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Intestinal Absorption and Blood Clearance of L-Histidine-Related Compounds after Ingestion of Anserine in Humans and Comparison to Anserine-Containing Diets

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Anserine is a bioactive dipeptide found in muscles and brains of vertebrates, but little is known about the kinetics of its absorption into blood and the clearance after the ingestion of anserine or anserine-containing diets. This study investigated time-dependent changes in the concentrations of L-histidine-related compounds from deproteinized blood. The concentration of anserine peaked and then decreased to zero, whereas the concentration of π-methylhistidine gradually increased, at which point anserine was not detected. Thus, ingested anserine is absorbed intact in human blood and is hydrolyzed to π-methylhistidine and β-alanine by serum and tissue carnosinases. Moreover, the crossover study suggests that there was no significant difference in absorption under curves of anserine between anserine alone and anserine-containing diet, whereas there was significant difference in the peak concentration of anserine. This is the first study to demonstrate intestinal absorption and blood clearance of anserine.

KEYWORDS: Anserine; histidine; carnosinase; dietary supplement; blood clearance

INTRODUCTION

Anserine (β-alanyl-π-methylhistidine) and carnosine (β-alanyl-L-histidine) are cytoplasmic dipeptides found in high concentrations in the muscles and brains of vertebrates (*1*), but they were not found in rice, wheat, and soybean (*2*). These dipeptides are particularly abundant in the white muscles of migratory marine fish (*3*) and the breast muscles of poultry (*4*). Anserine and carnosine have various physiological activities: acting as an antioxidant (*5*); activation of myosin ATPase (*6*); regulation of Ca^{2+} sensitivity and excitation-contraction coupling (*7*); pH buffering (*8*); protection of proteins against glycation (*9*); and controlling autonomic nerve activities (*10*). Thus, if ingested anserine and carnosine can be effectively absorbed and enter into the blood stream in humans, they may be useful as food ingredients that have a benefit to the human body.

The fate of orally ingested peptides is now well documented. Small peptides are not completely hydrolyzed by intestinal peptidase to be absorbed as free amino acids, but are transported by peptide transport systems in the small intestinal brush border (*11*). Carnosine is also thought to be transported across the brush border membrane via the peptide transporter PEPT1 (*12*). In fact, carnosine has been confirmed to be absorbed intact in human plasma and is excreted in urine after the ingestion of carnosine alone (*13*), white muscle from eels (*14*), and beef (*15*). Moreover, it has been confirmed that carnosine absorption is affected by hypertonic solutions (*13*) or certain proteins (*16*).

On the other hand, there are very few studies of anserine. Although Abe et al. (*14*) reported the urinary excretion of L-histidine-related compounds (HRC; anserine, carnosine, L-histidine, and π-methylhistidine) after the ingestion of skipjack tuna and chicken meats, intestinal absorption and blood clearance of HRC after the ingestion anserine alone were not elucidated. Therefore, the aim of this study was to determine the effects of anserine ingestion on intestinal absorption and blood clearance of HRC. Moreover, because purified anserine is not broadly marketed as food, we also assessed the effects of anserine-containing diets (a dietary supplement and the common Japanese cuisine) on the absorption and blood clearance of HRC in a crossover design.

MATERIALS AND METHODS

Subjects. Seven healthy volunteers (30.9 ± 7.2 years; 56.7 ± 8.9 kg; 5 males and 2 females) participated. The study was performed according to the Declaration of Helsinki and was approved by the Human Research Committee of our institutional review board. Written informed consent was obtained from all subjects prior to the beginning of the study.

Analysis of Postabsorptive Degradation. Human whole blood and plasma were collected from seven donors and stored at −80 °C until the degradation study. A 90 μL volume of sample (plasma, deprotein-

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ized plasma, and deproteinized whole blood) was preincubated on ice or at 37 °C for 10 min. The reaction was started by the addition of a 5 μL volume of anserine (prepared 4.2 mM in H_2O). After incubation, 10 μL of 20% trichloroacetic acid (TCA; Wako Pure Chemical Industries, Osaka, Japan) was added to the solution to stop the reaction. The solution was deproteinized by centrifugation, and the supernatant was analyzed for HRC by HPLC according to the method of Abe et al. (*17*).

Preparation of Anserine Samples. Edible-grade anserine was purified from fish extract with food-grade reagents. Muscle of skipjack tuna (800 g) was boiled in 1.5 L of tap water for 1 h, and the broth was collected and filtered through no. 2 paper (Advantec Toyo Kaisha Ltd., Tokyo, Japan). It was then ultrafiltered to remove macromolecules, such as proteins, through a CF-30 membrane (Amicon, Beverly, MA), desalted with an NTR-729 reverse osmotic membrane (Nitto Denko Co., Tokyo, Japan), and purified on an ion-exchange column of cation-exchange resin Dowex50-X4 (*18*). After lyophilization, anserine powder was obtained and analyzed for anserine content by HPLC.

Marine Active (Yaizu Suisankagaku Industry Co., Shizuoka, Japan) was used as a commercial dietary supplement. It contains 10.3% of anserine, 7.1% of L-histidine, 0.93% of carnosine, 1.9% of other amino acids, 5.0% of protein (derived from extract of skipjack and yellowfin tuna), and 70.0% of dextrin. Determination of HRC in this product was performed by HPLC.

The experimental diet consisted of chicken breast, white muscle of skipjack, *tataki* (in which the surface of the skipjack fillet is seared), ginger, Welsh onion, precooked rice, and salad (cherry tomatoes and green stems of fresh garlic). All ingredients were purchased from a local retailer, and meats were immediately stored at −80 °C until the day before the test. Meats were thawed at refrigeration temperatures for 16 h. Chicken breast, white muscle of skipjack, Welsh onion, and green stems of fresh garlic were weighed and cooked with small amounts of flavoring agents (Japanese *sake*, soy sauce, sugar) containing neither anserine nor carnosine. Precooked rice was heated with a microwave and weighed. Other ingredients were cut and weighed. Portions of the experimental diet were analyzed for HRC by HPLC.

Anserine Ingestion and Blood Sample Collection. Subjects fasted, except for water, for 12 h prior to the experiment. On the morning of the experiment, each fasted subject orally took anserine alone [2.0 g/60 kg of body weight (bwt)] in water (4% w/v), the dietary supplement (19.4 g/60 kg of bwt) in water (20% w/v), or the experimental diet containing 2.0 g of anserine/60 kg of bwt in a crossover design. Experiments were repeated at intervals of more than 30 days. Subjects were allowed water ad libitum during the experiment. Approximately 5 mL of venous blood was collected from the cubital vein before and at 20, 40, 60, 80, 120, and 240 min after the ingestion of anserine alone or dietary supplement. When each subject ingested the experimental diet, venous blood was collected before and at 1, 2, 3, 4, 5, and 7 h. Blood was dispensed into tubes containing lithium−heparin as an anticoagulant (BD Japan, Tokyo, Japan). Collected whole blood (1.8 mL) was immediately deproteinized with 0.2 mL of 20% TCA and then centrifuged. Harvested samples were stored at −80 °C until analysis.

Determination of HRC. Concentrations of HRC were determined by cation exchange chromatography on an HPLC system (Hitachi, Tokyo, Japan) after the method of Abe and Ohmama (*17*). Calibration curves of HRC were generated from the aqueous solution of anserine nitrate salt (Sigma-Aldrich, St. Louis, MO), carnosine (Sigma-Aldrich), L-histidine (Nacalai Tesque, Kyoto, Japan), and π-methylhistidine (Calbiochem, Los Angeles, CA). Anserine alone and the dietary supplement were dissolved in Milli-Q water, and 10-μL samples were injected into HPLC. Minced and mixed experimental diet (10 g) was homogenized with 70 mL of 1 M perchloric acid. After neutralization and centrifugation, 10 μL of the supernatant was injected into HPLC. Deproteinized whole blood (300 μL) was ultrafiltered through a membrane with a molecular weight cutoff of 10000 Da (Ultrafree MC, Millipore, Bedford, MA). Ultrafiltered samples (10−20 μL) were directly injected into HPLC.

Data and Statistics. Data are expressed as means ± SEM. Statistical analyses were performed with repeated measurements by ANOVA, and multiple comparisons were performed with Tukey's analysis. $P < 0.05$ was considered to be statistically significant.

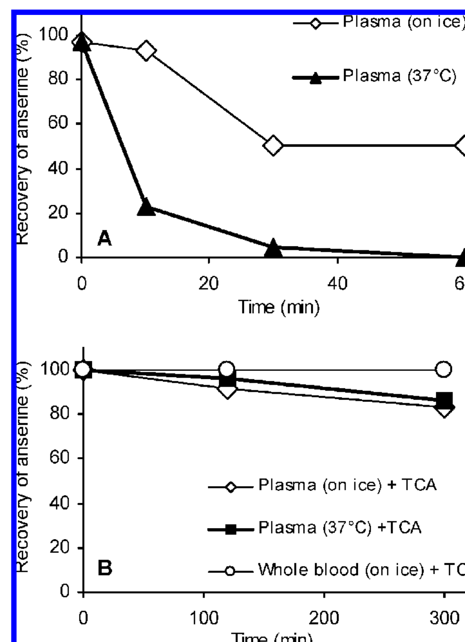

Figure 1. Degradation time course of the addition of 50 μg/mL anserine to serum or whole blood in vitro: (**A**) aliquots of plasma were incubated either at 37 °C or on ice; (**B**) aliquots of plasma or whole blood deproteinized with TCA were incubated either at 37 °C or on ice.

RESULTS AND DISCUSSION

Optimization of the Blood Handling Method. In the first study, we could not detect anserine in subjects' blood following 2 g of anserine ingestion. Therefore, we administered 10 g of anserine alone to subjects; however, we detected only trace levels of anserine. Thus, prior to the clinical trials, we attempted to develop a method to avoid rapid postabsorptive anserine degradation. When we analyzed the time course of anserine degradation following anserine addition to plasma in vitro and incubation either on ice or at 37 °C, no anserine was detected at 37 °C for 60 min and half of the added anserine was detected on ice for 30 min (**Figure 1A**). Next, we attempted to prevent anserine degradation by deproteinizing with TCA and substituting whole blood for plasma to shorten treatment time (**Figure 1B**). Whereas anserine was recovered completely from deproteinized whole blood, only about 90% was recovered from deproteinized plasma for 300 min at both 4 and 37 °C. Therefore, to recover anserine in the clinical trials, whole blood was collected, ice-chilled, and directly deproteinized with 20% TCA.

Serum carnosinase is reported to postabsorptively degrade carnosine. Gardner (*13*) performed a recovery study of carnosine added in vitro to plasma at either 4 or 37 °C, suggesting that plasma carnosinase hydrolyzed the added carnosine (200 nmol/mL) with a half-life of the order of 1 min at 37 °C. Furuta et al. (*19*) incubated [^{14}C]-carnosine added to human plasma at room temperature. They found that the total amount of [^{14}C]-carnosine decomposed β-alanine and [^{14}C]-histidine within 15 min. On the other hand, serum and tissue carnosinases have unique substrate specificities for dipeptides. Anserine was hydrolyzed much more slowly compared to carnosine; the rate of anserine hydrolysis was 52% that of carnosine (*20*). In this study, 4.8% of added anserine could be detected in the plasma at 30 min (37 °C). Because the activity of serum carnosinase was 18−72 μmol of carnosine/mlL/h (*21*), there is certainly

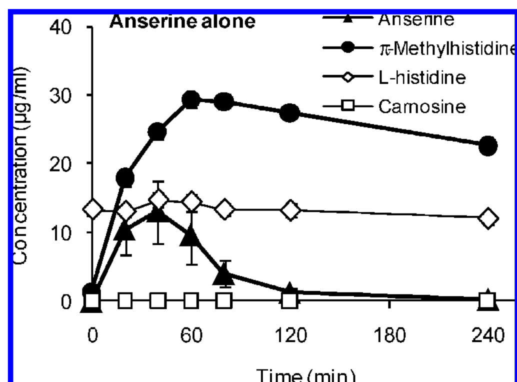

Figure 2. HRC in blood after ingestion of an aqueous solution of 2.0 g of anserine/60 kg of bwt. Values are means ± SEM ($n = 7$).

Table 1. Kinetic Parameters following Ingestion of Anserine Alone[a]

compound	AUC_{0-4} (μg·h/mL)	t_{max} (h)	$t_{1/2}$ (h)	C_{max} (μg/mL)
anserine	18.2 ± 7.2	0.52 ± 0.07	1.28 ± 0.21	13.4 ± 4.5
π-methylhistidine	184.7 ± 27.3	1.19 ± 0.07	7.29 ± 0.48	30.0 ± 1.0

[a] Values represent mean ± SEM ($n = 7$). Abbreviations used: AUC_{0-4}, area under the blood concentration−time curve from times 0 to 4 h; $t_{1/2}$, terminal phase half-life.

enough serum carnosinase to hydrolyze ingested anserine (8.3 mmol/60 kg of bwt) within an hour ex vivo. However, our results also showed that anserine is more stable in blood and tissues than carnosine after oral administration.

Intestinal Absorption and Blood Clearance of HRC. **Figure 2** shows the changes in the levels of HRC after the ingestion of anserine alone. Anserine was not detected in blood samples collected prior to administration, indicating that all subjects had fasted and that large amounts of anserine and π-methylhistidine were not endogenously released into blood. Anserine concentration of blood reached 13.1 μg/mL at 40 min after subjects were administered anserine alone and returned to an undetectable level after 4 h. On the other hand, an increase in π-methylhistidine concentration lasted until 60 min after ingestion and remained 76% of C_{max} at 4 h (**Table 1**). Moreover, the $t_{1/2}$ of anserine in blood was remarkably shorter than the $t_{1/2}$ of π-methylhistidine. These results suggest that intact anserine is absorbed across the human gastrointestinal tract and is sequentially hydrolyzed to π-methylhistidine and β-alanine by serum and tissue carnosinases in a manner similar to that for carnosine (*19*).

The time course curves of anserine and π-methylhistidine after the ingestion of the dietary supplement (**Figure 3**) were similar to those after the ingestion of anserine alone, and there were no significant differences in all of the kinetic parameters between dietary supplement and anserine alone (**Tables 3** and **4**). The data indicate that the intestinal absorption and blood clearance of anserine are hardly affected by the other contents (HRC, other amino acids, protein, and dextrin; **Table 2**). On the other hand, the AUC_{0-4} of π-methylhistidine (1.44 ± 0.14 μmol·h/mL) was 5.0-fold greater than that of L-histidine (0.29 ± 0.02 μmol·h/mL), although the amount of ingested anserine (8.3 mmol/60 kg of bwt) was smaller than the amount of L-histidine ingestion (9.0 mmol/60 kg of bwt). This result possibly emerged from the difference in utilization by the human body. Half the L-histidine was further utilized for incorporation of endogenous high molecular weight substances (*19*), whereas π-methylhistidine, after the ingestion of anserine, was excreted without being metabolized (*14, 22*). This is also why no increase

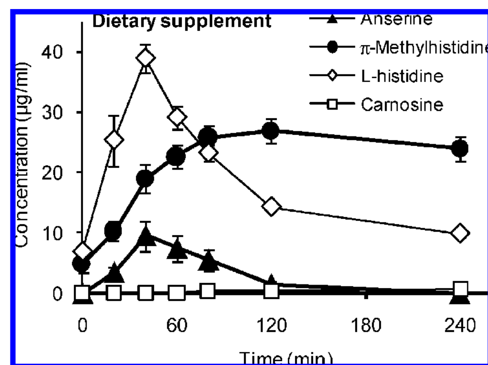

Figure 3. HRC in blood after ingestion on an aqueous solution of dietary supplement containing 2.0 g of anserine/60 kg of bwt. Values are means ± SEM ($n = 7$).

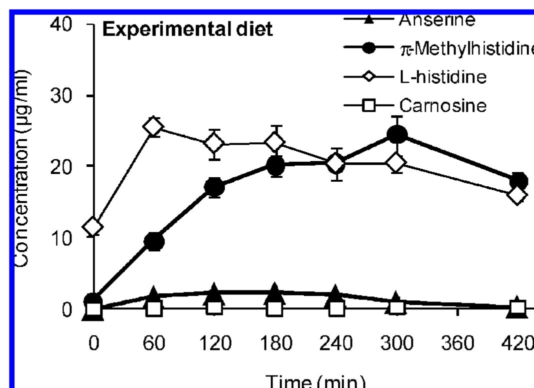

Figure 4. HRC in blood with time after ingestion of experimental diet containing 2.0 g of anserine/kg of bwt. Values are means ± SEM ($n = 7$).

Table 2. Composition (Grams per 60 kg of Body Weight) of Ingested Samples

component	anserine alone	dietary supplement	experimental diet
water	48.0	78.4	389.9
protein	2.0	5.0	68.0
fat	0.0	0.0	22.6
carbohydrate	0.0	13.5	115.1
ash	0.0	0.3	3.7
total	50.0	97.2	599.3
L-histidine	0.0	1.4	2.1
π-methylhistidine	0.0	0.0	0.0
carnosine	0.0	0.2	0.3
anserine	2.0	2.0	2.0

in L-histidine was detected in subjects administered anserine alone in this study.

There were also no significant differences in $AUC_{0\text{-last}}$ between the experimental diet and anserine alone. However, the experimental diet was significantly slower to reach t_{max} (2.7 h) (**Figure 4**; **Tables 3** and **4**) than anserine alone and the dietary supplement, and the C_{max} of the experimental diet was significantly lower than that of anserine alone and the dietary supplement. These phenomena were probably due to the form of anserine ingested, with anserine alone and the dietary supplement resulting in a more rapid absorption than would occur when anserine was ingested in the forms of muscle of chicken and fish. Because of the difference in C_{max} between the experimental diet and the others, the effects of anserine on physiological function after ingestion of the experimental diet are possibly less than expected from a dose of anserine. Ferraris

Table 3. Kinetic Parameters of Anserine following Anserine Ingestion[a]

ingested sample	$AUC_{0\text{-last}}$ (μg · h/mL)	t_{max} (h)	$t_{1/2}$ (h)	C_{max} (μg/mL)
anserine alone	18.2 ± 7.2 a	0.52 ± 0.07 b	1.28 ± 0.21 b	13.4 ± 4.5 b
dietary supplement	11.7 ± 3.1 a	0.76 ± 0.06 b	1.35 ± 0.11 b	9.99 ± 2.48 b
experimental diet	10.5 ±1.7 a	2.71 ± 0.42 a	4.58 ± 0.42 a	2.70 ± 0.59 a

[a] Values represent mean ± SEM ($n = 7$). The values of anserine are the same as in **Table 1**. Values with different letters have a significant difference of $P < 0.05$. Abbreviations used: $AUC_{0\text{-last}}$, area under the blood concentration−time curve from times 0 to 7 h (experimental diet) or 4 h (dietary supplement and anserine alone); $t_{1/2}$, terminal phase half-life.

Table 4. Kinetic Parameters of π-Methylhistidine following Anserine Ingestion[a]

ingested sample	$AUC_{0\text{-last}}$ (μg · h/mL)	t_{max} (h)	$t_{1/2}$ (h)	C_{max} (μg/mL)
anserine alone	184.7 ± 27.3 a	1.19 ± 0.07 b	7.29 ± 0.48 b	30.0 ± 1.0 b
dietary supplement	243.8 ± 24.3 a	1.81 ± 0.12 b	10.31 ± 0.87 b	27.3 ± 2.0 b
experimental diet	173.9 ± 7.9 a	4.43 ± 0.30 a	9.35 ± 0.34 a	24.2 ± 2.3 a

[a] Values represent mean ± SEM ($n = 7$). The values of anserine are the same as in **Table 1** again. Values with different letters have a significant difference of $P < 0.05$. Abbreviations used: $AUC_{0\text{-last}}$, area under the blood concentration−time curve from times 0 to 7 h (experimental diet) or 4 h (dietary supplement and anserine alone); $t_{1/2}$, terminal phase half-life.

et al. (*16*) found that carnosine uptake is stimulated by dietary levels of amino acids, peptides, and protein using everted intestinal sleeves of mice. However, in this study, there were no significant differences between anserine-containing diets and anserine alone with regard to $AUC_{0\text{-last}}$ of anserine. These results may be because there are far more individual differences in hydrolysis of anserine by carnosinase than stimulation effect on dipeptide uptake by coexisting substances.

To our knowledge, there are two studies of carnosine absorption into blood in human subjects. Gardner et al. (*13*) reported a plasma carnosine concentration of 183.1 μg/mL at 0.5 h after the ingestion of 4.0 g of carnosine alone. Park et al. (*15*) reported 32.4 μg/mL at 3.5 h after the ingestion of cooked beef patties containing 267 mg of carnosine. After careful consideration of the ingested doses and the differences of meats, the peak concentrations of ingested carnosine in both papers are 10 times greater than the levels of anserine in this study. Tomonaga et al. (*23*) suggested that a component of chicken breast extract other than carnosine accelerated the metabolism of histidine and increased the concentrations of anserine and carnosine in the brain of rats. Therefore, we consider that the difference of entry speed into tissues greatly contributes to the difference of peak concentrations between anserine and carnosine.

In conclusion, ingested anserine is absorbed intact into blood and is hydrolyzed by serum and tissue carnosinases in a manner similar to that of carnosine. On the other hand, it is suggested that there is a difference of entry speed into tissues between anserine and carnosine. In terms of ingested form, there is no significant difference in the AUC of anserine and π-methylhistidine between the experimental diet, the dietary supplement, and anserine alone. However, the C_{max} of HRC after the ingestion of the experimental diet was significantly lower than others and the phenomenon possibly lowered the effect on physiological function. This is the first study to show intestinal absorption and blood clearance of anserine. These results suggest that anserine could be a useful functional food ingredient, but further studies are needed to evaluate the component-derived anserine contributions to physiological activities.

ABBREVIATIONS USED

HRC, L-histidine-related compounds; HPLC, high-performance liquid chromatography; TCA, trichloroacetic acid; $AUC_{0\text{-last}}$, area under the blood concentration−time curve from times 0 to 7 h (experimental diet) or 4 h (dietary supplement and anserine alone); $t_{1/2}$, terminal phase half-life; bwt, body weight.

SAFETY

All experiments were closely supervised by a medical doctor and performed at a medical institution. The dietary supplement and anserine were confirmed to be safe by an acute toxicity test, a mutagenicity test, and a microorganism test.

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