Development and validation of a sensitive LC-MS/MS assay for the quantification of anserine in human plasma and urine and its application to pharmacokinetic study.

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Abstract

Carnosine (beta-alanyl-L-histidine) and its methylated analogue anserine are present in relevant concentrations in the omnivore human diet. Several studies reported promising therapeutic potential for carnosine in various rodent models of oxidative stress and inflammation-related chronic diseases. Nevertheless, the poor serum stability of carnosine in humans makes the translation of rodent models hard. Even though anserine and carnosine have similar biochemical properties, anserine has better serum stability. Despite this interesting profile, the research on anserine is scarce.

The aim of this study was to explore the bioavailability and stability of synthesized anserine by 1) performing *in vitro* stability experiments in human plasma and molecular modelling studies and by 2) evaluating the plasma and urinary pharmacokinetic profile in healthy volunteers following different doses of anserine (4-10-20 mg/kg body weight). A bio-analytical method for measuring anserine levels was developed and validated using liquid chromatography-electrospray mass spectrometry.

Both plasma (C_{MAX}: 0.54 - 1.10 - 3.12 µM) and urinary (C_{MAX}: 0.09 – 0.41 – 0.72 mg / mg creatinine) anserine increased dose-dependently following ingestion of 4 – 10 – 20 anserine mg/kg BW, respectively. The inter-individual variation in plasma anserine was mainly explained by the activity (R^2 = 0.75) and content (R^2 = 0.77) of the enzyme serum carnosinase-1. Compared to carnosine, a lower interaction energy of anserine with carnosinase-1 was suggested by molecular modelling studies. Conversely, the two dipeptides seems to have similar interaction with the PEPT1 transporter.

It can be concluded that nutritionally relevant doses of synthesized anserine are well-absorbed and that its degradation by serum carnosinase-1 is less pronounced compared to carnosine. This makes anserine a good candidate as a more stable carnosine-analogue to attenuate chronic diseases in humans.

Keywords

Anserine; Carnosine; pharmacokinetics; carnosinase-1

Introduction

Carnosine (beta-alanyl-L-histidine) and its methylated variant anserine (beta-alanyl-L-pi-methylhistidine) are two histidine-containing dipeptides (HCD) which are present in high concentrations in the skeletal muscle of mammals. The daily intake of carnosine and anserine in omnivorous subjects is estimated to be around 600 mg/day (60% anserine - 40% carnosine) (Hisatsune et al. 2015; Katakura et al. 2017).

Several animal experiments demonstrated the potential of carnosine as an agent for the attenuation of different oxidative stress and inflammation-related chronic diseases, such as metabolic syndrome (Aldini et al. 2011), diabetes (Sauerhofer et al. 2007), ischemia (Bae et al. 2013) and atherosclerosis (Barski et al. 2013). The molecular mechanisms explaining these effects of carnosine are still debated. Nevertheless, some mechanistic hypotheses have been reported about metal ion chelation, inhibition of protein glycation and scavenging of reactive carbonyl species, which are involved in the onset and propagation of several inflammatory-based diseases (summarized in Boldyrev et al. 2013).

In rodents, carnosine is highly bioavailable and stable in the circulation upon supplementation. On the contrary, the bioavailability is very low in humans (Gardner et al. 1991; Yeum et al. 2010) owing to the high activity of the enzyme serum carnosinase-1 (CN1), which quickly hydrolyzes carnosine to the amino acids beta alanine and histidine. The terms carnosinemia/anserinemia will be used in a physiological way to indicate the transient and intermittent increase in plasma carnosine or anserine following dietary intake of carnosine or anserine. This is not related to the pathological high carnosine levels in patients with serum carnosinase deficiency, which should rather be called hypercarnosinemia instead of carnosinemia (Perry et al. 1967). Carnosinemia has been consistently reported in rodents upon supplementation of diverse doses (Sauerhofer et al. 2007; Aldini et al. 2011; Stegen et al. 2015), whereas it is mostly absent following human supplementation (Gardner et al. 1991; Suzuki 2004; Yeum et al. 2010; Baguet et al. 2014), except if very high doses are used. Specifically, a pharmacological dose of 60 mg/kg BW of pure carnosine, which elicited some side-effects like headache and paresthesia, was needed to saturate the carnosinase enzyme and consequently induce a short elevation of plasma carnosine following carnosine supplementation in some, but not in all subjects (7 out of 23; Everaert et al. 2012). Interestingly, this carnosinemia was only found in the subjects with low activity of serum carnosinase.

The importance of serum bioavailability of carnosine for eliciting a protective effect is evident since genetic studies demonstrated that patients with gene polymorphisms associated with a low activity of serum carnosinase are less susceptible to the development of nephropathy (Janssen et al. 2005). This

indicates that the disease-protecting effect of low serum carnosinase activity probably relates to the higher stability of carnosine in the circulation.

The metabolic instability of carnosine in human serum on one hand and its potential therapeutic value on the other, have prompted great interest in the design and synthesis of stable carnosine derivatives (Aldini et al. 2011; Menini et al. 2015; Iacobini et al. 2018). Another strategy to overcome the poor bioavailability of carnosine is the use of natural carnosine analogues such as anserine, which is more resistant to degradation (Pegova et al. 2000; Yeum et al. 2010; Peters et al. 2011), but maintains most of the properties of the parent compound such as the antioxidant power, the metal ion chelating ability and the scavenging of reactive carbonyl species. However, since synthesized anserine is not commercially available for human studies, its pharmacokinetics is only partly known. To bypass this, Kubomura et al. (2009) extracted anserine from tuna extracts and showed that anserine (33 mg/kg BW) is absorbed in humans and then hydrolyzed within 2-4 h, since its two constituent amino acids are elevated in the circulation. It was suggested that the H(+)/peptide transporter 1 (PEPT1) is responsible for the absorption of anserine across the intestinal epithelium (Geissler et al. 2010).

Recently, different reports revealed that long-term carnosine supplementation (1-2 g/day) can mildly improve glucose metabolism in overweight subjects (De Courten et al. 2016) and type-2 diabetic patients (Houjeghani et al. 2018), ameliorate the urinary albumin excretion ratio in young type-1 diabetic patients with diabetic nephropathy (Elbarbary et al. 2017) and slightly improve some symptoms in children with autism spectrum disorder (Hajizadeh-Zaker et al. 2018; Mehrazad-Saber et al. 2018). Despite the beneficial effects, the amount of circulating histidine-containing dipeptides is, in all probability, very low in these studies. Therefore, the use of anserine instead of carnosine is expected to enhance the bioavailability therefore boost the supplementation-induced effects. Interestingly, supplementing carnosine together with purified anserine (1 g/day, 1:3 ratio, 3 months) has been shown to preserve verbal episodic memory and brain perfusion in healthy elderly subjects (Hisatsune et al. 2015). However, research on long-term anserine supplementation in different patient populations is lacking at this moment.

To better understand the bioavailability and metabolic stability of anserine in humans, the aim of the present paper was to evaluate the dose-dependent pharmacokinetic profile of anserine in human volunteers following oral administration of pure anserine obtained by synthesis. A bio-analytical method for assaying anserine levels in both plasma and urine was developed and validated using LC–MS/MS operating in positive electro-spray ionization (ESI+) mode. Moreover, the *in vitro* stability of anserine was further investigated and explained by molecular modelling studies, using carnosine as reference compound.

Materials and methods

Pharmacokinetic study

All subjects gave their informed consent and the study was approved by the Local Ethical Committee (Ghent University Hospital).

Seven young healthy subjects (mean ± SEM: 27 ± 1.2 years, 75 ± 4.8 kg body weight, 88.0 ± 3.0 mg/dl fasting plasma glucose, 131 ± 2.7 mmHg systolic and 78 ± 2.3 mmHg diastolic blood pressure, 4 females / 3 males) were orally supplemented with 4, 10 or 20 mg/kg BW anserine on 3 different occasions (> 1 week in between). Participants were asked to refrain from meat or fish 24h prior to the start of the experiments. After an overnight fast (at least 8 h), an indwelling catheter was inserted in an antecubital vein and blood was withdrawn before and 20, 40, 60, 80, 120, 180 minutes following oral supplementation of pure anserine (Flamma, dissolved in 250ml water). A fasting serum sample was collected for determination of serum carnosinase activity and content. Blood samples for determination of anserine were collected in pre-cooled (4°C) EDTA tubes and immediately centrifuged (4°C) to separate plasma. The anticoagulant EDTA was chosen for its ability to chelate Zn^{2+} ions which are essential for the catalytic activity of carnosinase (Yeum et al. 2010). Plasma samples were deproteinized with sulfosalicylic acid (35%) and stored immediately at -20°C until further analysis with LC-MS/MS. Urine was collected prior and 45, 90, 135, 180, 240 minutes after anserine supplementation. The subjects were allowed to drink water ad libitum and received a carnosine- and anserine-free meal after the last blood collection.

Serum carnosinase activity and concentration

Serum carnosinase activity was quantified by fluorometric determination of liberated histidine after carnosine addition (based on Teufel et al. 2003). Briefly, the reaction was initiated by addition of 10 mM carnosine (Flamma) to serum and stopped after 10 min incubation at 37°C by adding 600 mM trichloroacetic acid (TCA). For controls, TCA was added before carnosine. After centrifugation (4500 rpm, 15 min), supernatant was added to a mixture of OPA (incomplete phtaldehyde with 0.2% 2-mercaptoethanol) and 4M sodium hydroxide and fluorescence was determined after 40 min (excitation: 360 nm and emission: 465 nm). Serum carnosinase concentrations were determined by a sandwich ELISA (enzyme-linked immunosorbent assay) developed by Adelmann et al. (2012).

Anserine analysis by LC-ESI-MS/MS: method set-up and validation

Chemicals

LC-grade H2O (18 MΩ cm) was prepared with a Milli-Q H2O purification system (Millipore, Bedford, MA, USA). Nonafluoropentanoic acid (NFPA), 5-sulfosalicylic acid (SSA), trichloroacetic acid (TCA), anserine, carnosine and LC–MS grade solvents were purchased from Sigma–Aldrich (Milan, Italy). Tyrosyl-histidine (TH), used as internal standard, and anserine administered to human volunteers were synthesized by Flamma S.p.a. (Chignolo d'Isola, Bergamo, Italy).

Sample preparation

Preparation of standard and quality control samples.

Plasma and urine used to prepare standard and quality control samples were obtained from six healthy volunteers who followed a lacto-ovo-vegetarian diet free of HCD for two days in order to reduce anserine content in biological fluids as much as possible. Stock solutions of anserine and internal standard (IS) tyrosyl-histidine (TH) were prepared in water (1 mg/mL) and then diluted in the biological matrices (urine or plasma) in order to obtain the following standard concentrations: TH 7 µM and anserine 5.0, 25.0, 100.0, 200.0, 500.0 µM in urine; TH 5 µM and anserine 0.5, 1.0, 5.0, 10.0, 20.0, 100.0 µM in plasma. Urine standards with anserine concentrations of 5, 25 and 500 µM served as QC samples; the concentrations 0.5, 1 and 10 µM were used as plasma QC samples.

Preparation of urine and plasma samples for the pharmacokinetic study.

Urine samples were diluted 2, 5 or 10 fold with Milli-Q H_2O to obtain anserine concentration in the calibration range. After dilution, the IS and TCA were added in order to reach a final concentration of 7 µM and 2 % v/v, respectively. Samples were then centrifuged at 14000 g for 10 minutes and the supernatant was transferred to vials for analysis (20 µL injected).

Deproteinized samples were diluted 1:9 with a solution of NFPA 0.08% (in H_2O) and added to the internal standard to reach a final concentration of TH 5 µM. Samples were transferred to vials for analysis (100 µL injected). Anserine levels in urine were normalized as anserine (mg)/creatinine (mg). Creatinine amount was quantified by using a creatinine colorimetric assay kit (Cayman Chemical Company, Ann Arbor, MI, USA).

LC-MS/MS conditions and method validation

LC-MS/MS operating conditions and optimization.

Sample analysis was performed using a LC-MS/MS system consisting of an HPLC (Surveyor, ThermoFinnigan Italy, Milan, Italy), equipped with a quaternary pump, a degasser and a Surveyor autosampler, connected to a TSQ Quantum Ultra Triple Quadrupole (ThermoFinnigan Italy, Milan, Italy) mass spectrometer. The chromatographic separation was carried out on a Phenomenex Synergi 4 µm Polar-RP 80Å column (2 mm x 150 mm), protected by a Phenomenex Synergi 4 µm Polar-RP guard

column (2 mm x 4 mm), maintained at 25°C and working at a constant flow rate of 200 µL/min. 20 µL of urine sample was injected and anserine eluted with a 17 min multistep gradient of phase A (H_2O – NFPA 0.08%) and phase B (CH_3CN): 0-5 min, isocratic of 1% B; 5-6 min, from 1% B to 60% B; 6-12 min, isocratic of 60% B; 12-12.1 min, from 60% B to 1% B and then 12.1-17 min, isocratic of 1% B. Anserine and TH eluted at 10.5 and 10.7 min, respectively (Fig S1A of *Supplementary material*).

The same chromatographic conditions were initially applied for the quantitative analysis of anserine in plasma. However, the presence of SSA in plasma samples greatly reduced anserine and TH retention times (RT < 3 min). Attempts made to counter the reduced retention by increasing the injection volume of a diluted sample, did not significantly affect the RT values. A good separation and retention of the two analytes was then achieved by changing the gradient.

100 µL of plasma sample was injected and anserine eluted with a 12 min multistep gradient of phase A (H_2O – NFPA 0.08%) and phase B (CH_3CN): 0-3 min, isocratic of 1% B; 3-4 min, from 1% B to 30% B; 4-8 min, isocratic of 30% B; 8-8.1 min, from 30% B to 1% B and then 8.1-12 min, isocratic of 1% B. As shown in Fig S1C of *Supplementary material*, in such conditions anserine and TH eluted at 9.2 min and 10.3 min, respectively. The chromatographic conditions were obtained by optimizing those reported by Orioli et al. 2007. The mass spectrometer, equipped with an ESI source, was operated in positive ion mode and the source parameters were set as follows: spray voltage 4.5 kV, capillary temperature 270°C, sheath gas flow 40 units, auxiliary gas flow 5 units. A multiple reaction monitoring (MRM) method was adopted for the quantification of anserine. Fragmentation parameters were optimized as follows: scan with 1.0 *m/z*; scan time, 0.05 s; Q1 and Q3 peak full width at half-maximum (FWHM), 0.5 *m/z*: collision gas (Argon) pressure, 1.5 mTorr.

MS conditions for the MRM analysis of anserine and TH were optimized by the direct infusion of standard solutions (10 µM) into the ESI source in positive ion conditions. The collision energies and the transitions selected for the analysis were as follows:

ANS: *m/z* 241.20 → 109.00 + 96.00 (collision energy, 30 eV)

TH: *m/z* 319.00 → 156.00 + 110.00 (collision energy, 20 eV)

Method validation

The methods for anserine analysis in plasma and urine were validated according to the US FDA guidelines for bioanalytical method validation. The following parameters were considered: selectivity, matrix effect, linearity, precision and accuracy, and stability.

The selectivity was evaluated on six blank samples of biological matrix (plasma and urine) collected from volunteers after two days of anserine-free diet. The following restrictions were adopted in order to consider the method selective: each blank sample must not report a peak with the same RT of the analyte or, if present, it has to be characterized by an area under the curve lower than the 20% of the area of the lowest limit of quantification (LLOQ) sample.

Five replicates at three different concentrations were used to test the matrix effect: the LLOQ, a concentration near to the geometric mean of the linear range, and a high concentration. The samples were prepared in mobile phase A and the same concentrations were obtained by spiking anserine in matrix (plasma and urine). Matrix effect (%) was calculated as:

$$\frac{(AUC_{ANS}/AUC_{TH})_{Matrix}}{(AUC_{ANS}/AUC_{TH})_{Phase\ A}}\ x\ 100$$

The linearity of the method was evaluated over the concentration range of 5–500 µM in urine and 0.5-100 µM in plasma analyzing each calibration point in triplicate. The calibration curves were built by plotting the peak area ratios of ANS/IS versus the nominal concentrations of anserine by weighted ($1/x^2$) least-squares linear regression. The lowest limit of quantification (LLOQ) was the lowest plasma concentration in the calibration curve with a signal-to-noise ratio of ≥10. The back-calculated concentrations at LLOQ must be within ±20% deviation from the nominal value while at each point they have to be within ±15%.

Intra-batch, intra-day and inter-day precision and accuracy were calculated using five replicates for three different concentrations of QC samples. Precision was expressed as CV%, while accuracy as bias.

Anserine stability in matrices was tested at 4°C and after two freeze and thaw cycles from -80°C to 4°C.

Validation of the LC-ESI-MS/MS method for the quantitative analysis of anserine in urine

The mean value of the area under the curve of six blank samples was found to be 10.7% in respect to the mean value of three replicates of the LLOQ. Therefore, according to the criteria previously reported, the method can be defined as selective.

The matrix effect was calculated by comparing the peak areas of anserine and TH, when spiked in mobile phase A and in urine. The results, expressed as % (CV%), are: 133.4% (9.1%) at 5 µM; 79.4% (3.5%) at 25 µM; 101.5% (3.0%) at 500 µM. The matrix effect was < 20% at 25 and 500 µM and > 20% at the lowest concentration (LLOQ).

The method was found to be quite sensitive, the LLOQ being 0.025 µM, so the calibration range was initially built from 0.025 µM up to 500 µM. However, despite the precision's being very good even at the lowest concentrations, linearity was not satisfactory. By excluding the concentrations from 0.025 µM to 1 µM and setting 5 µM as the LLOQ, a satisfactory calibration equation was obtained *y= 0.03400(± 0.0003701) x + 0.1302(± 0.07175)* (the slope and intercept are mean ± standard deviation) with a correlation coefficient (r^2) of 0.9993. The anserine concentrations used to build the curve were 5, 25, 100, 200 and 500 µM.

Precision (CV %) and accuracy (bias) intra-batch, intra-day and inter-day were evaluated by analyzing the validation samples at three different concentrations injected five times. Results are reported in Table S1 of *Supplementary material*. The intra-batch precision was lower than 3.55% (4.43% for the LLOQ) and accuracy was in a range between -0.94% and +8.21% in respect to the nominal values (-17.76% for the LLOQ). The intra-day CV% values were lower than 11.58% (18.60% for the LLOQ) and the values relative to the inter-day precision were lower than 12.64% (2.83% for the LLOQ). The intra-day accuracy was in a range between -9.15% and -5.13% (-11.88% for the LLOQ), while the inter-day accuracy was between +11.46% and +14.72% (-19.60% for the LLOQ).

The stability tests were performed under working conditions and after two freeze and thaw cycles from -80°C to 4°C. Anserine and the IS were both stable in the auto sampler set at 4°C for 18 h, which is the time for each analysis cycle. The first freeze and thaw cycle did not affect the anserine and TH concentrations, while after the second cycle a reduction of both the compounds of around 10% of the initial concentrations was observed.

Validation of the LC-ESI-MS/MS method for the quantitative analysis of anserine in plasma

As found for urine, also in plasma, blank samples showed a detectable peak at the same retention time as anserine. However, the method can be deemed selective by considering that the area of the peak in the blank sample is lower than 20% of the area of anserine at the LLOQ (Fig S1D of *Supplementary material*). In particular the mean value of the area under the curve of six blank samples corresponds to 2.76% of the mean value of three replicates of the LLOQ (0.5 µM).

The data relative to the matrix effect calculated at three concentrations, expressed as mean % with the relative CV%, are: 128.9% (4.7%) at 0.5 µM; 117.9% (9.3%) at 5 µM; 95.5% (3.7%) at 100 µM. For the LLOQ an effect of more than 28% was observed. It was however impossible to reduce this effect.

The calibration curve is characterized by a satisfactory linearity (R^2 = 0.99) in the range of concentrations considered (0.5 - 100.0 µM). The equation (the slope and intercept are mean ± standard deviation) is *y*

= *0.0614(± 0.00045) x + 0.007(± 0.01891)*. The anserine concentrations used to build the curve were 0.5, 1.0, 5.0, 10.0, 20.0 and 100.0 µM, where 0.5 µM is the LLOQ.

Precision and accuracy intra-batch, intra-day and inter-day were evaluated by analyzing three different concentrations (0.5, 5 and 100 µM) injected in quintuplicate. The results are reported in Table S2 of *Supplementary material*. The intra-batch precision was lower than 6.25% and the accuracy was in a range between 0.12% and +9.66% in respect to the nominal values. The intra-day CV % values were lower than 4.99% and the values relative to the inter-day precision were lower than 5.96%. The intra-day accuracy was in a range between -5.80% and -5.67%, while the inter-day accuracy was between -8.40% and +4.82%.

Anserine and the IS were both stable in the autosampler set at 4°C for 11 h, which is the maximum length of time for each analysis cycle. After the first cycle of freeze and thaw, the analytes were reduced by about 10% of the initial concentrations and after the second cycle, the reduction was about 15%.

***In vitro* stability tests**

Initial stability experiments were performed with a concentration of 5 µM at 37°C, which reflects the *in vivo* condition and concentration upon supplementation. Serum stability was performed by spiking an aliquot of anserine or carnosine working solution down to a final concentration of 5 µM into human serum. Both serum and peptide solutions were pre-warmed at 37°C before starting stability test. Aliquots were sampled at 0, 5; 15; 30 minutes to determine the residual peptide concentration. Prior to injection, samples were deproteinized for 10 minutes at 4°C by a fivefold dilution into acetonitrile. Sample supernatants were then obtained by centrifugation at 18000 g for 10 minutes at 4°C. Residual peptide amount was determined on an LC-MS platform built with an Exion LC™ 100 chromatograph connected with an API4000™ triple quadrupole mass analyzer through a Turbo V™ electrospray source (AB SCIEX, Milan, Italy). Chromatographic separation was carried out with a Hypersil GOLD HILIC column (150 x 2.1 mm, particle size 3 µM, pore size 175 Å, Thermo Scientific) coupled with a Drop-In-Guard-Cartridge (5 µm) and heated at 40°C (additional details in Online Resources 1).

As the added dipeptides were rapidly hydrolysed in the above mentioned conditions, stability experiments were repeated with final concentration of 100 µM at room temperature. Heparin plasma of subjects with low (0.81 µmol/mL/h) or high (1.91 µmol/mL/h) serum carnosinase activity was pooled. Anserine or carnosine was added separately at a final concentration of 100 µM of each dipeptide and incubated at room temperature. The degradation of the dipeptide by the carnosinase enzyme was stopped on different time points (0, 1, 2, 3, 5, 7, 10, 15, 25, 40 min) by adding 35% of sulfosalicylic acid (9:1) and removing the proteins by centrifugation. The remaining amount of histidine-containing dipeptides was measured fluorometrically by HPLC. In short, deproteinized plasma supernatant (2.6 µL)

was mixed with 77.4 µL of AccQ Fluor Borate buffer and 20 µL of reconstituted Fluor Reagent from the AccQTag chemistry kit (Waters, Milford, MA, USA). The same method was applied to the combined standard solutions of carnosine and anserine. The derivatized samples were applied to a Waters high-performance liquid chromatography system compromised of an XBridge BEH column (4.6 × 150 mm, 2.5 µm) and fluorescence detector (excitation/emission wavelength: 250/395 nm). The column was equilibrated with eluent A (10%, Waters), acetonitrile and double distilled water at a flow rate of 1 mL/min at 37°C.

Molecular modelling

With regard to carnosinase recognition, docking simulations were based on the already reported putative complex between carnosine and carnosinase and were carried out by adopting the computational strategies previously described (Peters et al. 2017). Similarly, docking simulations on PEPT1, the transporter responsible for dipeptide transport across intestinal enterocytes, involved the homology model as generated by fragments (Pedretti et al. 2008) and were based on the computational procedures reported elsewhere (Vistoli et al. 2012).

Statistics

Repeated Measures Anova was used to test the variation in serum carnosinase activity or concentration over the different test days. Correlation were evaluated by means of bivariate Pearson correlations. Values are presented as mean ± SEM and significance assumed at $P \leq 0.05$.

Results

Pharmacokinetic study

The pharmacokinetic profile of anserine was determined in urine and plasma of seven human volunteers after an oral intake. Three different doses were used: 4, 10 and 20 mg/kg BW. None of the subjects reported side-effects at any given dose. No intra-individual changes in serum carnosinase activity (CV% = 14.7%; 1.7 ± 0.1 µmol/mL/h) or protein content (CV% = 8.7%; 157.9 ± 8.4 µg/mL) were observed between the different testing days (over a period of 3 to 6 weeks, $p > 0.05$). Serum carnosinase activity was found to be highly correlated with the serum carnosinase content in the circulation ($r = 0.86$, $p < 0.05$) in our small cohort of healthy young subjects.

Urine samples were collected before the treatment and 45, 90, 135, 180 and 240 min after administration. Despite the subjects followed a diet free from histidine-containing dipeptides the day before, 3 out of 7 subjects had traces of anserine in their urine before the anserine supplementation protocol. Fig 1 reports the excretion profile of anserine for the three dosages expressed as amount of anserine (mg) normalized to creatinine (mg) and suggested that the maximum concentration is dose-dependently reached 90 minutes after supplementation.

For each dosage the amount of urinary anserine is also reported as a cumulative curve (Fig 2). The excretion plateau is reached after 135 minutes for all the dosages, while the mean amount of excreted anserine was 4.3, 6.5 and 8.1% for an anserine intake of 4, 10 and 20 mg/kg, respectively. As shown in Fig 2, there is a high inter-individual variability. In fact, the individual amount of anserine excreted ranges from 0.02% to 9.92% for a dose of 4 mg/kg, from 1.88% to 11.80% for a dose of 10 mg/kg and from 2.80% to 15.03% for a dose of 20 mg/kg. The total urinary anserine excretion was negatively correlated ($r = -0.79$, $p < 0.05$) to serum carnosinase activity but not with protein content following ingestion of 4 mg/kg anserine. Nevertheless, no correlation was found after an anserine dose of 10 or 20 mg/kg.

Anserine plasma levels were also measured before anserine intake and after 20, 40, 60, 80, 120 and 180 minutes. A dose of 20 mg/kg of anserine results in a measurable increase of plasma anserine for all subjects. Similarly, also lower doses of anserine were able to increase anserinemia in the majority of subjects (4/7 and 5/7 responders for a dose of 4 and 10 mg/kg, respectively). Fig 3 shows the mean (± SD) plasma profiles relative to the three different dosages: a clear dose-dependency can be seen. The PK values, AUC, C_{MAX} and T_{MAX}, were then calculated with PK solver add-in program for Microsoft Excel (Table 1). The peak plasma concentration is reached between 24 and 31 minutes for all three doses. The AUC values after the oral intake of 4, 10 and 20 mg/kg are 27.35 µmol/L*min, 66.74 µmol/L*min and 223.57 µmol/L*min, respectively, and the C_{MAX} values were 0.538 µmol/L, 1.099 µmol/L and 3.117 µmol/L. The high inter-individual variability is strongly related to the inter-individual variability of serum

carnosinase activity and content. This hypothesis is supported by the inverse correlation between anserine AUC (20 mg/kg) and both plasma CN1 content (R^2 = 0.77, Fig 4A) and activity (R^2 = 0.75, Fig 4B). No correlation was found between the levels of anserine in plasma and urine.

	AUC 0-t (µM*min)	C_{MAX} (µmol/L)	T_{MAX} (min)
4 mg/kg	27.35 (12.78)	0.538 (0.26)	24.00 (3.38)
10 mg/kg	66.74 (30.33)	1.099 (0.51)	25.71 (3.42)
20 mg/kg	223.57 (71.39)	3.117 (0.88)	31.43 (5.51)

Table 1 PK parameters (n=7) of anserine after the oral supplement of 4, 10 and 20 mg/kg. Data are reported as mean (SEM).

A comparison was also made between the results herein reported and those reported by Yeum et al. (2010) who analyzed serum and urinary anserine content by LC-ESI-MS/MS after the consumption of anserine-rich food (beef and chicken broth). A good correlation is found between anserine concentrations in urine (R^2 = 0.93) and anserine doses (this study) and anserine amount contained in meat (Yeum et al. 2010) (Fig 5). The same is found for plasma (R^2 = 0.98) (Fig 5). However, a difference was observed for the T_{MAX} values being 24-31 minutes in this study, while 60 min and 100 min was reported after chicken broth and chicken meat consumption, respectively.

***In vitro* stability tests**

Anserine undergoes a fast degradation in human serum when spiked at a concentration comparable to the C_{MAX} observed after supplementation of 20 mg/kg. In fact, 5 µM anserine was undetectable after 15 minutes of incubation in serum at 37°C and only 20% of initial amount was detectable after 5 minutes, whereas at the same concentration and time point the remaining amount of carnosine was only 0.03% (data not shown). The estimated half-life of anserine and carnosine according to a one-phase decay model (R^2 = 0.99 for anserine and 0.93 for carnosine) were 2.1 minutes and 0.42 minutes, respectively. Repeating these experiments at room temperature and with an initial dipeptide concentration of 100 µM, the half-life of anserine was still about 4.5-fold longer than carnosine. Unlike carnosine, which was undetectable after 5 minutes of incubation, anserine was still detectable after 15 minutes (Fig 6A). Importantly, the degradation of anserine is delayed in plasma of subjects with a low carnosinase activity up to a three-fold increase of the half-life (6.0 vs. 18.2 minutes). Consequently, anserine content was below limit of detection after 10 minutes in plasma of high carnosinase activity subjects, while 40 minutes were required for an anserine disappearance in low carnosinase activity subjects (Fig 6B).

Molecular modelling

With a view to rationalizing the greater stability of anserine towards the carnosinase activity as well as its satisfactory oral bioavailability, docking simulations were performed involving the resolved structure of the enzyme (PDB Id 3DLJ) as well as the published homology model of PEPT1.

Fig 7A compares the calculated poses for carnosine and anserine within the catalytic cavity of human serum carnosinase and allows the key stabilizing interactions to be revealed. In detail and in both complexes, the peptide carbonyl group assumes a pose conducive to the catalysis since it suitably approaches the zinc ion, the C-terminus is engaged in a clear ion-pair with Arg350, while the N-terminus contacts the negatively charged residues surrounding the zinc ions such as Asp202 or Glu173. As expected, the major difference between the two compared complexes involves the imidazole ring, which assumes a shifted and overturned arrangement in anserine compared to that observed for carnosine. Specifically and while the carnosine's imidazole ring is engaged in a clear H-bond with Gln110 reinforced by π-π stacking interactions with His452, the anserine's imidazole ring loses both these contacts while approaching Asn220, with which it can at most stabilize π-π stacking interactions with the amide group. Notably, the anserine N-methyl group appears to be inserted within a markedly hydrophobic niche, as lined by Leu387, Leu389 and Pro426, where it can elicit clear apolar contacts. The above described differences are reflected in a decrease of the interaction energy as computed by PM7-based semi-empirical methods which shifts from -27.1 kcal/mol to -23.6 kcal/mol for carnosine and anserine, respectively. Although the enzyme possesses a well-known specificity for histidine-containing dipeptides, which is here rationalized by the mentioned interactions involving the carnosine's imidazole ring, the reported complexes suggest that the enzyme might tolerate a certain degree of structural variability in the C-terminal side chain. In contrast, the enzymatic cavity surrounding the common beta-alanyl residue appears to tightly wrap the substrate. This finding emphasizes that here the enzyme is unable to accommodate bulkier residues as indeed confirmed by the experimental results showing that beta-alanine can be at most replaced by Gly and Ala residues.

With regard to the PEPT1 recognition relevant for the intestinal absorption of the dipeptides, Fig 7B similarly compares the computed poses for carnosine and anserine within the PEPT1 binding cavity. While showing slightly different arrangements, both ligands are able to elicit the key contacts involving their N-terminus with Glu23, Glu26, Trp294 and Tyr588. Again, both carboxyl groups are engaged in a set of H-bonds involving the NH backbone atoms of Trp294, Ala295, Leu296 and Phe297. Likewise to what was observed for carnosinase, the major difference between carnosine and anserine concerns the arrangement of the imidazole ring since also here the imidazole ring of anserine shows shifted and overturned pose compared to that of carnosine. Nevertheless, such a different arrangement does not seem to have a detrimental role since both imidazole rings are similarly involved in hydrophobic and π-

π stacking contacts with the surrounding apolar residues such as Phe293, Leu296, Tyr588 and Leu591. The marginal effect of the N-methylation on the PEPT1 recognition is also supported by the similar scores computed for the two complexes (-23.99 kcal/mol and -24.51 kcal/mol, for carnosine and anserine, respectively).

Discussion

This study evidenced that pure anserine is absorbed and detectable into the blood circulation after an oral dose equivalent to an average food daily intake. Anserine bioavailability was found to be mainly determined by the activity of serum carnosinase, which was found to be able to determine the half-life of the peptide.

Different experiments have revealed that anserine can be quantified in human plasma upon the acute supplementation of both anserine containing foods or anserine purified from foods (Suzuki et al. 2006; Harris et al. 2006; Kubomura et al. 2009; Yeum et al. 2010). Based on the data herein reported on the absorption of pure anserine and the experiments of Yeum et al. (2010, Fig 5B) on the absorption of similar doses of anserine through foods, it can be concluded that the matrix does not affect much the C_{MAX} detectable in the circulation. On the contrary, the T_{MAX} is delayed when anserine is ingested through meat (plasma T_{MAX} = 24-31 min for pure anserine and 100 min for meat). The time to peak plasma anserine is also delayed following ingestion of chicken broth, in which anserine is soluble and directly available for absorption (plasma T_{MAX} = 60 min). This effect is possibly due to competition for anserine transporter PEPT1 by other small peptides (e.g. carnosine) contained in foods.

This is the first study elucidating the dose-response relationship of acute supplementation with chemically synthesized anserine. Despite the precautions taken to block carnosinase activity following blood withdrawal (cooling, EDTA tubes, immediate deproteinization), anserinemia could not be observed in subjects with a high serum carnosinase activity when the lowest doses (4 or 10 mg/kg) were ingested. Therefore, one can speculate that anserinemia is only possible when the enzyme serum carnosinase-1 is saturated. Logically, a low carnosinase activity stimulates a faster saturation of the enzyme. Since the maximum velocity of the carnosinase enzyme to hydrolyze carnosine is faster as for anserine (V_{MAX} 33.0 vs. 13.3 µmol/mL/h, respectively; Pegova et al. 2000), a higher dose of carnosine is required to reach measurable amount of carnosine in blood. This can evoke headache and paresthesia as reported in some participants taking 60 mg/kg of the peptide (Everaert et al. 2012). Interestingly, we demonstrated that a nutritionally-relevant dose of anserine (i.e. 20 mg/kg) evoked a significant increase in circulating anserine levels in all subjects, despite their carnosinase activity, and without any adverse side-effects. Based on the PK and molecular modelling data herein reported, we can conclude that the higher stability of anserine to carnosinase hydrolysis is responsible for his higher bioavailability, compared to carnosine.

The *in vitro* stability tests were initially carried out considering a value similar to the C_{MAX} of anserine after supplementation of 20 mg/kg (5 µM). Despite the first time point considered is higher than the t1/2

value, the high sensibility of the method allowed to detect low anserine and carnosine concentrations thus obtaining the half-life values. The experiment was repeated increasing anserine concentration up to 100 μM to verify the reliability of previous obtained results. Even if this high concentration can saturate the enzyme, comparable results were obtained. Such results confirm the higher stability of anserine stability to carnosinase hydrolysis.

Providing the evidence that the activity of serum carnosinase-1 affects anserine levels is important, since a genetic variation in the CNDP1, which is the gene coding the enzyme, is linked to the development of diabetic nephropathy in diabetic patients, while patients with a lower amount of leucine repeats in the promotor region of the gene have a lower circulating carnosinase activity (Janssen et al. 2005; Mooyaart et al. 2010) and are less susceptible to develop diabetic nephropathy (Janssen et al. 2005; Freedman et al. 2007; Mooyaart et al. 2010; Yadav et al. 2016). Therefore it can be speculated that patients with a lower amount of leucine repeats (5-5 vs. others) are protected against kidney damage owing to higher levels of circulating histidine-containing dipeptides.

In Asia, anserine and/or carnosine-rich extracts of chicken meat or fish, have a popular tradition as a fatigue-reducing (Suzuki 2004; Hirohiko et al. 2006) and health-preserving nutritional strategy (Matsumura et al. 2002; Konagai et al. 2013; Szcześniak et al. 2014; Katakura et al. 2017). Yet, given the current concern with environmental sustainability, the future of meat extracts seems limited, as a single daily dose of anserine-rich extract would require not less than 1 kg of chicken breast meat to start with. The supplementation with pure histidine-containing dipeptides has also the advantage to be able to identify the functionality of single nutrients and therefore support the development of histidine-containing dipeptides as nutraceuticals.

It can be concluded that supplementation of a nutritionally relevant dose of pure anserine is a promising strategy to overcome the poor bioavailability of carnosine without eliciting any side-effects. Further long-term supplementation studies in rodents and humans are nevertheless needed to better elucidate the therapeutic potential of anserine in chronic diseases and to support its development as novel nutraceutical compound.

Figures

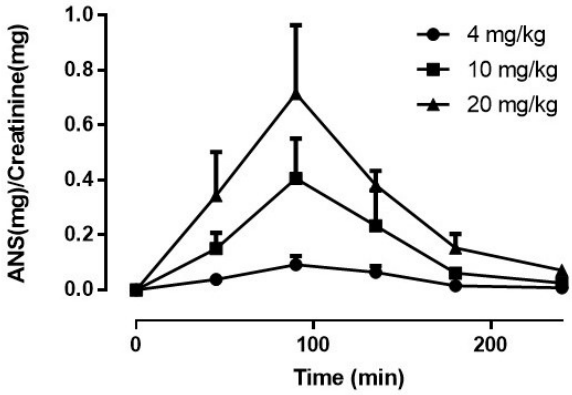

Fig. 1 Mean (± SEM) urinary excretion of anserine (expressed per mg creatinine) after the intake of anserine at three different dosages: 4, 10 and 20 mg/kg.

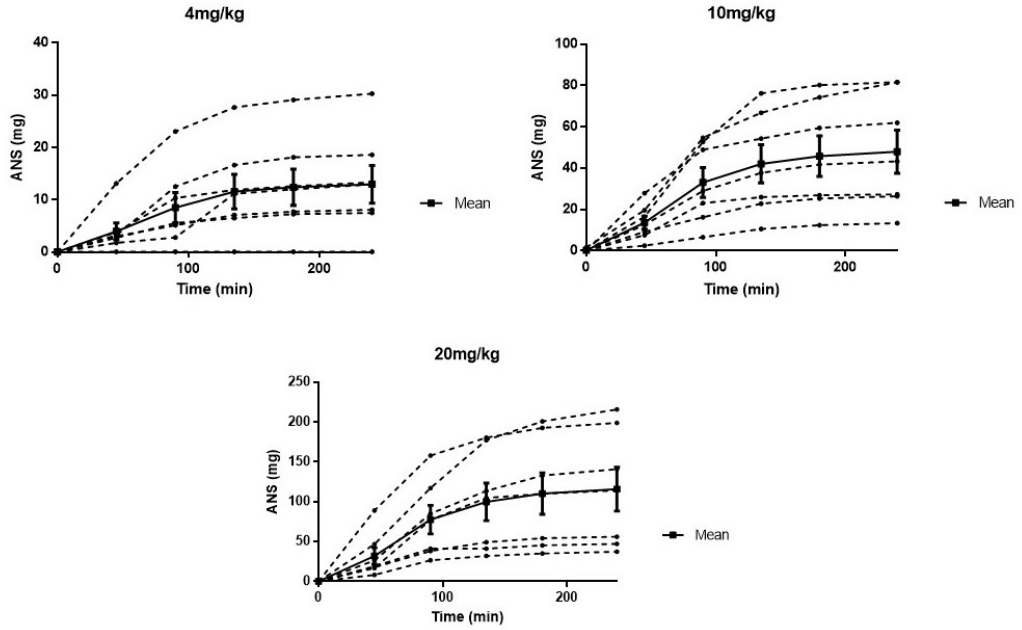

Fig. 2 Cumulative curves of mg anserine in urine after administration of 4, 10 and 20 mg/kg of anserine. Charts show the cumulative curve for each subject and the mean ± SEM (bold line).

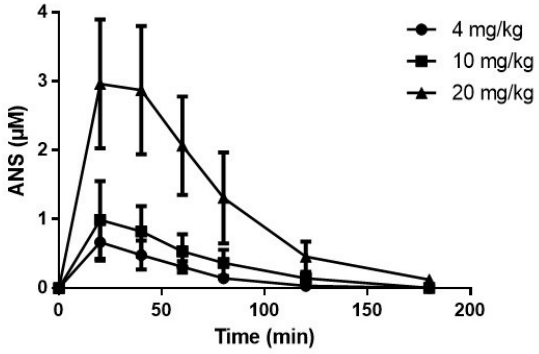

Fig. 3 Mean (± SEM) plasma profiles of anserine after the administration of 4, 10 and 20 mg/kg anserine.

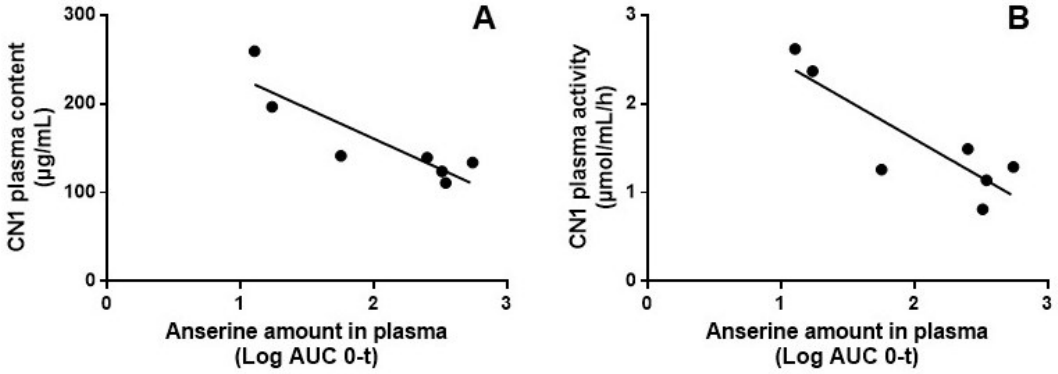

Fig. 4 Correlation between serum carnosinase-1 (CN1) protein content (A, $R^2 = 0.77$) and activity (B, $R^2 = 0.75$) and AUC of anserine (log) following ingestion of 20mg/kg.

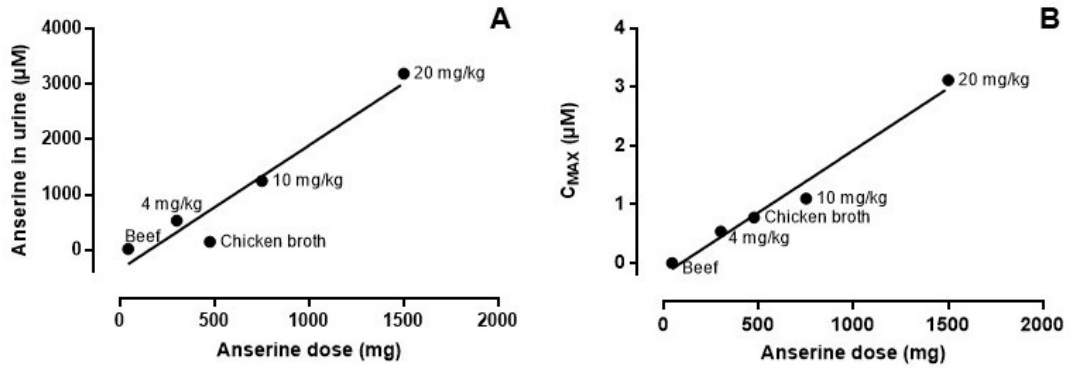

Fig. 5 Correlation between anserine levels in urine (A; total amount in µM) and plasma (B; Cmax) and the different anserine dosages of the present study and those of Yeum et al. (2010).

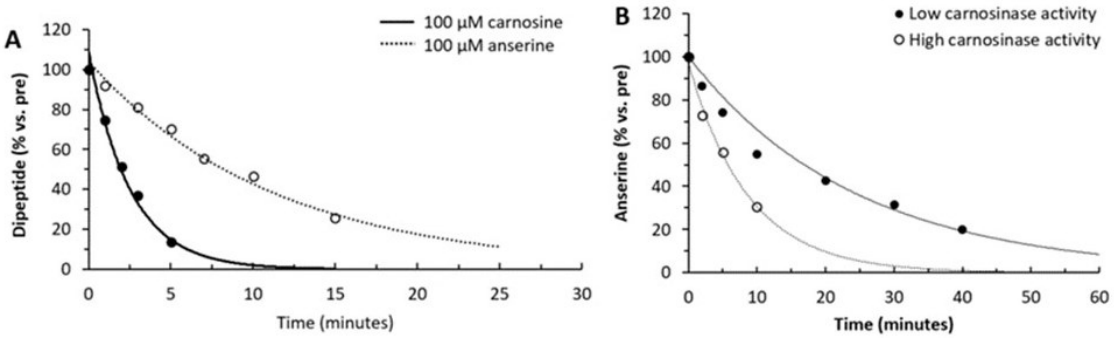

Fig. 6 *In vitro* hydrolysis of anserine (100 µM) in human plasma at room temperature. (A) The degradation of 100 µM anserine is much slower compared to 100 µM carnosine. (B) Hydrolysis of 100µM anserine is strongly delayed in plasma with a low vs. a high carnosinase activity (half-life 6.0 vs. 18.2 minutes).

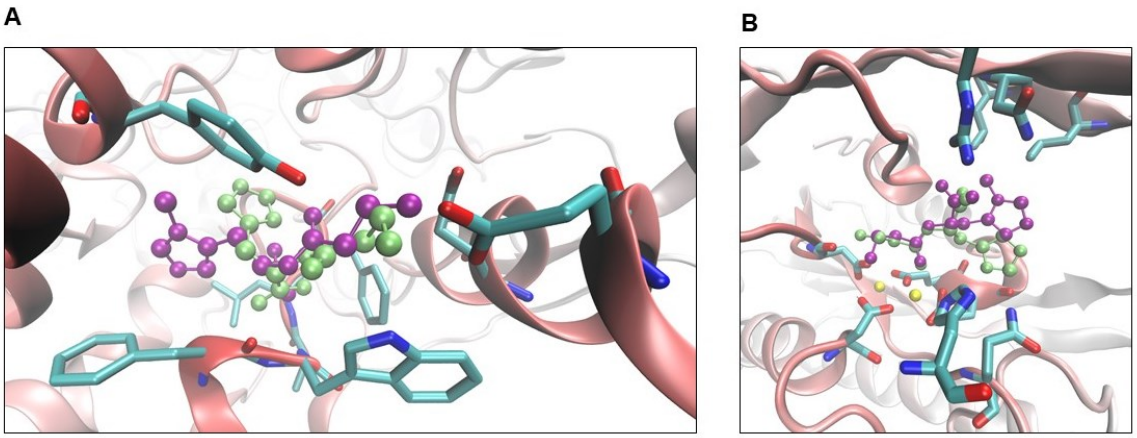

Fig. 7 Comparison of the poses of carnosine and anserine as computed within the binding cavity of CN1 (A) and PEPT1 (B). In both putative complexes carnosine and anserine are coloured in lime and purple, respectively. In CN1 complexes, the zinc ions are depicted as yellow spheres.

Conflict of Interest

The authors declare that they have no conflict of interest

Statement of human rights

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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