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Quantification of N^{τ}-Methylhistidine and N^{π}-Methylhistidine in Chicken Plasma by Liquid Chromatography–Tandem Mass Spectrometry

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The concentration of *N*$^{\tau}$-methylhistidine in plasma provides an index of skeletal muscle protein breakdown. This study aimed to establish a quantitative method for measuring the concentrations of *N*$^{\tau}$-methylhistidine and its isomer *N*$^{\pi}$-methylhistidine in chicken plasma, using liquid chromatography–tandem mass spectrometry with stable isotope dilution analysis. The acceptable linear ranges of detection were 1.56–50.00 μmol/L for *N*$^{\tau}$-methylhistidine and 0.78–25.00 μmol/L for *N*$^{\pi}$-methylhistidine. The proposed method detected changes in the plasma levels of *N*$^{\tau}$-methylhistidine and *N*$^{\pi}$-methylhistidine in response to fasting and re-feeding. These results suggest that the method developed in this study can be used for the simultaneous measurement of *N*$^{\tau}$-methylhistidine and *N*$^{\pi}$-methylhistidine in chicken plasma.

Key words: LC–MS/MS, method validation, *N*$^{\tau}$-methylhistidine, *N*$^{\pi}$-methylhistidine

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Introduction

The amino acid *N*$^{\tau}$-methylhistidine is abundant in actin and myosin, the major proteins in skeletal muscle. Urinary excretion and/or plasma concentration of *N*$^{\tau}$-methylhistidine have been used to evaluate the rate of myofibrillar protein degradation in small animals and cattle[1–4], as well as overall skeletal muscle protein breakdown in birds[2,5,6].

The presence of *N*$^{\tau}$-methylhistidine is typically determined via high-performance liquid chromatography with ninhydrin derivatization and visible light detection[7], *ortho*-phthalaldehyde (OPA) derivatization and fluorescence detection[2,8], and phenyl isothiocyanate derivatization and ultraviolet light detection[9]. Recently, liquid chromatography–mass spectrometry (LC–MS) and gas chromatography–mass spectrometry have been used for the determination of *N*$^{\tau}$-methylhistidine levels in mammals and chickens[10–14]. However, in MS and especially LC–MS, the co-elution of similar compounds in biological samples (e.g., plasma, serum, and urine) has raised questions about the efficiency and reproducibility of the ionization source (i.e., the so-called matrix effect). Although stable-isotope dilution analysis, which uses isotopic analogs as internal standards, effectively suppresses the matrix effect, the methods mentioned above for measuring *N*$^{\tau}$-methylhistidine do not use stable-isotope dilution analysis or tandem mass spectrometry (MS/MS).

Given the above limitations, we developed and validated a method for the quantification of *N*$^{\tau}$-methylhistidine in chicken plasma that combines stable-isotope dilution and liquid chromatography-MS/MS (LC-MS/MS). The proposed protocol was compared with ultra-high-performance liquid chromatography (UHPLC) coupled with OPA derivatization and fluorescence

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detection for the quantification of N^{τ}-methylhistidine in amino acids. N^{π}-Methylhistidine, an isomer of N^{τ}-methylhistidine, is a component of the imidazole dipeptide anserine (β-alanyl-N^{π}-methylhistidine)[15–17]. Given that chicken meat contains more anserine than beef or pork[18], we developed and validated an LC–MS/MS protocol for quantifying N^{π}-methylhistidine in chicken plasma.

Materials and Methods

Reagents

N^{τ}-Methylhistidine (N^{τ}-methyl-L-histidine) was purchased from Millipore (Burlington, MA, USA). N^{π}-Methylhistidine (N^{π}-methyl-L-histidine) and Wako Amino Acids Mixture Standard Solutions Type H were purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan). N^{τ}-Methyl-d_3-histidine (N^{τ}-methyl-d_3-L-histidine) was purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA).

Preparation of stock solutions and working solution

Mixed solutions of standards and internal standards were prepared separately. Solutions of N^{τ}-methylhistidine and N^{τ}-methyl-d_3-histidine internal standards were prepared at a final concentration of 5 mmol/L, along with 5 mmol/L N^{π}-methylhistidine standard. To avoid repeated freezing and thawing of these standard solutions, 100-µL aliquots were stored separately at -80 °C until use.

Animals and sample preparation

All experimental protocols and procedures were reviewed and approved by the Animal Care and Use Committee of Kagoshima University (approval number: A21003). Eight male Ross 308 broiler chicks (*Gallus gallus domesticus*) at 0 days of age were provided by a commercial hatchery (Kumiai Hina Center, Kagoshima, Japan). Chicks were housed in an electrically heated battery brooder in a temperature-controlled room at 30 °C, and the thermostat was turned down by 1 °C every 2 days. Once the chicks reached 10 days of age, the room was maintained at 25 °C. A continuous lighting program (20 h light, 4 h darkness) was used. The chicks were provided with *ad libitum* water and a semi-purified diet with no animal protein (Table 1) until 25 days of age. Then, they were individually housed in wire-bottomed aluminum cages (40 × 50 × 60 cm) located in the temperature-controlled room at 25 °C. All the chicks had free access to food and water. At 28 days of age, the chicks were fasted for 24 h and allowed free access to food for another 24 h. During this 48-h period, blood (500 µL) was collected from the wing vein, and body weight was measured every 12 h (five times). Blood samples were immediately centrifuged at 5900 × *g* for 10 min at 4 °C with heparin sodium (10–20 IU/mL) to separate the plasma. The plasma (100 µL) from individual chicks was stored at -80 °C and was used to determine N^{τ}-methylhistidine and N^{π}-methylhistidine levels under fasted and re-fed conditions. The remaining plasma samples were mixed into pooled plasma samples for use in method validation.

LC–MS/MS analysis

The N^{τ}-methyl-d_3-histidine standard solution (5 mmol/L) was diluted to 400 nmol/L immediately prior to the experiment. Aliquots of plasma sample (5 µL) were mixed with 250 µL water, 182.5 µL phosphate-buffered saline, 62.5 µL N^{τ}-methyl-d_3-histidine standard solution (400 nmol/L), and 500 µL acetonitrile. After vigorous shaking, the samples were centrifuged at 20,000 × *g* and 4 °C for 5 min. The supernatant was filtered through a sterilized 0.22-µm membrane (TORAST Disc; Shimadzu, Kyoto, Japan).

LC–MS/MS analysis was performed on an LCMS-8050 instrument (Shimadzu). Samples were analyzed using multiple reaction monitoring (MRM) and electrospray ionization. For LC analysis, an Intrada Amino Acid column (3 µm, 100 × 3.0 mm; Imtakt, Kyoto, Japan) was used. Mobile phases A (acetonitrile/formic acid, 100:0.3, v/v) and B (acetonitrile/100 mM ammonium formate, 20:80, v/v) were used for gradient elution as follows: 0–4 min, 20% B; 4–14 min, 20%–100% B; and 14–16 min, 100% B. The flow rate was 0.6 mL/min, column temperature was 37 °C, and sample injection volume was 5 µL. MS analysis conditions were as follows: positive ionization mode, 3 L/min nebulizer gas flow rate, 10 L/min heating gas flow rate, 300 °C interface temperature, 400 °C DL temperature, and 10 L/min drying gas flow rate. MRM transitions and collision energies were individually optimized for N^{τ}-methylhistidine, N^{π}-methylhistidine, and the internal standard using Shimadzu LabSolutions software. Optimal dwell time was experimentally determined for each component (Table 2). Two quantitative ions (*m*/*z* 170.20>96.10 and 170.20>124.10) were used for N^{τ}-methylhistidine, *m*/*z* 170.20>96.10 for N^{π}-methylhistidine, and *m*/*z* 173.20>127.10 for the internal standard.

UHPLC analysis

Aliquots of plasma samples (50 µL) were mixed with 250

Table 1. **Composition and analysis of the basal diet**

Ingredients (g/100 g)	
Corn meal	57.90
Soybean meal	34.00
Corn oil	4.30
$CaCO_3$	0.66
$CaHPO_4$	2.00
NaCl	0.50
DL-Methionine	0.14
Mineral and vitamin premix[a]	0.50
Calculated analysis	
Crude protein (%)	20.0
Metabolizable energy (Mcal/kg)	3.1

[a] Content per kilogram of vitamin and mineral premix: vitamin A, 90 mg; vitamin D3, 1 mg; DL-alpha-tocopherol acetate, 2000 mg; vitamin K3, 229 mg; thiamin nitrate, 444 mg; riboflavin, 720 mg; calcium D-pantothenate, 2174 mg; nicotinamide, 7000 mg; pyridoxine hydrochloride, 700 mg; biotin, 30 mg; folic acid, 110 mg; cyanocobalamin, 2 mg; calcium iodate, 108 mg; MgO, 198,991 mg; $MnSO_4$, 32,985 mg; $ZnSO_4$, 19,753 mg; $FeSO_4$, 43,523 mg; $CuSO_4$, 4019 mg; and choline chloride, 299,608 mg.

Table 2. **MS/MS conditions during MRM transition for the analyzed compound**

Compound name	Polarity	Precursor ion (*m/z*)	Product ion (*m/z*)	Dwell time (ms)	Collision energy (V)
N^{τ}-Methylhistidine	+	170.20	124.10	100	−17.0
N^{τ}-Methyl-d_3-histidine	+	173.20	127.10	100	−14.0
N^{π}-Methylhistidine	+	170.20	96.00	100	−19.0

μL water, 200 μL norvaline solution (50 μmol/L) as internal standard, and 500 μL acetonitrile. After vigorous shaking, the samples were centrifuged at 20,000 × *g* and 4 °C for 5 min. The supernatant was filtered through a sterilized 0.22-μm membrane (TORAST Disc).

UHPLC analysis was performed using a NexeraX2 system (Shimadzu) with a Kinetex EVO C18 column (2.6 μm × 100 × 3.0 mm). Amino acids were separated using a pre-column, according to a previously described method for plasma-free amino acids[19,20]. Gradient elution was performed using mobile phase A (17 mM potassium dihydrogen phosphate, 3 mM dipotassium hydrogen phosphate) and mobile phase B (distilled water/acetonitrile/methanol, 15:45:40, v/v/v) according to the following program: 0–1.5 min, 11% B; 1.5–6 min, linear increase from 11% to 22% B; 6–8 min, from 22% to 30% B; 8–10.5 min, from 30% to 53% B; 10.5–12.5 min, 53% B; 12.5–13 min, from 53% to 100% B; 13–17 min, 100% B; 17–17.5 min, linear decrease from 100% to 11% B, followed by a re-equilibration step of 5.5 min under the initial conditions. Pre-column derivatization was performed using the UHPLC system with 45 μL mercaptopropionic acid, 22 μL OPA, and 7.5 μL sample. The mixture was allowed to rest for 2 min; then, 5 μL fluorenylmethyl chloroformate was added, the mixture was allowed to rest for 2 min, and 1 μL of the mixture was injected in the column. The flow rate was 0.85 mL/min and the column temperature was 35 °C. The RF-20Axs high-sensitivity fluorescence detector was set to Ch1 (excitation wavelength, 350 nm; emission wavelength, 450 nm) and Ch2 (excitation wavelength, 266 nm; emission wavelength, 305 nm).

Validation method

Validation conformed to the guidelines for analytical procedures and method validation provided by the Food and Drug Administration[21].

Linearity of the calibration curve

The ranges of standard solutions were 0.10–50.00 μmol/L for N^{τ}-methylhistidine and 0.05–25.00 μmol/L for N^{π}-methylhistidine. To ensure accurate linearity of the calibration curve, three replicates of each standard solution were prepared and loaded onto LC–MS/MS and UHPLC columns on the same day. Calibration curves were constructed using linear regression of plotted data, after which the coefficient of determination was calculated. The linearity of the calibration curve was considered acceptable when the mean coefficient of determination (r^2) for three replicates was greater than 0.995.

Intraday and inter-day repeatability

To calculate the accuracy and precision of the methods, intraday repeatability was calculated as the total value of six replicates of the pooled chicken plasma samples in one day. Inter-day repeatability (i.e., accuracy) was defined as the total value of six replicates on three consecutive days. Inter-day repeatability was validated in the same way as intraday repeatability.

Recovery test

Three concentrations (low, mid-range, and high) of the standard substances were prepared upon dilution with ultrapure water: 5, 10, 20 μmol/L for N^{τ}-methylhistidine and 2.5, 5, 10 μmol/L for N^{π}-methylhistidine. Each standard substrate at the above concentrations was added to individual pooled chicken plasma samples. The recovery percentage of each standard sample was calculated using the following equation: recovery percentage = (standard-added plasma - standard-free plasma) / (theoretical value) × 100. Recovery tests were conducted in triplicate.

Accuracy and precision

Accuracy and precision of the methods were evaluated using three concentrations of N^{τ}-methylhistidine (5, 10, and 20 μmol/L) and N^{π}-methylhistidine (2.5, 5, and 10 μmol/L). Accuracy was defined as the percentage recovery obtained using the following equation: accuracy = (measured mean value - theoretical value) / (theoretical value) × 100. Precision was defined as the relative standard deviation (RSD), calculated using the following equation: RSD = (standard deviation / measured mean value) × 100. Accuracy and precision values were considered acceptable if within 85%–115%.

Statistical analysis

All data are presented as the mean ± standard error. Two-way repeated analysis of variance, with factors of fasting or re-feeding and elapsed hours after each treatment (0, 12 or 24 h), was used to analyze broiler chicken body weight and plasma concentrations of N^{τ}-methylhistidine, as well as plasma-free amino acids. Subsequently, multiple paired *t*-tests were conducted between each time point (0, 12, 24, 36, and 48 h), with *P* values adjusted using the Benjamini-Hochberg method[22]. An adjusted P value < 0.05 was considered statistically significant. All analyses were performed using R software[23].

Results and Discussion

Peak specificity

Figure 1 shows representative chromatograms of N^{τ}-methylhistidine and N^{π}-methylhistidine samples, as well as the internal standard N^{τ}-methyl-d_3-histidine, obtained via LC–MS/MS. Retention times of the detected peaks were as follows: 15.43 min (N^{τ}-methylhistidine), 16.15 min (N^{π}-methylhistidine),

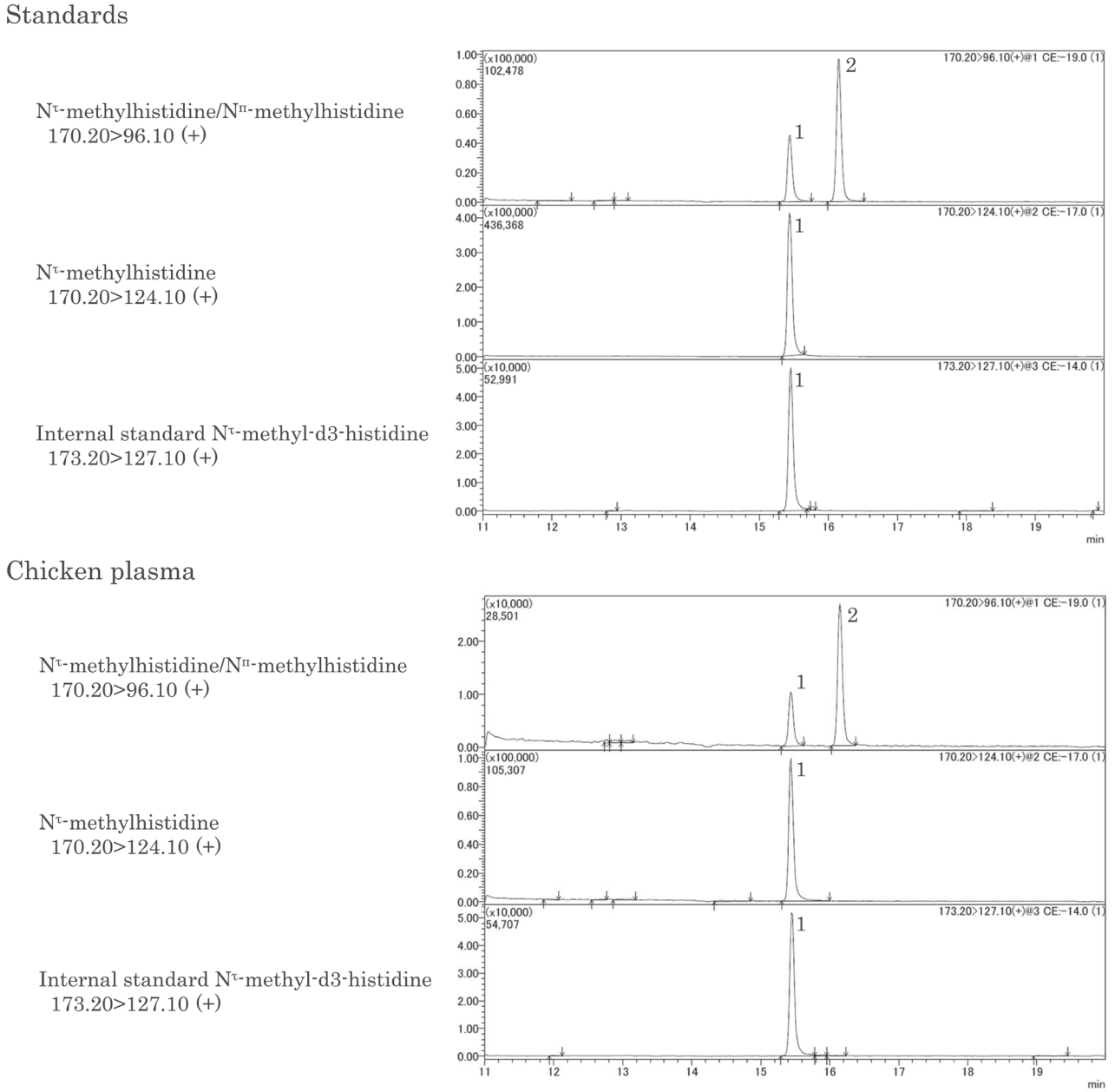

Fig. 1. **Representative LC–MS/MS chromatograms of solutions prepared with N^{τ}-methylhistidine, N^{π}-methylhistidine, and internal standard (N^{τ}-methyl-d_3-histidine), as well as chromatograms of chicken plasma samples.**
1, N^{τ}-Methylhistidine; 2, N^{π}-methylhistidine.

and 15.45 min (N^{τ}-methyl-d_3-histidine). The quantitative ions of N^{τ}-methylhistidine were m/z 170.20>96.10 and 170.20>124.10, that of N^{π}-methylhistidine was m/z 170.20>96.10, and that of the internal standard was m/z 173.20>127.10. No peaks were observed for ultrapure water at these retention times (data not shown). Clearly distinguishable peaks for N^{τ}-methylhistidine and N^{π}-methylhistidine were detected in pooled chicken plasma sample (Fig. 1), suggesting that LC–MS/MS allowed for successful determination of both compounds and their differentiation from other endogenous components.

Figure S1 shows the representative chromatograms of commercial N^{τ}-methylhistidine and N^{π}-methylhistidine standard samples obtained by UHPLC; their retention times were 3.15 and 2.35 min, respectively. Whereas no amino acid peak overlapped with that of N^{τ}-methylhistidine; the glycine peak was found to overlap with that of N^{π}-methylhistidine. No peaks for

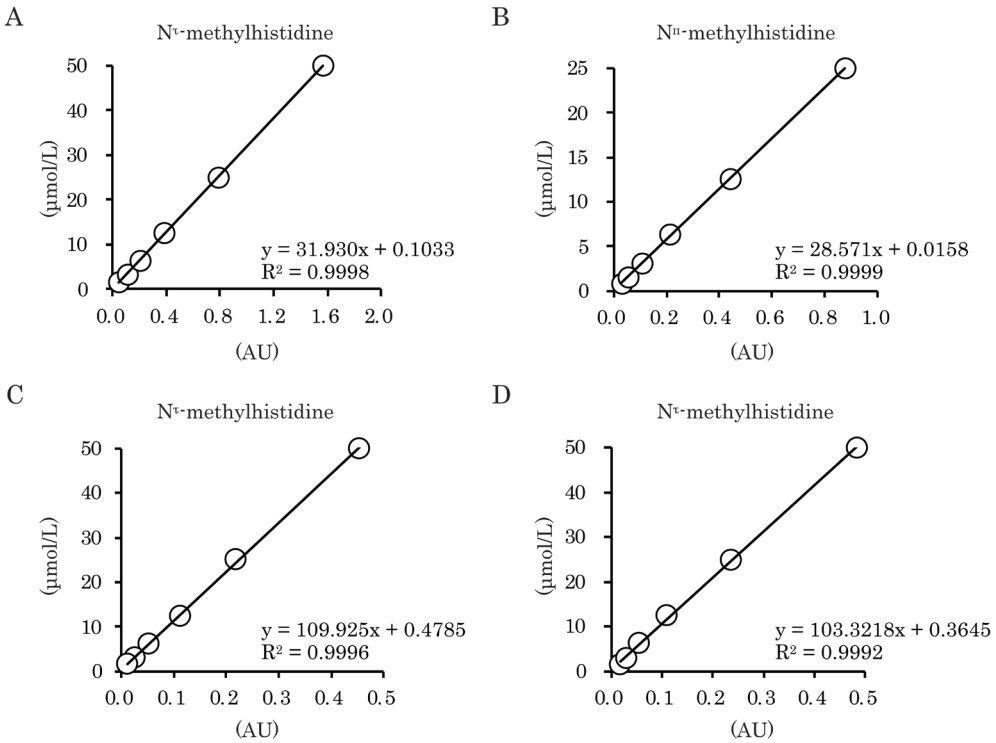

Fig. 2. **Linearity of calibration curves obtained via (A–C) LC–MS/MS and (D) UHPLC.**
(A) N^{τ}-Methylhistidine, *m*/*z* 170.20>96.10; (B) N^{π}-methylhistidine, *m*/*z* 170.20>96.10; (C) N^{τ}-methylhistidine, *m*/*z* 170.20>124.10; (D) N^{τ}-methylhistidine. AU, arbitrary unit.

ultrapure water were detected at these retention times (data not shown). A clear peak for N^{τ}-methylhistidine was observed also in pooled chicken plasma samples; whereas that for N^{π}-methylhistidine overlapped again with the one for glycine. These findings indicated that UHPLC was not reliable for quantifying N^{π}-methylhistidine and/or glycine when the sample contained both compounds (e.g., plasma or meat samples). Therefore, in subsequent experiments, UHPLC was applied only for the detection of N^{τ}-methylhistidine.

Linearity of the calibration curve

The acceptable ranges of the calibration curves obtained by LC–MS/MS were as follows: 1.56–50.0 μmol/L, r^2 = 0.9998 (N^{τ}-methylhistidine, *m*/*z* 170.20>96.10, Fig. 2A); 0.78–25.00 μmol/L, r^2 = 0.9999 (N^{π}-methylhistidine, *m*/*z* 170.20>96.10, Fig. 2B); 1.56–50.00 μmol/L, r^2 = 0.9996 (N^{τ}-methylhistidine, *m*/*z* 170.20>124.10, Fig. 2C). All concentrations above or below these ones were rejected. For UHPLC, the acceptable range of the calibration curve for N^{τ}-methylhistidine was 1.56–50.00 μmol/L, with r^2 = 0.9992 (Fig. 2D). Concentrations above or below these were rejected.

Intraday and inter-day repeatability and recovery test

Table 3 lists intraday and inter-day repeatability calculated for N^{τ}-methylhistidine (*m*/*z* 170.20>96.10 and 170.20>124.10) and N^{π}-methylhistidine (*m*/*z* 170.20>96.10) using LC–MS/MS. The accuracies were considered acceptable if they fit within the range of 85%–115%. Values were comparable with that for N^{τ}-methylhistidine obtained by UHPLC.

Table 4 lists the recovery percentages of three different concentrations of N^{τ}-methylhistidine and N^{π}-methylhistidine using LC–MS/MS. The recovery percentage ranges were 90.81%–94.67% for N^{τ}-methylhistidine (*m*/*z* 170.20>96.10), 88.00%–100.14% for N^{τ}-methylhistidine (*m*/*z* 170.20>124.10), and 92.23%–102.04% for N^{π}-methylhistidine (*m*/*z* 170.20>96.10). The recovery percentages and precision values (RSD) of N^{τ}-methylhistidine were comparable to those obtained by UHPLC and were considered acceptable when they fit within the range of 85%–115%. In contrast, the precision of N^{π}-methylhistidine detection by LC–MS/MS fell outside the acceptable range. Therefore, the results of the N^{τ}-methylhistidine recovery test using LC–MS/MS were acceptable; whereas those for N^{π}-methylhistidine require further improvement.

N^{τ}-Methylhistidine and N^{π}-methylhistidine detection in the plasma of fasted and re-fed chickens

Given that 24 h of fasting boosts N^{τ}-methylhistidine release in chicken muscle[24], we used LC–MS/MS to detect changes in plasma N^{τ}-methylhistidine in chickens fasted for 24 h and re-fed for another 24 h. Body weight dropped significantly in response to 24 h of fasting, but returned rapidly to the pre-

Table 3. **Intraday repeatability and inter-day reproducibility of assays**

	Intraday						Inter-day	
	Day 1		Day 2		Day 3			
	Measured concentration (μmol/L)	RSD (%)	Measured concentration (μmol/L)	RSD (%)	Measured concentration (μmol/L)	RSD (%)	Measured concentration (μmol/L)	RSD (%)
N^{τ}-Methylhistidine								
LC–MS/MS (*m/z* 170.20>96.10)	12.64	5.97	11.34	4.66	12.11	3.13	11.84	1.45
LC–MS/MS (*m/z* 170.20>124.10)	12.25	5.44	11.60	6.93	12.00	4.37	11.95	1.17
UHPLC	10.37	4.21	10.80	3.86	9.80	4.95	10.32	5.77
N^{π}-Methylhistidine								
LC–MS/MS (*m/z* 170.20>96.10)	7.99	9.83	7.69	6.52	8.37	9.68	8.01	2.14

Intraday and inter-day repeatability was measured as the mean value of six and three replicates, respectively. RSD (%) = (standard deviation / measured mean value) × 100.

Table 4. **Recovery by LC–MS/MS and UHPLC of plasma samples fortified with a standard solution of N^{τ}-methylhistidine or N^{π}-methylhistidine at three spiking levels**

	Recovery					
	Low		Middle		High	
	Recovery ratio (%)	RSD (%)	Recovery ratio (%)	RSD (%)	Recovery ratio (%)	RSD (%)
N^{τ}-Methylhistidine						
LC–MS/MS (*m/z* 170.20>96.10)	94.67	14.57	93.93	6.01	90.81	7.30
LC–MS/MS (*m/z* 170.20>124.10)	100.14	13.60	93.11	2.92	88.00	8.68
UHPLC	99.19	3.57	97.31	5.78	98.10	2.06
N^{π}-Methylhistidine						
LC-MS/MS (*m/z* 170.20>96.10)	94.07	30.97	102.04	9.22	94.03	15.65

The recovery ratio represents the mean value of three replicates. RSD (%) = (standard deviation / measured mean value) × 100. The three spiking levels were set to 5, 10, and 20 μmol/L (N^{τ}-methylhistidine) and 2.5, 5, and 10 μmol/L (N^{π}-methylhistidine).

Table 5. **Changes in plasma concentrations of N^{τ}-methylhistidine and N^{π}-methylhistidine in fasted and re-fed chickens**

	Fasting			Re-feeding	
	0 h	12 h	24 h	36 h	48 h
Body weight	1337.37 ± 35.96 ^{B}	1282.27 ± 38.27 ^{C}	1245.43 ± 38.24 ^{D}	1353.67 ± 38.58 ^{B}	1386.42 ± 42.73 ^{A}
N^{τ}-Methylhistidine					
LC–MS/MS (*m/z* 170.20>96.10)	6.72 ± 0.76 ^{C}	11.34 ± 0.48 ^{B}	14.60 ± 1.03 ^{A}	12.29 ± 1.18 ^{B}	11.89 ± 1.12 ^{B}
LC–MS/MS (*m/z* 170.20>124.10)	7.02 ± 0.78 ^{C}	11.69 ± 0.51 ^{B}	14.87 ± 1.06 ^{A}	12.67 ± 1.19 ^{B}	12.41 ± 1.23 ^{B}
UHPLC	6.18 ± 0.92 ^{B}	9.70 ± 0.89 ^{A}	11.93 ± 1.43 ^{A}	10.56 ± 1.26 ^{A}	10.35 ± 1.42 ^{A}
N^{π}-Methylhistidine					
LC–MS/MS (*m/z* 170.20>96.10)	6.19 ± 0.62 ^{B}	8.77 ± 0.53 ^{A}	9.21 ± 0.63 ^{A}	9.55 ± 0.71 ^{A}	8.73 ± 0.82 ^{A}

Results are expressed as the mean ± standard error ($n = 8$). Means with the same superscript letter within rows are not significantly different at $P < 0.05$.

fasting value after 12 h of re-feeding (Table 5). In contrast, the plasma N^{τ}-methylhistidine concentration determined by LC–MS/MS increased rapidly in response to 12 or 24 h of fasting, and then decreased after 12 h of re-feeding. A fasting-induced increase in plasma N^{τ}-methylhistidine was detected also by UHPLC, along with changes in other amino acids (Table S1), but a re-feeding-induced decrease could not be observed. The N^{τ}-methylhistidine concentration did not return to the level observed before fasting until after the chickens had been re-fed for 24 h. Plasma N^{τ}-methylhistidine values obtained by LC–MS/MS (m/z 170.20>96.10 and 170.20>124.10) were slightly higher than those obtained by UHPLC. These results suggest that LC–MS/MS is reliable for quantifying plasma N^{τ}-methylhistidine.

Even though the precision of N^{π}-methylhistidine analysis by LC–MS/MS fell outside the acceptable criteria, its accuracy was acceptable, suggesting that LC–MS/MS could be used to detect varying plasma N^{π}-methylhistidine levels. To this end, we attempted to detect changes in plasma N^{π}-methylhistidine in chickens, and found that they rapidly increased in response to 12 or 24 h of fasting (Table 5). This result is consistent with that of a study on Atlantic salmon following a 2-day fasting period[25]. It is likely that degradation of N^{π}-methylhistidine-containing anserine in skeletal muscles contributed to this result, as suggested by a decrease in anserine concentration in the skeletal muscle of skipjack tuna fasted for 3 days[26]. However, in murine skeletal muscles, both N^{π}-methylhistidine and anserine levels increased in response to fasting for 24 h[27]. Interestingly, a higher level of N^{π}-methylhistidine in the plasma was maintained until 24 h after re-feeding (Table 5); whereas plasma-alanine levels decreased after 24 h of re-feeding (Table S1). These results suggest that anserine and carnosine synthesis may be suppressed by the paucity of β-alanine. However, because only a few reports have focused on the biological roles and/or kinetics of N^{π}-methylhistidine in chickens and other animals, further research is necessary to gain insight into the physiological significance of higher N^{π}-methylhistidine plasma levels under fasting and re-feeding conditions. N^{π}-methylhistidine-containing anserine has several physiological functions, such as buffering and antioxidation[28], and is enriched in chicken meat[18]. Given that the turnover of anserine and its components (N^{π}-methylhistidine and β-alanine) in chickens remains controversial, we believe that LC–MS/MS could resolve such issues and help characterize the metabolism of these compounds in chickens and other animals.

In the present study, we evaluated the performance of LC–MS/MS for the quantification of N^{τ}-methylhistidine and N^{π}-methylhistidine in plasma. The detection range for N^{τ}-methylhistidine was 1.56–50.00 nmol/L and that for N^{π}-methylhistidine was 0.78–25.00 nmol/L. We report that LC–MS/MS can successfully detect changes in N^{τ}-methylhistidine and N^{π}-methylhistidine in chicken plasma in response to fasting and refeeding. Taken together, our results show that LC–MS/MS can be used to reliably quantify N^{τ}-methylhistidine and N^{π}-methylhistidine in chicken plasma. However, because the collision energies for N^{τ}-methylhistidine, N^{τ}-methyl-d_3-histidine, and N^{π}-methylhistidine were optimized separately using Shimadzu LabSolutions software, their ionization efficiencies may be different in other studies. Further research on the collision energies and ionization efficiencies of these compounds may improve their quantitative analysis. Moreover, the proposed LC–MS/MS method can measure amino acids without the need for derivatization, and can separate and detect target compounds from co-eluting compounds using the MRM mode. Hence, LC–MS/MS might be more appropriate than other methods and could potentially measure both N^{τ}-methylhistidine and N^{π}-methylhistidine even in the presence of interfering co-eluting compounds.

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Author Contributions

Jun-ichi Shiraishi, Shozo Tomonaga, Saki Shimamoto, and Daichi Ijiri conceived and designed the study. Jun-ichi Shiraishi, Shinya Ishihara, Ayumi Katafuchi, and Daichi Ijiri performed the experiments and contributed reagents, materials, and analytical tools. Ayumi Katafuchi, Saki Shimamoto, and Daichi Ijiri wrote the manuscript. Jun-ichi Shiraishi, Shozo Tomonaga, Hanwool Do, Shinya Ishihara, and Akira Ohtsuka reviewed and revised the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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