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ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · NOVEMBER 2005

Impact Factor: 2.91 · DOI: 10.1021/jf0516078 · Source: PubMed

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Quantitation of Clovamide-Type Phenylpropenoic Acid Amides in Cells and Plasma Using High-Performance Liquid Chromatography with a Coulometric Electrochemical Detector

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A high-performance liquid chromatography (HPLC) method was developed for measuring the concentrations of clovamide-type phenylpropenoic acid amides (*N*-caffeoyldopamine and *N*-caffeoyltyramine) in cell and plasma samples. The separation was performed on a Nova-Pak C18 column using an isocratic buffer with a coulometric electrochemical detector with four electrode channels. Using the HPLC method, *N*-caffeoyldopamine and *N*-caffeoyltyramine could be detected with good peak resolutions at respective retention times (4 and 6.4 min). The calibration curves were linear over the ranges (0.1 and 100 μM), and their lower limit of detection was as little as 100 fmol. For quantifying *N*-caffeoyldopamine and *N*-caffeoyltyramine in cell and plasma samples, the samples were extracted by extraction methods with more than 95% recoveries. After extraction, the amides were detected with the same sensitivity, peak resolutions, and retention times. Using this method, plasma concentrations of *N*-caffeoyltyramine were determined in blood samples collected at 12, 24, 30, 36, 48, 60, and 75 min after the oral administrations of *N*-caffeoyltyramine (0.5 mg and 2 mg/30 g body weight). This HPLC method with an electrochemical detector is the first reported method able to quantify *N*-caffeoyldopamine and *N*-caffeoyltyramine in biological samples with excellent detection limits, peak resolutions, discrete retention times, and consistent reproducibility.

KEYWORDS: ***N*-Caffeoyldopamine; *N*-caffeoyltyramine; HPLC; biological sample; mice**

INTRODUCTION

Clovamide-type phenylpropenoic acid amides are phytochemicals produced via forming an amide bond between carboxyl groups of phenylpropenoic acids and the amine group of tyrosine related compounds (*1*−*5*). They have been found in many plants including *Capsicum* spp. and Cocoa. They are believed to play important roles in protecting plants from the pathogen attacks (*6*−*10*). However, potential effects of clovamide-type phenylpropenoic acid amides on human health have not been investigated until lately. In recent studies, beneficial effects of clovamide-type phenylpropenoic acid amides on human diseases were suggested, and their mechanisms are being investigated to a greater extent (*11*−*16*).

Currently, there is a great need for quantifying clovamide-type phenylpropenoic acid amides in biological samples (cells and plasma), but no method is available for the purpose. Therefore, in this study, a high-performance liquid chromatography (HPLC) method was developed to determine concentrations of two clovamide-type phenylpropenoic acid amides (*N*-caffeoyltyramine and *N*-caffeoyldopamine) (**Figure 1**) in biological samples. Because the developed HPLC method provides excellent peak resolutions of clovamide-type phenylpropenoic acid amides with high sensitivity and reliability, the method was applied for analyzing not only *N*-caffeoyltyramine and *N*-caffeoyldopamine, but also other clovamide-type phenylpropenoic acid amides in cell and plasma samples.

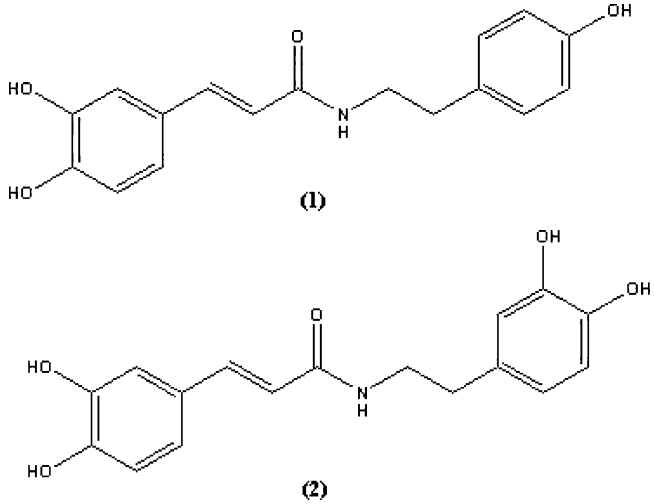

Figure 1. Chemical structures of *N*-caffeoyltyramine (**1**) and *N*-caffeoyldopamine (**2**).

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10.1021/jf0516078
Published on Web 09/16/2005

MATERIALS AND METHODS

Materials. *N*-Caffeoyltyramine, *N*-caffeoyldopamine, and *N*-feruloyltyramine were synthesized and purified as described previously (*15*−*17*). U937 and Jurkat cells were purchased from ATCC (Rockville, MD).

Cell Culture Conditions. U937 and Jurkat cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, respectively. Cell viability was determined microscopically by trypan blue exclusion (*18*). The cells were cultured up to 1×10^6 cells/mL for the experiments. The number of cells was counted with a hematocytometer.

HPLC Measurement. *N*-Caffeoyldopamine and *N*-caffeoyltyramine standards were prepared in the range from 0.1 to 100 μM in 40% methanol. A 2.1 mm × 150 mm i.d. Nova-Pak C18 (Waters, Milford, MA) was used as the stationary phase to analyze two clovamide-type phenylpropenoic acid amides (*N*-caffeoyldopamine and *N*-caffeoyltyramine), and an isocratic buffer of 50 mM NaH_2PO_4 (pH 4.3) containing 40% methanol was used at 1 mL/min as the mobile phase for the HPLC analyses. The clovamide-type phenylpropenoic acid amides in standard and biological samples were injected by an autosampler into an Alliance 2690 HPLC system (Waters, Milford, MA) and then were detected by CoulArray electrochemical detector with four electrode channels (ESA, Chelmsford, MA) and quantified by its software (v.1.0). For optimal measurement of *N*-caffeoyldopamine and *N*-caffeoyltyramine, the four channels were set at 100, 300, 550, and 800 mV.

Cell Samples Preparation. U937 or Jurkat cells (2×10^6) were suspended in 1 mL of HEPES/phosphate buffer containing 147 mM NaCl, 5 mM KCl, 1.9 mM KH_2PO_4, 1.1 mM Na_2HPO_4, 5.5 mM glucose, 0.3 mM $MgSO_4 \cdot 7H_2O$, 1 mM $MgCl_2 \cdot 6H_2O$, 0.3 mM $CaCl_2 \cdot 2H_2O$, and 10 mM HEPES, pH 7.4. *N*-Caffeoyldopamine and *N*-caffeoyltyramine were added to the cell suspensions to give final concentrations of 1, 5, 10, 20, and 40 μM. After the incubation (15 min), the cells were washed twice with cold phosphate-buffered saline (pH 7.4) by the centrifugation at 14 000*g* for 10 min. *N*-Caffeoyldopamine and *N*-caffeoyltyramine in cell pellets were extracted with 500 μL of 50 mM phosphate buffer (pH 4.3) containing 40% methanol. The samples were then centrifuged at 14 000*g* for 10 min, and the supernatants were saved. The extraction was performed twice, and both supernatants were separately analyzed using the HPLC method described above.

Plasma Samples Preparation. For measuring *N*-caffeoyldopamine and *N*-caffeoyltyramine in plasma samples, Swiss Webster mice 25−30 weeks old (Charles River, Wilmington, MA) were used for collecting blood. All animal procedures were performed according to an animal protocol approved at BARC (Beltsville Agricultural Research Center). Individual mice were placed in standard cages and housed in the environmentally controlled Beltsville Human Nutrition Research Center Animal Facility. Mice were fed AIN-76A purified diet that provided the recommended allowance of all nutrients required for maintaining optimal health, but lacking *N*-caffeoyldopamine and *N*-caffeoyltyramine tested in the study (the diet was analyzed by HPLC for confirming that these compounds were not in the diet). Mice in the experimental trial were divided into three groups (control, and two oral dosing groups), and each group consisted of 5 mice. For the oral dosing group, *N*-caffeoyltyramine (0.5 mg and 2 mg/30 g body weight) was administrated into the animal using a dosing needle. Blood was collected via the tail bleeding technique, at 12, 24, 30, 36, 48, 60, and 75 min after the oral dosing. Whole blood was centrifuged at 2000*g* for 10 min, and the plasma was precipitated with methanol (final concentration: 40%) and centrifuged at 14 000*g* for 10 min. The final supernatant was used for the assay.

Data Analyses. All statistic analyses were performed with the StatView program of SAS institute Inc. (Cary, NC). The linearity of regression line was determined by the correlation coefficient (*R*). Data points in all figures represent the mean ± SD of more than three samples.

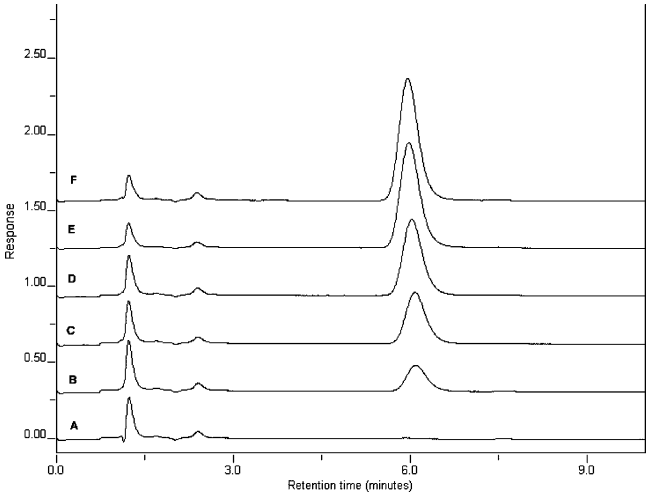

Figure 2. Chromatograms of *N*-caffeoyltyramine. *N*-Caffeoyltyramine standards (**A** (0 pmol), **B** (0.2 pmol), **C** (0.4 pmol), **D** (0.6 pmol), **E** (0.8 pmol), and **F** (1 pmol)) were injected into a high-performance liquid chromatograph (HPLC), and chromatograms were collected by using an electrochemical detector poised at 800 mV.

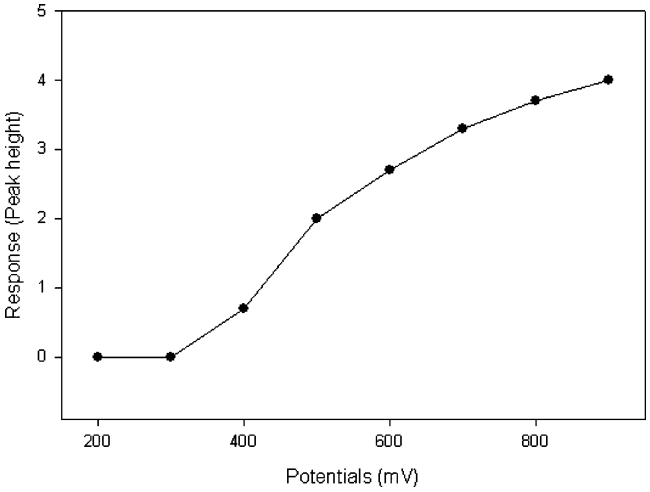

Figure 3. Hydrodynamic voltammogram of *N*-caffeoyltyramine. The voltammogram was generated by using the electrochemical detector with four channels poised at 100–900 mV.

RESULTS AND DISCUSSION

Measurement of *N*-Caffeoyltyramine and *N*-Caffeoyldopamine in Standard Samples. *N*-Caffeoyltyramine in standards was determined using the high-performance liquid chromatography (HPLC) equipped with an electrochemical detector with four electrodes. The chromatograms of *N*-caffeoyltyramine standards (0.2, 0.4, 0.6, 0.8, and 1 pmol) are shown in **Figure 2**. Total HPLC running time for the assay was 10 min, and *N*-caffeoyltyramine was detected at a retention time of 6.4 min. To optimize applied potentials at the sequential electrodes, hydrodynamic voltammograms of *N*-caffeoyltyramine were generated (**Figure 3**). The potential at which half of the *N*-caffeoyltyramine is oxidized is approximately 500 mV, and the potential at which *N*-caffeoyltyramine is fully oxidized is greater than 800 mV. Therefore, all of the assays in this study were performed using the electrochemical detector with four electrodes sequentially set at potentials of 100, 300, 550, and 800 mV. Using the detector, a satisfactory linear response for *N*-caffeoyltyramine was also obtained at the concentrations between 0.1 and 100 pmol (correlation coefficient (R) = 0.9988).

In the samples, *N*-caffeoyltyramine and its precursors (caffeic

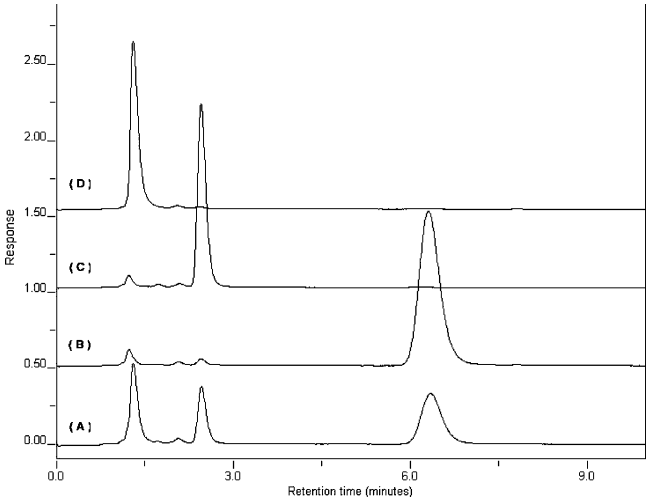

Figure 4. Chromatograms (**A**: tyramine (70 pmol), caffeic acid (3 pmol), and *N*-caffeoyltyramine (3 pmol)); (**B**: *N*-caffeoyltyramine (10 pmol)); (**C**: caffeic acid (10 pmol)); (**D**: tyramine (200 pmol)) were collected using HPLC equipped with an electrochemical detector poised at 800 mV.

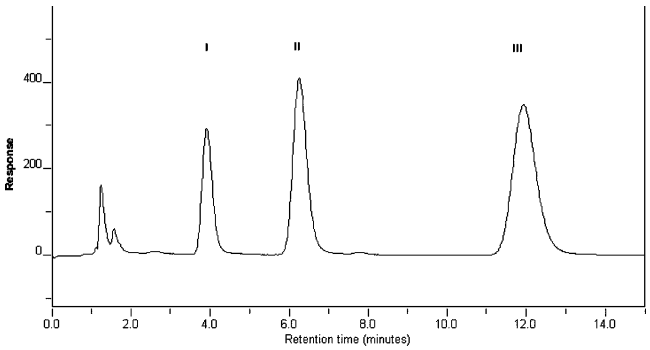

Figure 5. Chromatograms of *N*-caffeoyldopamine (3 pmol) (**A**), *N*-caffeoyltyramine (3 pmol) (**B**), and *N*-feruloyltyramine (3 pmol) (**C**). Chromatograms were detected using an electrochemical detector at 800 mV.

acid and tyramine) may be found together. Thus, the mobile phase was first formulated to optimize a separation condition for *N*-caffeoyltyramine from its precursors. Several buffer conditions were evaluated, and an isocratic buffer (50 mM NaH_2PO_4, pH 4.5, containing 40% methanol) was found suitable for the analysis of the sample containing *N*-caffeoyltyramine, and its precursors, for which retention times were 6.4, 2.5, and 1.4 min, respectively (**Figure 4**). Because the isocratic buffer developed herein demonstrated an excellent separation condition for *N*-caffeoyltyramine from its precursors, the buffer was further examined to find out whether it was applicable for separating *N*-caffeoyltyramine from its clovamide-type phenylpropenoic acid amide analogues (*N*-caffeoyldopamine and *N*-feruloyltyramine), often found together in plants. Using the buffer, *N*-caffeoyltyramine, *N*-caffeoyldopamine, and *N*-feruloyltyramine were adequately separated with discrete retention times of 4.0, 6.4, and 12 min, respectively (**Figure 5**). These discrete retention times enabled *N*-caffeoyltyramine, *N*-caffeoyldopamine, and *N*-feruloyltyramine to be analyzed simultaneously in one sample. However, the HPLC analysis time had to be extended beyond 10 min, because the retention time for *N*-feruloyltyramine was 12 min, later than the other two.

Measurement of *N*-Caffeoyltyramine and *N*-Caffeoyldopamine in Cell Samples. Although all of the assay conditions were optimized to separate *N*-caffeoyltyramine from its precursors and the analogues in standard samples, the method still

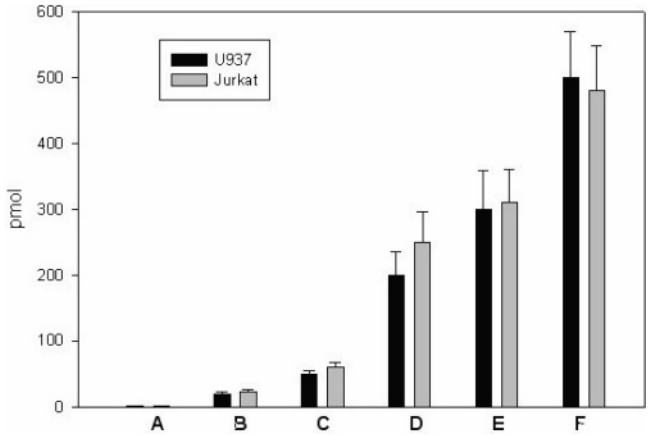

Figure 6. Determination of *N*-caffeoyltyramine in U937 and Jurkat cells treated with *N*-caffeoyltyramine (**A** (0.1 μM), **B** (0.5 μM), **C** (1.0 μM), **D** (5.0 μM), **E** (10 μM), and **F** (20 μM)) for 15 min. The samples were extracted with the buffer containing 40% methanol.

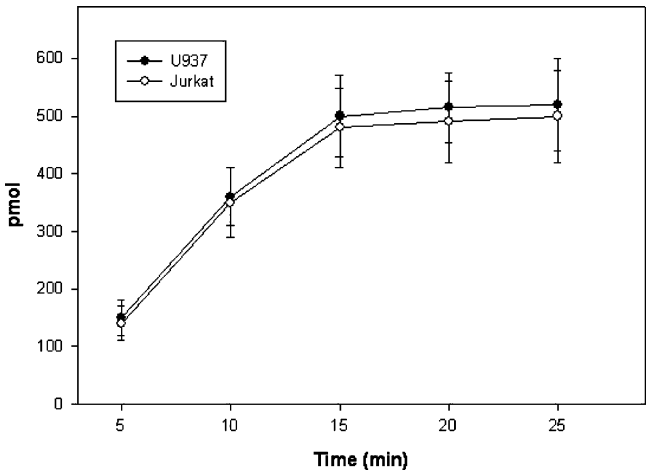

Figure 7. Accumulation of *N*-caffeoyltyramine in U937 and Jurkat cells treated with *N*-caffeoyltyramine (20 μM), over time. The samples were extracted with the buffer containing 40% methanol.

needed to be validated in biological samples. Human monocytic and lymphocyte cells (U937 and Jurkat cells) were used for preparing cell samples, because these cells are likely to be exposed to *N*-caffeoyltyramine and *N*-caffeoyldopamine, if these compounds are consumed by humans. The cells were treated with various concentrations of *N*-caffeoyltyramine (0.1, 0.5, 1, 5, 10, and 20 μM), incubated for 15 min, and extracted with the buffer containing 40% methanol. The supernatant was analyzed using the same HPLC method. As expected, *N*-caffeoyltyramine was detected with the same retention time (6.4 min) in the cell samples. The amount of *N*-caffeoyltyramine detected in the cell samples increased proportionally with the amount of the *N*-caffeoyltyramine added to the samples (**Figure 6**). *N*-Caffeoyltyramine accumulated in the cells also increased proportionally with the length of the incubation times between 0 and 15 min, but no significant increase could be observed after 15 min (**Figure 7**). Because an extraction procedure was employed in preparing cell samples, the recovery of *N*-caffeoyltyramine was determined after the first and second extractions. U937 and Jurkat cells were incubated with *N*-caffeoyltyramine (50 μM) for 15 min, and extracted with the buffer containing 40% methanol. The recovery of *N*-caffeoyltyramine in the first extract was more than 95%, thus necessitating no second extraction. Identical cell experiments were also performed using *N*-caffeoyldopamine, and similar results

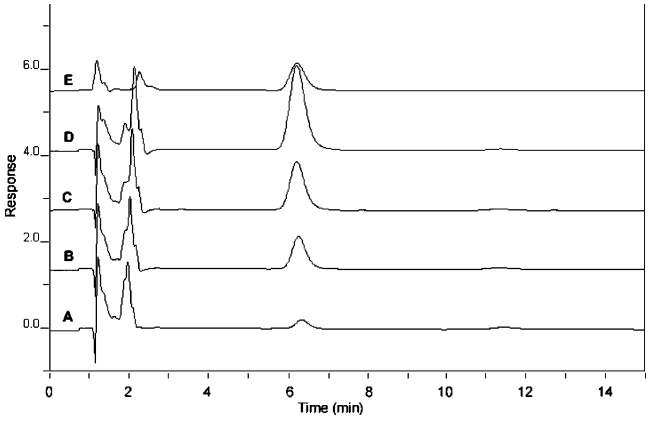

Figure 8. HPLC assay of *N*-caffeoyltyramine in plasma samples from whole blood collected from Swiss Webster mice. *N*-Caffeoyltyramine was added to the blood samples to give final concentrations of 0.5 μM (**A**), 2.5 μM (**B**), 5 μM (**C**), and 10 μM (**D**), and 2 μM *N*-caffeoyltyramine standard (**E**).

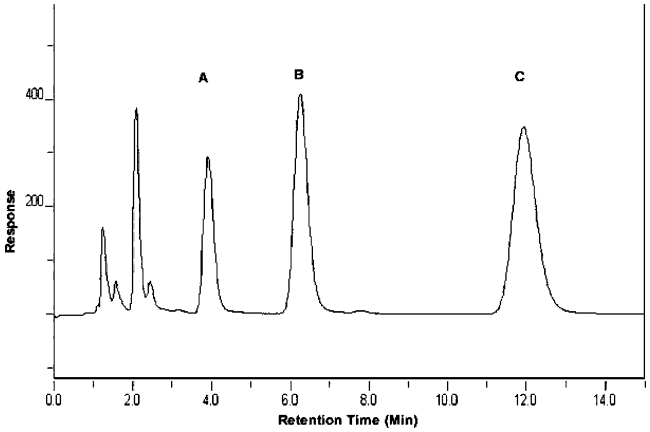

Figure 9. Chromatograms of *N*-caffeoyldopamine (3 pmol) (**A**), *N*-caffeoyltyramine (3 pmol) (**B**), and *N*-feruloyltyramine (3pmol) (**C**) in plasma samples. Chromatograms were detected using an electrochemical detector at 800 mV.

were obtained, except for the different retention time (4 min) of *N*-caffeoyldopamine (data not shown).

Measurement of *N*-Caffeoyltyramine and *N*-Caffeoyldopamine in Plasma Samples. The HPLC assay described above was further validated in plasma samples. For preparing plasma samples, whole blood was collected from Swiss Webster mice, and *N*-caffeoyltyramine was added to the blood samples to provide final concentrations of 0.5, 2.5, 5, and 10 μM. The chromatograms of *N*-caffeoyltyramine in plasma samples are shown in **Figure 8**. As expected, *N*-caffeoyltyramine was detected at the same retention time of 6.4 min as standard and cell samples. To determine the extraction recovery from the plasma samples, *N*-caffeoyltyramine was added to the plasma (1 pmol/μL) and analyzed as described above. The recovery of *N*-caffeoyltyramine in the plasma samples was more than 95% in five replicate preparations. This indicates that the supernatant obtained after the precipitation with the extraction buffer contains approximately 95% *N*-caffeoyltyramine in mice plasma. As shown in standard and cell samples, the HPLC method also demonstrated satisfactory resolutions of *N*-caffeoyltyramine and its analogues in plasma samples (**Figure 9**). Several replicate analyses of plasma samples incubated with *N*-caffeoyltyramine, *N*-caffeoyldopamine, or *N*-feruloyltyrmaine (10 μM) also demonstrated that the variation of the mean retention times was less than 0.1 min and the area of an individual peak was less than

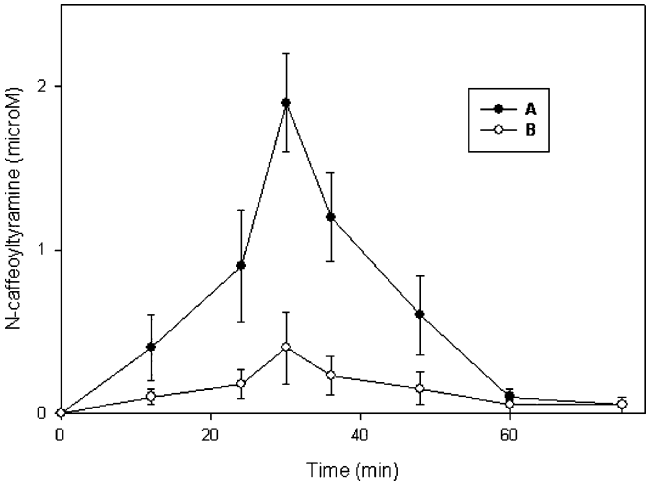

Figure 10. Determination of *N*-caffeoyltyramine in mice plasma following oral administration to Swiss Webster mice. *N*-Caffeoyltyramine (0.5 mg (**A**) and 2 mg (**B**)/30 g body weight) was orally provided to the mice.

Table 1. Concentrations of *N*-Caffeoyltyramine in Mice Plasma Following Oral Administration to Swiss Webster Mice[a]

time	*N*-caffeoyltyramine
30 min	50 ± 10 nM

[a] *N*-Caffeoyltyramine (0.1 mg/30 g body weight) was orally provided to the mice.

1%, indicating that the HPLC method can provide excellent precision for determining *N*-caffeoyltyramine, *N*-caffeoyldopamine, and *N*-feruloyltyramine in plasma samples.

Determination of *N*-Caffeoyltyramine in Mouse Plasma Following Oral Administration. Clovamide-type phenylpropenoic acid amides have been reported to demonstrate their specific activities depending on the concentrations (*16*, *17*). Therefore, plasma concentrations of the phenylpropenoic acid amides following the oral intakes should be determined as an index for determining their biological activities. Because the HPLC assay developed herein was sensitive enough to detect *N*-caffeoyltyramine in as little as 100 fmol in the plasma samples, the plasma concentrations after oral administrations to mice were determined. *N*-Caffeoyltyramine was detected only in plasma samples prepared from the oral dosing groups. The average concentrations of *N*-caffeoyltyramine ($n = 5$) in the plasma collected at 12, 24, 30, 36, 48, 60, and 75 min after the first and second oral administrations were 0.10, 0.18, 0.40, 0.23, 0.15, 0.05, and 0.03 μM, and 0.40, 0.90, 1.90, 1.20, 0.60, 0.10, and 0.05 μM, respectively. As shown in **Figure 10**, a fair amount of *N*-caffeoyltyramine could be detected around 24 min after the oral administration, and the highest amount was detected at around 30 min. After that, plasma concentrations began to decrease, suggesting that *N*-caffeoyltyramine taken orally may be metabolized quickly after 30 min. Also, the plasma concentrations were fairly well correlated to orally administrated amounts, suggesting that plasma concentrations may be used for calculating oral administration doses. For instance, the plasma concentrations of *N*-caffeoyltyramine are expected to be around 0.01−0.05 μM, after its oral administration at 0.1 mg/30 g body weight based on **Figure 10**. Indeed, the plasma concentration was approximately 0.05 μM in the plasma samples collected at 30 min after the oral administration of 0.1 mg/30 g body weight (**Table 1**).

In this study, a high-performance liquid chromatography (HPLC) method was developed to determine the clovamide-type phenylpropenoic acid amides, *N*-caffeoyltyramine and

N-caffeoyldopamine, in biological samples. The HPLC analyses were performed using an isocratic buffer, which provides several advantages over gradient buffer conditions. First, an isocratic condition provides consistent currents in the sample chamber of an electrochemical detector, which keeps the signal background stable. Second, there is no need for column equilibrium because the buffer condition is continuous, which enables the method to perform high-throughput measurement more efficiently than does a gradient buffer. Although the peak resolutions of *N*-caffeoyltyramine and *N*-caffeoyldopamine in biological samples were sufficient for this study, the resolution can be improved by adopting different gradient buffer conditions. However, any variation of buffer conditions might lengthen the total analysis time. Also, different buffer conditions might cause some of the clovamide-type phenylpropenoic acid amides to be insoluble because *N*-caffeoyltyramine is more likely to precipitate in aqueous buffers than *N*-caffeoyldopamine. Therefore, the solubility of clovamide-type phenylpropenoic acid amides should be considered, when new buffer conditions are formulated.

As described above, good peak resolutions for *N*-caffeoyltyramine and *N*-caffeoyldopamine could be obtained using this isocratic buffer condition, but their peak heights were different even though the same amount of each compound was analyzed. The peak height difference is likely due to the different redox potentials and electro-reactivity of the individual compounds. None of the compounds exhibited a peak at 100 mV, only *N*-caffeoyldopamine began to show a peak at 110−280 mV, and both compounds (*N*-caffeoyltyramine and *N*-caffeoyldopamine) exhibited peaks at higher than 300 mV. Actually, the difference in the electro-reactivity may be useful in concurrently analyzing compounds with similar retention times as well as evaluating the purity of each compound in the samples, because pure chemicals have inherent and consistent ratios between peak heights shown at different potentials as compared to impure chemicals.

Excellent linearity, sensitivity, precision, and recovery of *N*-caffeoyltyramine and *N*-caffeoyldopamine were observed in cell and plasma samples. The detection limit in cell and plasma samples can be as low as 100 fmol, and the chromatograms in cell and plasma samples were identical to those of *N*-caffeoyltyramine and *N*-caffeoyldopamine standards. The recovery of *N*-caffeoyltyramine and *N*-caffeoyldopamine extracted from cell samples was evaluated using the HPLC method, to optimize conditions for their extraction. The first and second extractions were performed and analyzed, and the recovery was determined by measuring three different samples ($n = 5$): the two samples of supernatants from the first and second extractions, and the sample of final precipitate. In five replicate preparations, the recovery of *N*-caffeoyltyramine and *N*-caffeoyldopamine in the first extraction was more than 95%, and the recovery was very consistent. These data indicate that the first extraction may be enough for preparing cell samples. Also, the stability in the cell samples was measured. Two cell samples (*N*-caffeoyltyramine and *N*-caffeoyldopamine; 50 μM) were prepared and stored at −20 °C, for 24 h, 48 h, 1 week, and 2 weeks, and their concentrations were determined. The concentration changes over time were not greater than a standard deviation. These data indicate that the samples can be stored at −20 °C to yield consistent and reproducible measurements.

For preparing plasma samples, whole blood was collected from Swiss Webster mice fed AIN-76A purified mice diet. For confirming *N*-caffeoyltyramine and *N*-caffeoyldopamine not in the diet, the mice diet was extracted and analyzed. Neither could be detected in the samples extracted from the mice diet and plasma samples. For preparing plasma samples, an extraction method different from the cell extraction method was used. Therefore, the recovery from plasma samples was again evaluated using the HPLC method. The recovery of *N*-caffeoyltyramine and *N*-caffeoyldopamine in the plasma samples was better than 95% in five replicate preparations. This indicates that the supernatant obtained after the precipitation with methanol contained approximately 95% *N*-caffeoyltyramine in mice plasma.

The HPLC method developed herein provided a superior separation condition for two clovamide-type phenylpropenoic acid amides in biological samples. Because of the capability of the method to provide good peak resolutions, this method may be applicable for analyzing not only *N*-caffeoyltyramine and *N*-caffeoyldopamine, but also other clovamide-type phenylpropenoic acid amides in cell and plasma samples. Indeed, *N*-feruloyltyramine was satisfactorily separated from *N*-caffeoyltyramine and *N*-caffeoyldopamine in biological samples using the HPLC method. The retention times of the three clovamide-type phenylpropenoic acid amides were discrete enough to analyze them simultaneously in plasma samples. However, HPLC analysis time had to be extended beyond 10 min, because the retention time for *N*-feruloyltyramine was around 12 min, later than those of *N*-caffeoyldopamine and *N*-caffeoyltyramine. In fact, *N*-caffeoyltyramine can be methylated at hydroxyl groups of tyramine and caffeic acid moieties in the compound and converted to several methylated forms of *N*-caffeoyltyramine. In particular, methylation at the 4-hydroxyl group of the caffeic acid moiety in the compound converts *N*-caffeoyltyramine into *N*-feruloyltyramine. Due to this methylation, *N*-feruloyltyramine is likely to be less hydrophilic than *N*-caffeoyltyramine, thereby making the retention time of *N*-feruloyltyramine longer than that of *N*-caffeoyltyramine. *N*-Caffeoyltyramine and its analogues have been reported to have significant effects on molecular and cellular events such as apoptosis and platelet activation, and the effects are dependent on its cellular and plasma concentrations. For instance, at concentrations between 0.05 and 0.5 μM, *N*-caffeoyltyramine has more effects on platelet activation than others (*15*−*17*). Therefore, plasma concentrations of *N*-caffeoyltyramine should be determined to find out the effects it might provide following oral consumption. Based on the data, the oral administrations of *N*-caffeoyltyramine (0.1, 0.5, and 2 mg/30 g body weight) can provide its plasma concentrations of 0.05−0.22 μM, where platelet activation can be inhibited. This method may be a useful tool in future studies for investigating cellular and/or molecular functions of *N*-caffeoyltyramine analogues, such as their absorption, metabolism, and bio-availability.

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Received for review July 6, 2005. Revised manuscript received August 15, 2005. Accepted August 16, 2005.

JF0516078