Application of ^<13>C stable isotope labeling liquid chromatography-multiple reaction monitoring-tandem mass spectrometry method for determining intact absorption of bioactive dipeptides in rats

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Title: Application of ^{13}C-stable-isotope labeling LC-MRM-MS/MS method for determining intact absorption of bioactive di-peptides in rats

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Short title: Absorption of di-peptides by LC-MRM-MS/MS method

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Abstract

Liquid chromatography-multiple reaction monitoring-mass/mass spectrometry (LC-MRM-MS/MS) method using ^{13}C-stable isotope labeled di-peptides was newly developed to simultaneously determine the absorption of three anti-hypertensive peptides (Val-Tyr, Met-Tyr and Leu-Tyr) into blood of spontaneously hypertensive rats in one run-in assay. After extracting ^{13}C-peptides in blood sample with a C_{18} cartridge, the extract was applied to a ^{13}C-mono-isotopic transition LC-MRM-MS/MS system, with D-Val-Tyr included as internal standard. An excellent separation of each di-peptide in LC was achieved at the elution condition of 5-100% methanol in 0.1% formic acid at a flow rate of 0.25 ml/min. The ^{13}C-peptides ionized by electron spray were detected in the positive ion mode within 15 min. The established method showed a high reproducibility with <10% coefficient of variation as well as high accuracy of >85%. After the administration of a mixture containing the three ^{13}C-di-peptides to rats at each dose of 30 mg/kg, we could successfully determine the intact absorption of each ^{13}C-peptide with the maximal absorption amount of 1.1 ng/ml-plasma for Val-Tyr by the proposed LC-MRM-MS/MS method.

Keywords: di-peptide; absorption; mass spectrometry; multiple reaction monitoring; ^{13}C-isotope labeling

Abbreviations used: ACE, angiotensin I-converting enzyme; RA, renin-angiotensin; MeOH, methanol; CH_3CN, acetonitrile; FA, formic acid; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid choromatography-mass/mass spectrometry; ESI, electron spray ionization; MRM, multiple reaction monitoring; LOD, limit of detection; LOQ, limit of quantitation; *S/N*, signal/noise; CV, coefficient of variation; AUC, area under the curve; SHR, spontaneously hypertensive rat.

Peptide research has become to be one of the growing fields for preventing-medicinal chemistry, since some clinical evidences provide the efficiency of peptide-intake for improving hypertension disease [1,2]. In a series of our study on underlying mechanism(s) of anti-hypertensive effect of small peptides, we have demonstrated that they play a potential role in lowering blood pressure through inhibiting local angiotensin I-converting enzyme (ACE) activity [3], blocking L-type Ca^{2+} channel [4] or relaxing vascular constriction [5,6]. In a recent report [7], an alternative vascular regulating mechanism of di-peptides was revealed, in which Met-Tyr might be involved in CO production via the stimulation of heme oxygenase (HO)-1 activity to enhance soluble guanylyl cyclase/cyclic GMP vasorelaxation pathway. In addition to these findings concerning anti-hypertensive effect of small peptides, other health-benefits of peptides have been demonstrated; e.g., peptides in soy protein could inhibit monocyte chemoattractant protein-1 expression in apolipoprotein E-deficient mice [8], suggesting that the intake of peptides may be efficient in preventing the onset of atherosclerosis. Anti-obestic effect of soy protein hydrolysate or peptides was also reported [9], in which they lowered blood cholesterol level by stimulating LDL-receptor transcription at the liver.

In order to make clear the mentioned health-benefits of peptides, verification for intact absorption and the subsequent physiological action at targeting organs must be required. However, less research was conducted to clarify absorption behavior or bioavailability of peptides, due to the lack of high-sensitive and/or high-selective analytical techniques. In our previous studies regarding absorption of small peptides into blood system, we have established a fluorimetric heart-cut column-switching high performance liquid chromatography (HPLC) method [10,11], with which an intact absorption of anti-hypertensive peptide, Val-Tyr, into human blood system at a picomole level was demonstrated [12,13]. However, the proposed specific assay for bioactive peptides would provide great effort, because it still requires tedious pre-extraction and pre-separation steps prior to the assay.

Recent analytical methods for peptide determination have been focused on the application of LC-MS/MS method, by which desired peptides could be assayed sensitively and rapidly. Platerink *et al.* [14] have proposed a sensitive and simultaneous assay of small ACE inhibitory peptides using an LC-MS/MS method, but no data on plasma level of each peptide were provided. Recently, Foltz *et al.* [15] have reported the

increase in plasma level of ACE inhibitory peptide of Ile-Pro-Pro in picomolar concentrations after the intake by an LC-MS method, but they failed to distinguish between endogenous and exogenous (administered) peptides. A stable-isotope labeling method has been developed and capable of selective quantification of flavor [16], drug [17] or pesticide [18] in biological systems, because of its higher stability rather than radioactive isotopes and easy differentiation in atomic weight between natural (unlabeled) and labeled compounds. Thus, it seems likely that an LC-MS method using stable-isotope labeled peptides makes it possible to clarify whether administered bioactive peptides can be absorbed intact into blood without any tedious procedures.

In this study, we undertook to develop a ^{13}C-stable-isotope labeling LC-MRM (multiple reaction monitoring)-MS/MS method for successive determination of some bioactive di-peptides in blood in one run-in assay. Val-Tyr [4], Met-Tyr [7] and Leu-Tyr [19] were targeted for ^{13}C-stable isotope labeling, because the three di-peptides are the candidates of sardine peptides [19] that elicited an apparent anti-hypertensive effect in mild hypertensive subjects [2]. For a better evaluation of accuracy and robustness, D-Val-Tyr, a diasteromer of Val-Tyr, was used as internal standard.

Materials and methods

Chemicals

Fmoc-Val-OH-1-^{13}C (isotope purity>99%), Fmoc-Met-OH-1-^{13}C (isotope purity>99%) and Fmoc-Leu-OH-1-^{13}C (isotope purity>99%) were purchased from ISOTEC Co. (Tokyo, Japan). Fmoc-Tyr-*O*-polymer was obtained from Kokusan Chemical Works (Osaka, Japan). Synthetic ^{13}C-stable-isotope labeled Val-Tyr, Met-Tyr and Leu-Tyr were synthesized using an Fmoc-solid phase synthesis method according to manufacturer instructions (Kokusan Chemicals, Osaka, Japan). Their sequences were confirmed on a PPSQ-21 amino acid sequencer (Shimadzu Co., Ltd., Kyoto, Japan), and their stable-isotope labeling was confirmed by a ^{13}C-NMR measurement (JNM A400, JEOL, Tokyo, Japan; 100 scans in D_2O were accumulated with 12 Hz spinning and 3.69 μs pulse width at 35˚). Distilled water, acetonitrile (CH_3CN), methanol (MeOH) and formic acid (FA) were of LC-MS grade (Merck, Darmstadt, Germany). All other chemicals were of analytical-reagent grade and used without further purification.

Instrumentation

A system for successive determination of bioactive peptides in one run-in assay was performed on an Agilent 1200 HPLC (Agilent Technologies, Waldbronn, Germany) coupled to an Esquire 6000 ESI (electron spray ionization) Ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany).

Preparation of standard solutions

Stock solutions of three di-peptides were individually prepared by dissolving each peptide in distilled water to a concentration of 1.0 mg/ml and stored at 4˚C. Working standards were prepared daily prior to experiments by combination of three stock solutions and further dilution with distilled water to give appropriate standard solutions. Three separate replicates of each of five different concentrations between 0.1 and 5.0 ng/ml of three peptides were conducted for obtaining the calibration curve. The internal standard D-Val-Tyr, which was synthesized by Fmoc-D-Val-OH with Fmoc-Tyr-*O*-polymer, was added with a final concentration of 1.0 ng/ml.

LC-MS/MS analysis

A chromatographic separation was performed either on a BioSuite C_{18} 3 μm PA-A column (2.1 mm x 150 mm) (Waters, Milford, MA, USA), Cosmosil MS-II column or Cosmosil 5C_{18}-ARII column (both columns: 5 μm, 2.0 mm x 150 mm, Nacalai Tesque, Kyoto, Japan). Each column was operated at 40˚C. Mobile phase (CH_3CN-water containing 0.1% FA or MeOH-water containing 0.1% FA) and a flow rate (0.20-0.30 ml/min) were investigated in the text to establish optimal elution conditions for individual transition ion of three peptides. An injection volume to the system was fixed at 25 μl.

For experiments on optimization of LC separation of three ^{13}C-di-peptides, the conditions of MRM-MS/MS measurements in positive ionization mode were as follows: nebulizer gas (N_2), 40 psi; dry gas (N_2), 8 ml/min; dry temperature, 330˚C; HV capillary, -2,750 V; HV end plate offset, -500 V; resolution; 0.25 *m/z*, target ion trap; 50,000. The width for mono-isotopic isolation of targeted precursor ions ($[M + H]^+$, *m/z*) of ^{13}C-di-peptides was set at 0.8 *m/z* for 282.6 of [^{13}C]Val-Tyr, 314.6 of [^{13}C]Met-Tyr and 296.6 of [^{13}C]Leu-Tyr, followed by the MRM analysis at 182.2 ($[Tyr + H]^+$) with the width of 4.0 *m/z* as a targeted product ion for all ^{13}C-peptides. After the

optimization of LC separation, MS/MS parameters set individually for each ^{13}C-di-peptide via time segment definitions; i.e., three segmented time-definitions (4.5-6.0 min for [^{13}C]Val-Tyr, 7.0-8.5 min for [^{13}C]Met-Tyr and 10.5-12.5 min for [^{13}C]Leu-Tyr) were programmed under their corresponding MS/MS conditions given in Table 1. The data obtained represent the mean ± SD.

Recovery

Recoveries of three ^{13}C-peptides were examined by spiking the below-mentioned rat plasma with known amounts of the peptides. Although the control plasma may possess the corresponding ^{12}C-peptides endogenously [10], the recovery test was performed without any consideration of endogenous peptides, because of their low plasma level of e.g., <0.02 ng/ml of ^{12}C-Val-Tyr [10] and of high selectivity of ^{13}C-peptide precursor ion in the mono-isotopic MS/MS analysis. A high selective isolation of mono-isotopic precursor ion was confirmed under the MRM-MS/MS conditions (width: 0.8 *m/z* for targeted ^{13}C-precursor ion) in either a solution of the three ^{13}C-di-peptides (^{13}C (+)/^{12}C (-)), corresponding ^{12}C-di-peptides (^{13}C (-)/^{12}C (+)) or a mixture of ^{13}C - and ^{12}C- di-peptides (^{13}C (+)/^{12}C (+)) at the concentration of 5.0 ng/ml for each peptide (see Supplemental Fig. 1): e.g., peak area of [^{13}C]Val-Tyr transition (282.4>182.0); ^{13}C (+)/^{12}C (-), 37,245; ^{13}C (+)/^{12}C (+), 38,459; ^{13}C (-)/^{12}C (+), 1,014. Namely, over-estimation of [^{13}C]Val-Tyr by the interference from ^{12}C-Val-Tyr was less than 5% in the mono-isotopic MRM-MS/MS analysis, even when endogenous ^{12}C-Val-Tyr occurred at the same amount of [^{13}C]Val-Tyr in sample solution. Intra- and inter-day accuracy and precision of the assay procedure as well as the recovery test were assessed in spiked concentrations of 0.5 and 1.0 ng/ml of each ^{13}C-peptide to rat plasma. Four replicates were analyzed on each of three separate days. The data obtained in this study were represented as mean ± SD.

Administration study of ^{13}C-di-peptides in rats

Male 18-week-old spontaneously hypertensive rats (SHR/NCrj, Japan SLC, Shizuoka, Japan) were fed on a laboratory diet (CE-2, Clea Japan, Tokyo, Japan) and given water *ad libitum*. All rats were housed for 1 week at 21 ± 1˚C and 55 ± 5% humidity under controlled lighting from 8:30 to 20:30. Each rat (n=4, 331.4 ± 2.2 g) was unfed for 16 h before a single oral administration of peptides by gavage. One ml of

a mixture containing the three ^{13}C-peptides at each dose of 30 mg/kg was administered to each rat. Control rats were administered the same volume of water without peptide. At each time point up to 24 h (0, 0.25, 0.5, 1, 1.5, 2, 4, 6 and 24 h), about 100 μl of blood was collected from the tail vein into a heparinized tube, immediately centrifuging at 3,500 *g* for 15 min (4˚C) to obtain plasma sample.

An aliquot (50 μl) of obtained plasma was then added 10 μl of internal standard and diluted with 50 μl of a 30% CH_3CN in 0.1% NaCl solution. After one hour under refrigeration (4˚C), 100 μl of 100% CH_3CN was added to sample and centrifuged at 10,000 *g* for 15 min (4˚C). This process was repeated twice. The supernatant (300 μl) was applied to a solid-phase extraction with a C_{18} Sep-pak Cartridge (Waters). The elution of ^{13}C-peptide fraction from the cartridge was carried out with 3 ml of 40% CH_3CN on a Waters Sep-Pak concentrator (Waters) at an elution rate of 1.0 ml/min. The eluate was dried and dissolved in 50 μl of 0.1% FA solution for LC-MS/MS analysis. The rat experiment was carried out under the Guidance for Animal Experiments in Faculty of Agriculture and in the Graduate Course of Kyushu University and the Law (No. 105, 1973) and Notification (No. 6, 1980 of the Prime Minister's Office) of the Japanese Government.

Pharmacokinetic analysis of concentration-time data was performed using Excel and GraphPad Prism (GraphPad, La Jolla, CA, USA) software. Maximum plasma concentration (C_{max}) and time of maximum concentration (t_{max}) were directly obtained from the plasmatic concentration-time plots. Elimination rate constant (k) was determined by linear regression analysis of data points plotted between the respective C_{max} and 24 h. The elimination half-life ($t_{1/2}$) was obtained from the equation: $t_{1/2}$ = 0.693/k. The area under the plasmatic concentration-time curve up to 24 h ($AUC_{0\text{-}24}$) was calculated using the trapezoidal rule. Data obtained in this study were represented as mean ± SEM.

Results and discussion

^{13}C-stable-isotope labeled di-peptides

^{13}C-isotope labeling MS method has been reported by Jacob *et al*. [20], who used [^{13}C]Tyr as an internal standard to determine endogenous oxidative stress marker, di-Tyr. In the present study, we applied ^{13}C-stable-isotope labeled peptides for

determining plasma bioactive di-peptide level for the first time. Fmoc-solid phase synthesis using Fmoc-Val-, Met- or Leu-OH-1-^{13}C was performed to obtain ^{13}C-stable-isotope labeled di-peptides that can distinguish exogenous (absorbed) di-peptides with endogenous di-peptides by a mono-isotopic transition MRM-MS/MS analysis. As a result of ^{13}C-NMR measurement, only a single carbon signal at around 160 ppm for each synthesized di-peptide was observed at 100 scans (Fig. 1), indicating that the ^{13}C-labeled carbon was successfully and specifically incorporated into the carbonyl position of di-peptide skeleton.

Optimization of LC-MRM-MS/MS analysis

Since three ^{13}C-di-peptides responsible for anti-hypertensive action [2,7] possess a structural similarity with Tyr moiety, the selection of packaging materials to separate each di-peptide must be greatly important. In our reports on isolation of ACE inhibitory peptides from natural proteins, C_{18} reversed-phase materials in combination to a CH_3CN-water mobile phase system was found to be the most retentive for small peptides [10,11]. In this study, hence we primarily examined whether a good separation of three ^{13}C-di-peptides was achieved on three different C_{18} materials (BioSuite, Cosmosil MS-II or Cosmosil 5C_{18}-AR II column) and in two different mobile phases (CH_3CN or MeOH) at a flow rate of 0.25 ml/min at 40˚C. As shown in Fig. 2, in CH_3CN-water/0.1% FA mobile phase system a BioSuite column provided the most sufficient separation of each peptide among the three columns. By changing the mobile phase to MeOH-water/0.1% FA, we found further improvement of their separation in each column without any apparent reduction in peak intensity compared with those in CH_3CN-water/0.1% FA mobile phase system. Although the ionization efficiency of morphine in CH_3CN solvent was reported to be higher than in MeOH [21], the present finding revealed that solvent characteristics such as viscosity or conductivity was not a limiting factor for ESI-ionization of di-peptides. As shown in Fig. 2, a 5C_{18}-AR II column in MeOH-water/0.1% FA system was selected for further experiments to be feasible for overall separation of ^{13}C-labeled Val-Tyr, Met-Tyr and Leu-Tyr with a significant difference in LC retention time (ΔRT) between each peptide.

Our next attempt was to optimize LC-elution parameters including flow rate and gradient mode of solvent, which may greatly affect the elution behavior of retention and/or sharpness of peak on 5C_{18}-AR II column. As summarized in Table 2, the flow

rate of 0.25 ml/min gave a significant ΔRT for segmentation analysis as well as high signal to noise (*S/N*) ratio. Similarly, a linear gradient elution of 5-100% MeOH in 20 min at 0.25 ml/min was regarded to be the best LC-separation condition in terms of appropriate ΔRT and *S/N* ratio, at which a three-step segmented MRM-MS/MS analysis for the ^{13}C-di-peptides was performed at individual optimal ionization conditions shown in Table 1. The analytical time, established on the basis of the above conditions for separating three di-peptides was within 15 min, being much faster than the time (<120 min) for determining small peptides by our reported fluorimetric column-switching HPLC method [10].

Linearity

Aliquots of 5% MeOH solution containing 0.1% FA spiked with known concentrations of [^{13}C]Val-Tyr, [^{13}C]Met-Tyr and [^{13}C]Leu-Tyr were used for calibration, along with D-Val-Tyr as internal standard. Under the optimal LC-MS/MS conditions described above, their correlation was linear with the coefficient of correlation (*r*) of greater than 0.995 over the concentration range of 0.1-5.0 ng/ml (Fig. 3): a typical calibration graph for [^{13}C]Val-Tyr; y=2.3465x + 0.5195 (*r*=0.9985), [^{13}C]Met-Tyr; y=0.8133x + 0.0634 (*r*=0.9948), [^{13}C]Leu-Tyr; y=2.1367x + 0.1971 (*r*=0.9966), where y is the peak area ratio (observed peak area of peptide against that of D-Val-Tyr) and x is the peptide concentration (ng/ml).

The limit of detection (LOD: 3.3 SD/slope) and the limit of quantitation (LOQ: 10 SD/slope) were calculated to be 0.04 and 0.12 ng/ml for [^{13}C]Val-Tyr, 0.02 and 0.06 ng/ml for [^{13}C]Met-Tyr and 0.03 and 0.11 ng/ml for [^{13}C]Leu-Tyr, respectively.

Validation performance

Under the established LC-MS/MS conditions, we determined the accuracy and precision by replicate analysis of rat plasma spiked with 0.5 and 1.0 ng/ml of each ^{13}C-peptide. Four replicates of each spiked concentration were analyzed on each of three separate days. As summarized in Table 3, we could determine the ^{13}C-peptide levels in plasma without any interference in ionization efficiency with plasma matrix in one run-in assay. Under the established conditions, each ^{13}C-peptide determined with high reproducibility of both average within-run and between-day CV (coefficient of variation) values of less than 10%. Table 3 also shows a high precision of the

solid-phase extraction/LC-MS/MS techniques with >85% of recoveries of each ^{13}C-peptide from spiked rat plasma. This indicates that the pre-extraction treatment of rat plasma with a C_{18}Sep-pak Cartridge would be sufficient to overwhelm any matrix effect [22] of plasma on ionization of ^{13}C-peptides, with high reproducibility and precision.

Application of LC-MRM-MS/MS method for determining absorption of ^{13}C-di-peptides into the circulating system of Sprague-Dawley rats

Fig. 4 shows absorption profiles of each ^{13}C-di-peptide ([^{13}C]Val-Tyr, [^{13}C]Met-Tyr and [^{13}C]Leu-Tyr) after oral administration of their mixture to rats up to 24 h by the mono-isotopic LC-MRM-MS/MS method (^{12}C- or ^{13}C-mono-isotopic transition monitoring for each targeted precursor ion). As shown in Fig. 4A, no significant detection in MS peak of their corresponding endogenous ^{12}C-peptides monitored at ^{12}C-mono-isotopic transition during the 24 h-protocol was observed after the ^{13}C-peptide administration, demonstrating that the stimulation or unexpected production of endogenous peptides by any administration stress to SHRs was excluded, and the characteristic MS profiles monitored at ^{13}C-mono-isotopic transition in Fig 4B were derived from the administered ^{13}C-di-peptides, even if endogenous peptides occurred in the plasma samples. Within the present assay conditions, we failed to detect endogenous ^{12}C-Val-Tyr, ^{12}C-Met-Tyr and ^{12}C-Leu-Tyr due to a small volume of plasma (50 μl), suggesting that these peptides may occur at the level of less than their experimental LODs (e.g., <0.02 ng/ml in human [10] for endogenous Val-Tyr) or may not be endogenously produced in rat blood system, in particular for Met-Tyr or Leu-Tyr.

As summarized in Table 4, we successfully evaluated exogenous di-peptide absorption into rat circulating system by our proposed ^{13}C-stable isotope labeling LC-MRM-MS/MS method for the first time. After the administration of ^{13}C-di-peptide mixture, a maximal plasma level (C_{max}) of each ^{13}C-di-peptide was observed at 1 h in the descending order of Val-Tyr>Leu-Tyr>Met-Tyr (C_{max}, ng/ml-plasma: [^{13}C]Val-Tyr, 1.14 ± 0.32; [^{13}C]Met-Tyr, 0.12 ± 0.03; [^{13}C]Leu-Tyr, 0.16 ± 0.06) (Fig. 5). The higher absorption of Val-Tyr was assumed to be due to its higher transport ability across the small intestine via PepT1 transporter [23] or less enzymes involved in the hydrolysis of Val-Tyr [24]. Further experiments should be, however, needed to clarify the difference in absorption of each di-peptide in this combined administration study, since the

absorption of Val-Tyr in combined administration with more absorbable peptidic ACE inhibitory drug (captopril) was suppressed by their competitive transport via the PepT1 transporter [25]. The $AUC_{0\text{-}24}$ of each peptide also supported the favorable absorption of Val-Tyr. The $AUC_{0\text{-}24}$ of Val-Tyr (5.46 ng·h/ml-plasma) obtained in this study was higher than the result (2.30 ng·h/ml-plasma) assayed by our previously reported column-switching HPLC method [26]. It would be caused by the dosage administered to rats (30 mg/kg-rat), three-fold higher than the previous assay [26]. After reaching the C_{max} of each peptide, their plasma levels declined to a baseline thereafter with a $t_{1/2}$ of about 2 h (Table 4). The relatively slower elimination of di-peptides from the circulating blood system than those of longer peptides (e.g., tri-peptides: $t_{1/2}$ of <30 min, [15]) was consistent with our previous reports [13, 26] and the result by Pentzien and Meisel [27], who demonstrated high stability of di-peptides including Val-Tyr in a 30 min-incubation with human serum. Taken together, it was demonstrated by our proposed ^{13}C-isotope labeling LC-MRM-MS/MS method that administered di-peptides of Val-Tyr, Met-Tyr and Leu-Tyr could be absorbed intact into blood with the maximal level of 1.1 ng/ml-plasma for Val-Tyr. Although the level of di-peptides was much lower than that of captopril (C_{max} at 25 mg-dose; ~ 200 ng/ml-plasma) [28], the proposed selective peptide assay technique allows us to clarify overall bioavailability including accumulation at local organs [26] and possible vascular regulation mechanisms [29] of bioactive peptides, like L-type Ca^{2+} channel blocking action [4, 30] and HO-1 stimulation [7].

Conclusion

In this study, we developed a ^{13}C-stable isotope labeling LC-MRM-MS/MS method for selective monitoring of exogenous bioactive peptides in blood. By combination with a reversed-phase column species and mobile phase of MeOH containing 0.1% FA, we successfully resolved the puzzles of the separation of [^{13}C]Val-Tyr, [^{13}C]Met-Tyr and [^{13}C]Leu-Tyr. A rapid (<15 min) and reproducible (<5% CV) determination of administered di-peptides in rat plasma in one run-in assay was achieved. The proposed method also revealed that the ^{13}C-di-peptides could be absorbed intact into blood at the C_{max} of 1.1 ng/ml-plasma for [^{13}C]Val-Tyr.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Footnotes

Fig. 1. ^{1}H- and ^{13}C-NMR spectra of synthesized [^{13}C]Val-Tyr, [^{13}C]Met-Tyr and [^{13}C]Leu-Tyr.

^{1}H- and ^{13}C-NMR spectra of ^{13}C-di-peptides in D_2O were obtained at 16 and 100 scans, respectively. Arrowed peaks indicate carbonyl carbon from the peptide bond of each ^{13}C-di-peptide.

Fig. 2. LC-MRM-MS/MS chromatograms of ^{13}C-di-peptides.

MRM analysis was performed at 182.0 *m/z* of $[Tyr + H]^+$ from each precursor ion. LC separations were performed on either BioSuite C_{18} 3 μm PA-A column (2.1 mm x 150 mm), Cosmosil MS-II column or Cosmosil 5C_{18}-ARII column (both columns: 5 μm, 2.0 mm x 150 mm) with 5-100% CH_3CN or MeOH in 0.1% FA at a flow rate of 0.25 ml/min at 40˚C. Each ^{13}C-peptide was injected at the concentration of 5 ng/ml. MS/MS conditions were described in Table 1.

Fig. 3. Calibration curves of [^{13}C]Val-Tyr, [^{13}C]Met-Tyr and [^{13}C]Leu-Tyr ranged from 0.1 to 5.0 ng/ml by ^{13}C-mono-isotopic LC-MRM-MS/MS method.

MRM analysis was performed at 182.0 *m/z* of $[Tyr + H]^+$ from each precursor ion at the width of 0.8 *m/z*. LC separation was performed on Cosmosil 5C_{18}-ARII column with 5-100% MeOH in 0.1% FA at a flow rate of 0.25 ml/min at 40˚C. Each value is expressed as mean ± SEM (*n*=3).

Fig. 4. A) Stacked intensity-time chromatograms (waterfall plot) of [^{12}C]Val-Tyr, [^{12}C]Met-Tyr, [^{12}C]Leu-Tyr and the internal standard D-Val-Tyr arranged by sampling time of rat plasma (15 min to 24 h). ^{12}C-mono-isotopic transition monitoring of ^{13}C-di-peptides-administered plasma was performed. B) Stacked intensity-time chromatograms (waterfall plot) of [^{13}C]Val-Tyr, [^{13}C]Met-Tyr, [^{13}C]Leu-Tyr and the internal standard D-Val-Tyr arranged by sampling time of rat plasma (15 min to 24 h). ^{13}C-mono-isotopic transition monitoring of ^{13}C-di-peptides-administered plasma was performed.

Fig. 5. Time-course of plasma concentrations of [^{13}C]Val-Tyr, [^{13}C]Met-Tyr and

[^{13}C]Leu-Tyr after a single oral administration of a mixture containing the three ^{13}C-di-peptides at each dose of 30 mg/kg to 18-week-old SHRs. One hundred μl of blood was taken from the tail vein at a fixed time of 0 to 24 h. Plasma sample was subjected to a C_{18} Sep-pak Cartridge, and the extract was applied to LC-MRM-MS/MS system. Each value is expressed as mean ± SEM (*n*=4).

Supplemental Figures

Supplemental Fig. 1. Detection of [^{13}C]Val-Tyr, [^{13}C]Met-Tyr and [^{13}C]Leu-Tyr by the ^{13}C-mono-isotopic LC-MRM-MS/MS method in the presence or absence of corresponding ^{12}C-di-peptides.

Concentration of each peptide was set at 5.0 ng/ml. ^{13}C(+)/^{12}C(-), only ^{13}C-di-peptides; ^{13}C(+)/^{12}C(+), ^{13}C-di-peptides + ^{12}C-di-peptides; ^{13}C(-)/^{12}C(+), only ^{12}C-di-peptides. LC separation was performed on Cosmosil 5C_{18}-ARII column with 5-100% MeOH in 0.1% FA at a flow rate of 0.25 ml/min at 40˚C. MS/MS conditions were described in Table 1.