supernatant with a membrane filter (0.45 µm) was appropriately diluted with MeOH.

2.3. HPLC conditions

The HPLC system for the determination of AA and DAA

consisted of an LC-10AD_{VP} chromatographic pump (Shimadzu, Kyoto, Japan), a rheodyne 7125 injector (Sigma-Aldrich Co.) with a 20 µL of sample loop, a ZIC[®]-HILIC separation column (250×4.6 mm, i.d., 5 µm, Sigma-Aldrich Co.) and an SPD-10AV spectrophotometric detector (Shimadzu). The mixture of CH₃CN and 0.1% formic acid aqueous solution (87:13, v/v) flowed at 0.5 mL/min, and CH₃CN, MeOH and 5% formic acid aqueous solution (70:25:5, v/v/v) flowed at 0.75 mL/min was used for both *Morinda citrifolia* juice and rat plasma. The wavelength at 235 nm was used to monitor the eluate.

To identify AA and DAA in plasma, Waters Alliance 2695 separation module with 2996 photodiode array detector (Waters, Milford, MA, USA) was used. The absorption spectra ranging from 200 to 400 nm between standard and the observed sample peak were checked.

2.4. Method validation

The method was validated according to the US Food and Drug Administration Guideline for Bioanalytical Methods as detailed below [15]. Calibration curves for AA and DAA were prepared using rat plasma spiked with standard ranging from 0.8 to 20 µg/mL for AA, and from 4 to 100 µg/mL for DAA. The limits of detection (LOD) and quantification (LOQ) were defined as the concentration of analytes giving a signal to noise (S/N) ratio of 3 and 10, respectively. The method's recovery, accuracy and precisions were assessed by analyzing the plasma samples spiked with three concentrations (0.8, 3 and 20 µg/mL for AA and 4, 15 and 100 µg/mL for DAA). Analyte recovery was determined by comparing the peak area for each extracted analyte spiked at three concentrations to that of the corresponding standard (n=3). Accuracy is expressed as a percentage of the calculated concentration divided by the nominal concentration (n=3). Both intra- and inter-day precisions were indicated as the relative standard deviation (RSD, n=5).

2.5. Stability of analytes in plasma

The stability of analytes was assessed after storage at room temperature (25°C) for up to 6 h or three freeze-thaw cycles. The stability of the analytes with triplicate-measurements was assessed by analyzing plasma samples spiked with standards.

2.6. Administration study

The plasma samples used in the administration study were obtained from male Wistar rats (275–285 g) purchased from Kyudo Experimental Animal (Saga, Japan). Rats were anesthetized with 1.5 g/kg ethyl carbamate injected intraperitoneally and a cannula for blood sampling was placed in femoral artery. For the administration study, *Morinda citrifolia* juice with a volume of 50 mL/kg of body

weight which is corresponding to 37.4 mg/kg of AA and 146 mg/kg of DAA, was administered to rats *per oral*. Following administration of *Morinda citrifolia* juice, blood samples (1 mL each) were drawn at various intervals between 2 and 8 h. Plasma samples were prepared by centrifugation (2000 g) of whole blood for 10 min at 4°C. Plasma samples were stored at –30°C until analysis. All animal procedures and care protocols were approved by the Nagasaki University Animal Care and Use Committee (No. 1406021153).

3. Results and discussions

3.1. HPLC conditions and sample preparation

To separate AA and DAA, HyPURITY C18 (150×4.6 mm, i.d., 3 µm, ThermoFisher Scientific, Yokohama, Japan), Develosil 300 C4-HG-5 (150×2.0 mm, i.d., 5 µm, Nomura Chemical Co., Seto, Japan) or the ZIC®-HILIC columns was examined. The analytes due to their hydrophilicity could be retained by only the ZIC®-HILIC column and separated from interfering peaks. As mobile phase, a mixture of CH₃CN and 0.1% formic acid aqueous solution was used. Increasing of CH₃CN content in mobile phase prolonged the retention time of the analytes. By using mixture of CH₃CN and 0.1% formic acid aqueous solution (87:13, v/v) gave satisfied separation of analytes in *Morinda citrifolia* product. On the other hand, the separation of AA and DAA in rat plasma was achieved using the mixture of CH₃CN, MeOH and 0.1% formic acid aqueous solution (70:25:5, v/v/v) (Fig. 2). The HPLC analysis completed within 25 min and the retention times of AA and DAA were 9.1 and 21.5 min, respectively. A good agreement of absorption spectra of AA and DAA between standard and the appearing peaks corresponding retention time of AA and DAA was obtained.

For the determination of AA and DAA in *Morinda citrifolia* product and rat plasma, the sample could be applied for HPLC analysis with a simple deproteinization with MeOH. Using CH₃CN as a deproteinization agent gave the same result with that of MeOH.

Fig. 2. Chromatograms of rat plasma (A) and that spiked with standards (B). Sample: rat plasma (A) and that spiked with 3 µg/mL of AA and 15 µg/mL of DAA (B).

3.2. Method validation

The calibration curves using rat plasma spiked with standards indicated good linearity ($r \geq 0.996$) in the range of 0.8–20 µg/mL for AA and 4–100 µg/mL for DAA, respectively. The LODs and LOQs at an S/N ratio of 3 and 10 for AA and DAA were 0.33, 0.09 µg/mL and 1.1 and 0.3, µg/mL, respectively. Since this is the first report to determine AA and DAA in plasma to our best knowledge, there is no data to compare the sensitivity of the method. However, the sensitivities of the proposed method with 0.06 and 0.017 µg/mL of LOD (=S/N ratio of 3) for AA and DAA in *Morinda citrifolia* product was comparable with the conventional HPLC-PDA method [14].

The recoveries for AA and DAA were ranging from 94.9±3.5 to 101.1±8.2 % and from 95.9±4.2 to 100.8±1.6 %, respectively. Other validation parameters such as accuracy, precision for intra- and inter-day measurements were summarized in Table 1. The accuracy (ranging from 95.0±3.4 to 100.9±1.6 %, n=3), intra-day (less than 7.1 %, n=5) and inter-day assay precisions (less than 9.8 %, n=5) were acceptable.

Table 1. Accuracies and precisions for the proposed method.

Compound	Spiked concentration (µg/mL)	Accuracy % Mean ± SD (n = 3)	Precision RSD % (n = 5)	
			Intra-day	Inter-day
DAA	4	95.7 ± 1.8	7.1	9.8
	15	100.9 ± 1.6	3.6	5.7
	100	95.9 ± 4.2	1.7	2.0
AA	0.8	97.2 ± 5.1	6.4	7.5
	3	95.0 ± 3.4	4.3	5.2
	20	97.4 ± 14.6	1.6	2.1

3.3. Stability of analytes in plasma

The stabilities of AA and DAA in rat plasma stored under different conditions were examined. The data was indicated as a percentage of initial concentration with triplicate-measurements. All analytes could be safely stored at room temperature for up to 6 h or subjected to three freeze-thaw cycles as shown in Fig. 3.

3.4. Administration study

Furthermore, the proposed method was applied to monitor AA and DAA concentrations after *Morinda citrifolia* juice administration to rat. Before administration, the concentration of AA and DAA in *Morinda citrifolia* juice was determined. As result, 746.6±20.4 µg/mL of AA

and 2915 ± 63 $\mu\text{g/mL}$ of DAA were found ($n=3$). These contents in *Morinda citrifolia* juice agree with those of previous reports [10,13].

The concentration–time profiles of the iridoids are shown in Fig. 4. The concentrations of AA and DAA in plasma were in the range of 2.38–3.67 $\mu\text{g/mL}$ and 8.23–44.94 $\mu\text{g/mL}$, respectively. The plasma concentration of AA increased slowly, reached maximum (3.67 $\mu\text{g/mL}$) at 4 h after administration, and then, decreased slowly. On the other hand, concentration behavior of DAA was similar with that of AA for up to 4 h, but, then the concentration was increasing until 8 h after administration. The reasons for this phenomenon might be, 1) the one of major metabolites of AA is DAA, 2) several iridoid derivatives existing in *Morinda citrifolia* juice might be metabolized to DAA. In the previous report, 19 iridoid derivatives including AA and DAA in *Morinda citrifolia* were indicated [16]. However, the amount of DAA may be much higher than those [10,13,16]. To clarify this, further study to identify and determine iridoid derivatives is required.

Fig. 3. Stability of AA and DAA in rat plasma. Sample: rat plasma at room temperature (A) and that in freeze-thaw cycles (B). Data was indicated as mean + SD (for AA) and mean – SD (for DAA) SD.

On the other hand, the proposed method has several limitations: 1) only 2 derivatives (AA and DAA) could be monitored, 2) the sensitivity of the method is insufficient for human, because much higher dose of *Morinda citrifolia* juice compared to the recommended one by the food company is needed, 3) and the monitoring point is not enough to evaluate pharmacokinetic parameter. Therefore, it may be concluded that at this stage the proposed method is suitable for preclinical study by using experimental animals.

Fig. 4. Time courses of AA and DAA concentration after oral administration of *Morinda citrifolia* juice. Data indicates as Mean $\pm$ SE ($n=3$).

4. Conclusion

The simple HPLC method with sensitivity for the determination of AA and DAA in rat plasma sample after administration of *Morinda citrifolia* juice could be established. The method has acceptable validation for bioanalysis, and thus, might be useful to clarify the contribution of iridoid derivatives for desirable health effects of *Morinda citrifolia* in preclinical study.

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Conflict of interest

The authors have declared no conflict of interest.

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