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Quantification of nitrated tryptophan in proteins and tissues by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry

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Abstract

Aromatic amino acids are targets of reactive nitrogen species (RNS) such as peroxynitrite ($ONOO^-$) and nitrogen dioxide. It is known that tryptophan (Trp) as well as tyrosine is nitrated, generated isomers. However, no quantitative method to determine nitrotryptophan (NO_2Trp) in proteins has been developed so far.

In this study, we have developed a method for the quantification of Trp and NO_2Trp isomers, 2-, 4- and 6-NO_2Trp, which uses liquid chromatography with electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS). In order to confirm the applicability of our method to *in vitro* and *in vivo* system, we measured protein-bound NO_2Trp levels in $ONOO^-$ treated bovine serum albumin (BSA) and in liver of B6C3F1 mice at 2, 4, and 8 h after administration of 300 mg/kg acetaminophen (APAP). A mass spectrometer equipped with an electrospray ionization source using a crossflow counter electrode and ran in the positive ion mode (ESI^+) was used for multiple reaction monitoring (MRM) of transitions 205 → 188, 250 → 130, 250 → 159 and 250 → 233 for Trp, 2-, 4- and 6-NO_2Trp, respectively. The recoveries from mice liver samples were 98.3–105.9% for each compound. The limits of quantification were 50, 3.0, 10 and 4.0 nM for Trp, 2-, 4- and 6-NO_2Trp, respectively. In *in vitro* experiments demonstrated that all isomers of NO_2Trp were detectable from BSA treated with $ONOO^-$ and the amount generated decreased in the order of 6-, 4- and 2-NO_2Trp. In *in vivo* experiments, 4- and 6-NO_2Trp were detected in the liver of mice administered APAP. The concentration range of 4- and 6-NO_2Trp per mol of Trp in the sample was 2.24–3.92 and 26.96–32.71 nmol/mol of Trp, and its existence *in vivo* was confirmed for the first time with our method. The LC–ESI-MS/MS method was able to determine protein-bound NO_2Trp in a small amount of tissue sample, and is therefore applicable not only as a biomarker of RNS, but also as a mean to clarify novel mechanisms underlying RNS-related tissue damage.

Keywords: Nitrotryptophan; Peroxynitrite; Reactive nitrogen species; LC–ESI-MS/MS; Acetaminophen

1. Introduction

Nitrotyrosine (NO_2Tyr) is well known as a biomarker of reactive nitrogen species (RNS) such as peroxynitrite ($ONOO^-$) and nitrogen dioxide, which are produced in a variety of pathophysiological conditions such as neurodegenerative diseases, bacterial and viral infections, chronic inflammation and cancer [1–5]. $ONOO^-$ is produced by the direct reaction of nitric oxide with superoxide anion ($^{\bullet}O_2^-$) at an almost diffusion-limited rate (i.e., $6.7 \times 10^9\,l\,mol^{-1}\,s^{-1}$) [6]. Since the electrophilic nitronium species such as RNS is able to react with aromatic amino acids in protein, it is found that site-specific nitration is induced not only in tyrosine (Tyr) but also in tryptophan (Trp) [7]. Therefore, nitrated Trp is considered to be a biomarker of RNS-related disease like nitrotyrosine (NO_2Tyr). Additionally, Ikeda et al. [17] and Alvarez et al. [9] have reported that human Cu,

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Zn-superoxide disumutase (SOD), which catalyzes the disproportionation of $^{\bullet}O_2^-$ to H_2O_2 and oxygen, reacted with $ONOO^-$ in the presence of sodium bicarbonate, resulting in the loss of single Trp in the SOD structure and reduced enzyme activity. Based on these reports about NO_2Trp, the formation of NO_2Trp is expected not only as a biomarker of RNS similar to NO_2Tyr, but also as the key to clarify new mechanisms of RNS-related toxicity and carcinogenicity.

Several nitrated products such as 2-, 4-, 5-, 6- and 7-NO_2Trp have been produced in the reaction of Trp with $ONOO^-$ or a combination of myeloperoxidase (MPO), H_2O_2 and nitrite (NO_2^-) *in vitro* [7,8,10–13]. Ikeda and coworkers [13,14] have shown that 6-NO_2Trp is the major nitration product in the reactions of $ONOO^-/CO_2$ and $MPO/H_2O_2/NO_2^-$ with SOD. It has been reported that the formation of 6-NO_2Trp as the major product in myoglobin or hemoglobin via the reaction with $ONOO^-/CO_2$ [15], and in bovine serum albumin (BSA) via the reaction with $ONOO^-$ or $MPO/H_2O_2/NO_2^-$ [16]. However, no quantitative method to determine 6-NO_2Trp in proteins has been reported so far. Recently, Ikeda et al. [17] developed a method to detect 6-NO_2Trp in proteins, which involved western blot analysis with anti-6-NO_2Trp. Although immnochemical techniques can clarify the existence of NO_2Trp, its quantification is difficult. To clarify the biological function of NO_2Trp, a quantitative method having high sensitivity and precision is needed.

In this study, we developed a quantitative method to determine NO_2Trp in proteins, which uses liquid chromatography with electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS). The isomers of the target compound were found to be 2-, 4-, 6-NO_2Trp from the results of pre-experiments using BSA treated with $ONOO^-$ (data not shown). Furthermore, a suitable enzymatically protein digestion for the preparation of tissue samples without the artifact formation was used, which involved enzymatic protein digestion to detect NO_2Trp in tissue protein. In order to confirm the applicability of our method to the detection of NO_2Trp levels in protein, we measured protein-bound NO_2Trp levels in BSA treated with $ONOO^-$ and in liver of acetaminophen (APAP)-treated mice, an NO-mediated hepatotoxicity animal model [18–21].

2. Materials and methods

2.1. Chemicals and reagents

Tryptophan (Trp), trichloroacetic acid (TCA), hydrochloric acid (HCl), nitric acid, acetic acid, HPLC-grade acetonitrile and bovine serum albumin (BSA) were obtained from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Diethylenetriaminepentaacetic acid (DTPA) as a chelating agent, Protease Type XIV (Bacterial, from *Streptomyces griseus*) and acetaminophen (APAP) were purchased from Sigma (St. Louis, MO). L-[$^{15}N_2$]-tryptophan ([$^{15}N_2$]-Trp) was acquired from Spectra Stable Isotopes (Columbia, MD). Purified water was obtained with Milli-Q gradient A 10 equipped with an EDS polisher (Millipore, Bedford, MA).

2.2. Synthesis and purification of NO_2Trp standard and internal standards

In order to synthesize NO_2Trp as an analytical standard, 5.0 g of Trp was dissolved in 10 ml of acetic acid, followed by the addition of 2.0 ml of nitric acid. The mixture was stirred for 1.0 h at room temperature and neutralized with aqueous NaOH. The reaction mixture was separated and confirmed by liquid chromatography with photodiode-array detector and mass spectrometry (LC-PDA-MS). The LC-PDA-MS system (SHIMADZU: LC-10ADvp pump, SIL-HTC autosampler, SPD-M10Avp PDA detector, LCMS-2010A, Kyoto, Japan) was equipped with an electrospray ionization (ESI) source. Twenty microliters of the reaction mixture was injected directly onto a reversed-phase C_{18} column (CAPCELL PAK C_{18} MG, 2.0 mm × 250 mm, 5 μm, Shiseido, Kyoto, Japan) and eluted with 5.0% acetonitrile in 0.1% acetic acid (flow rate 0.2 ml/min). Mass analysis was performed in selected ion monitoring (SIM) and scan modes, respectively. The fragmentor voltage was 120 V in the positive ion mode for NO_2Trp.

To purify the products, 50 μl of the reaction mixture was injected directly onto a reversed phase C_{18} column (CAPCELL PAK C_{18} MG, 4.6 mm × 250 mm, 5 μm, Shiseido, Kyoto, Japan). The products were eluted at 21.1, 26.3 and 29.0 min (UV absorbance at 254 nm) with 5.0% acetonitrile in 0.1% acetic acid (flow rate 1.0 ml/min) and were collected with a SHIMADZU fraction collector (FRC-10A, Kyoto, Japan). The fractions obtained were dried *in vacuo* and weighed. The product's identity was confirmed by HPLC with MS (SHIMADZU LCMS-2010A, Kyoto, Japan) and ^{1}H NMR.

Synthesis of the stable isotopically labeled surrogate standard was also performed on the small scale by the same method. Briefly, 200 mg of [$^{15}N_2$]-Trp was dissolved in 2.0 ml of acetic acid, followed by the addition of 50 μl of nitric acid. The mixture was stirred for 1 h at room temperature and neutralized with aqueous NaOH. The reaction mixture was purified by HPLC.

2.3. Standard solutions

Stock solutions (1.0 mM) of Trp, 2-, 4- and 6-NO_2Trp were prepared in 10 mM HCl, respectively. Working solutions for calibration (4–1000 nM for Trp and 6-NO_2Trp, 3–1000 nM for 2-NO_2Trp and 10–1000 nM for 4-NO_2Trp) were prepared by the adding an adequate amount of surrogate standard (aqueous solution) and diluting with purified water to appropriate concentrations.

2.4. LC–ESI-MS/MS conditions

LC–MS/MS analyses were performed using an Alliance HT model 2795 liquid chromatographic system (Waters Corp.) coupled to a Micromass Quattro Ultima mass spectrometry system (Micromass, Beverly, MA). The mass spectrometer was operated using an ESI source in the positive ion mode (ESI^+) for multiple reaction monitoring (MRM). An aliquot (20 μl) of the sample was injected into a SHISEIDO CAPCEL PAK C_{18} MG (250 mm × 2.0 mm, 5 μm) maintained at 40 °C. Solvent A was

0.005% acetic acid and solvent B was acetonitrile. The column was equilibrated with a mixture of solvent A/solvent B (9/1, v/v). The compounds were eluted isocratically with solvents A and B at a flow rate of 0.2 ml/min.

In the assay for 2-NO_2Trp, the precursor ion ($[M+H]^+$) had a mass of *m/z* 250 and the selected product ion ($[M+H{-}H_2O{-}CO{-}CH_2N_2O_2]^+$) had a mass of *m/z* 130. Correspondingly for [$^{15}N_2$]-2-NO_2Trp, the precursor ion had a mass of *m/z* 252 and the selected product ion had a mass of *m/z* 132. The cone voltage used was 18 V and the collision energy was 10 eV. In the assay for 4-NO_2Trp, the precursor ion ($[M+H]^+$) had a mass of *m/z* 250, and the selected product ion ($[M+H{-}NH_3{-}C_3H_6O_2]^+$) had a mass of *m/z* 159. The detection at *m/z* 204 ($[M+H{-}H_2O{-}CO]^+$) of the product ion was more sensitive but less selective than the detection at the *m/z* of the other product ions. Correspondingly for [$^{15}N_2$]-4-NO_2Trp, the precursor ion had mass of *m/z* 252 and the selected product ion had a mass of *m/z* 160. The cone voltage used was 30 V and the collision energy was 19 eV. In the assay for 6-NO_2Trp, the precursor ion ($[M+H]^+$) had a mass of *m/z* 250 and the selected product ion ($[M+H{-}NH_3]^+$) had a mass of *m/z* 233. Correspondingly for [$^{15}N_2$]-6-NO_2Trp, the precursor ion had mass of *m/z* 252 and the selected product ion had a mass of *m/z* 234. The cone voltage used was 20 V and the collision energy was 11 eV. In the assay for Trp, the precursor ion ($[M+H]^+$) had a mass of *m/z* 205 and the selected product ion ($[M+H{-}NH_3]^+$) had a mass of *m/z* 188. For [$^{15}N_2$]-Trp, the precursor ion had a mass of *m/z* 207 and the product ion had a mass of *m/z* 189. The cone voltage used was 20 V and the collision energy was 11 eV. The source block temperature was 150 °C and the desolvation temperature was 350 °C. The flow rate of the cone gas was set at 200 l/h while that of the desolvation gas was set at 600 l/h. In order that Trp exists in protein at high concentration, all samples were diluted at the ratio of 1:10,000 before Trp assay.

2.5. Recovery

Recovery was evaluated by calculating the mean of the responses at each concentration. The spiked concentrations (low and high doses) of Trp, 2-, 4- and 6-NO_2trp were determined from the concentrations of each compound in the liver protein of control mice, using LC–ESI-MS/MS. A standard sample was spiked into control mouse sample at 50, 250 and 500 nM for Trp, at 5.0, 50 and 500 nM for 2- and 6-NO_2Trp, and at 10, 50 nM and 500 nM for 4-NO_2Trp.

2.6. NO_2Trp formation in BSA by the addition of $ONOO^-$

Ten milligrams of commercially available BSA was dissolved in 1.0 ml of 1.0 mM DTPA containing 100 mM sodium phosphate buffer (pH 7.4). $ONOO^-$ at several concentrations (0–1000 μM) was added to the BSA solutions and reaction mixtures were incubated at 37 °C after mixing for 1.0 h. After the reaction, protein was precipitated by adding 0.5 ml of 10% TCA, followed by centrifugation at 14,000 × *g* for 10 min at 4 °C. The residue was washed with 1.0 ml of digestion buffer, dispersed in 1.0 ml of digestion buffer containing 2.0 mg of protease and each surrogate standard, and incubated for 20 h at 50 °C. The digested protein was treated with 0.5 ml of 10% TCA and centrifuged at 14,000 × *g* for 10 min at 4 °C, and the supernatants were passed through 0.45 μm polyvinylidine difluoride (PVDF) filter. Then, 20 μl portion of the digested solution was injected into the LC–MS/MS for NO_2Trp analysis. After the dilution at the ratio of 1:10,000 of remaining digested sample, 20 μl portion of sample was injected into the LC–MS/MS for Trp analysis.

2.7. Animals, diet and housing conditions

Five-week-old male B6C3F1 mice (specific-pathogen-free) were purchased from SLC Japan (Shizuoka, Japan) and housed in polycarbonate cages (5 mice per cage) with hardwood chips as bedding in a conventional animal facility maintained under conditions of controlled temperature (23 ± 2 °C), humidity (55 ± 5%), air change (12 times/h) and lighting (12 h light/dark cycle). The animals were given free access to CRF-1 basal diet (Oriental Yeast, Tokyo, Japan) and tap water, and were used after a one-week acclimation period.

2.8. Animal treatment

The protocols for this study were approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences, Japan. Fifteen mice at the age of 6 weeks were administered 300 mg/kg APAP by i.p. injection and sacrificed 2, 4 and 8 h after the APAP administration. Five mice that received saline were used as control. Samples were immediately frozen in liquid nitrogen and stored at −80 °C until measurements of Trp and NO_2Trp.

2.9. Sample preparation

Tissue digestion was performed according to the method of Hensley et al. and our previous report [22,23]. Enzymatic protein digestion was applied to our method in the preparation of tissue samples. The samples were homogenized with digestion buffer (50 mM sodium acetate, pH 6.5). The mixture was centrifuged at 3000 × *g* for 10 min at 4 °C, and the supernatant was removed for analysis and quantified for protein in the supernatant. After adjusting the concentration of protein to 10 mg/ml of digestion buffer, the protein was precipitated by the addition of 0.5 ml of 10% TCA, followed by centrifugation at 14,000 × *g* for 10 min at 4 °C. The residue was washed with 1.0 ml of digestion buffer, dispersed in 1.0 ml of digestion buffer containing 2.0 mg of protease and each surrogate standard, and incubated for 20 h at 50 °C. The digested protein was treated with 0.5 ml of 10% TCA and centrifuged at 14,000 × *g* for 10 min at 4 °C, and the supernatants were passed through 0.45 μm PVDF filter. Then, 20 μl portion of the digested solution was injected into the LC–ESI-MS/MS for NO_2Trp analysis. After the dilution at the ratio of 1:10,000 of remaining digested sample, 20 μl portion of sample was injected into the LC–ESI-MS/MS for Trp analysis.

3. Results and discussion

3.1. Identification of nitrated Trp

As shown in Fig. 1A, three peaks (Peak1–3) were observed in the SIM chromatogram (*m/z* 250) of the reaction mixture of Trp nitration. Simultaneously, mass spectra and UV–vis absorption spectra were obtained with ESI/MS and PDA. Although three peaks showed *m/z* 250 in their MS spectra (Fig. 1B –D), their absorbance differed: 365, 340 and 400 nm, respectively. It identified that those peaks are 2-NO_2Trp (Peak 1), 6-NO_2Trp (Peak 2) and 4-NO_2Trp (Peak 3) as a result of the ^{1}H NMR (500 MHz) analysis. The chemical shifts and coupling constants of aromatic region were as follows: those of 2-NO_2Trp (D_2O with 30 mM NaOD) were H-4 (7.53 ppm, 1H, d, $J_{4-5} = 8.2$ Hz), H-7 (7.30 ppm, 1H, d, $J_{6-7} = 8.55$ Hz), H-5 (7.07 ppm, 1H, dd, $J_{5-6} = 7.6$ Hz, $J_{4-5} = 8.2$ Hz), H-6 (6.86 ppm, 1H, dd, $J_{6-7} = 8.55$ Hz, $J_{5-6} = 7.6$ Hz). Those of 4-NO_2Trp (D_2O with 30 mM NaOD) were H-5 (7.64 ppm, 1H, dd, $J_{5-6} = 7.9$ Hz, $J_{5-7} = 0.9$ Hz), H-7 (7.55 ppm, 1H, dd, $J_{5-7} = 0.9$ Hz, $J_{6-7} = 8.2$ Hz), H-2 (7.21 ppm, 1H, s), H-6 (7.00 ppm, 1H, dd, $J_{5-6} = 7.9$ Hz, $J_{5-7} = 8.2$ Hz). Those of 6-NO_2Trp (CD_3OD) were H-7 (8.37 ppm, 1H, d, $J_{5-7} = 1.9$ Hz), H-5 (7.98 ppm, 1H, dd, $J_{4-5} = 8.8$ Hz, $J_{5-7} = 1.9$ Hz), H-4 (7.75 ppm, 1H, d, $J_{4-5} = 8.8$ Hz), H-2 (7.60 ppm, 1H, s). The chemical structures of Trp (A), 2-NO_2Trp (B), 4-NO_2Trp (C), 6-NO_2Trp (D) and each stable isotopically labeled compound as a surrogate standard are shown in Fig. 2.

3.2. Optimal conditions for LC–MS/MS detection

Fig. 3A – D show the product ion spectra for Trp, 2-, 4- and 6-NO_2Trp. The mass spectrometer equipped with an ESI source using a crossflow counter electrode and ran in the positive ion mode (ESI^+) was used for MRM of the transitions 205 → 188, 250 → 130, 250 → 159 and 250 → 233 for Trp, 2-, 4- and 6-NO_2Trp, respectively.

The critical parameters affecting LC–MS/MS, namely, cone voltage, collision energy and mobile phase, were investigated. In order to establish the optimum cone voltage and collision energy for the detection of Trp and NO_2Trp isomers, the signal of *m/z* 205 and 250 precursor ions versus cone voltage were investigated, respectively. The optimal cone voltages were 20, 18, 20 V and 23 V in the positive ion mode for Trp, 2-, 4- and 6-

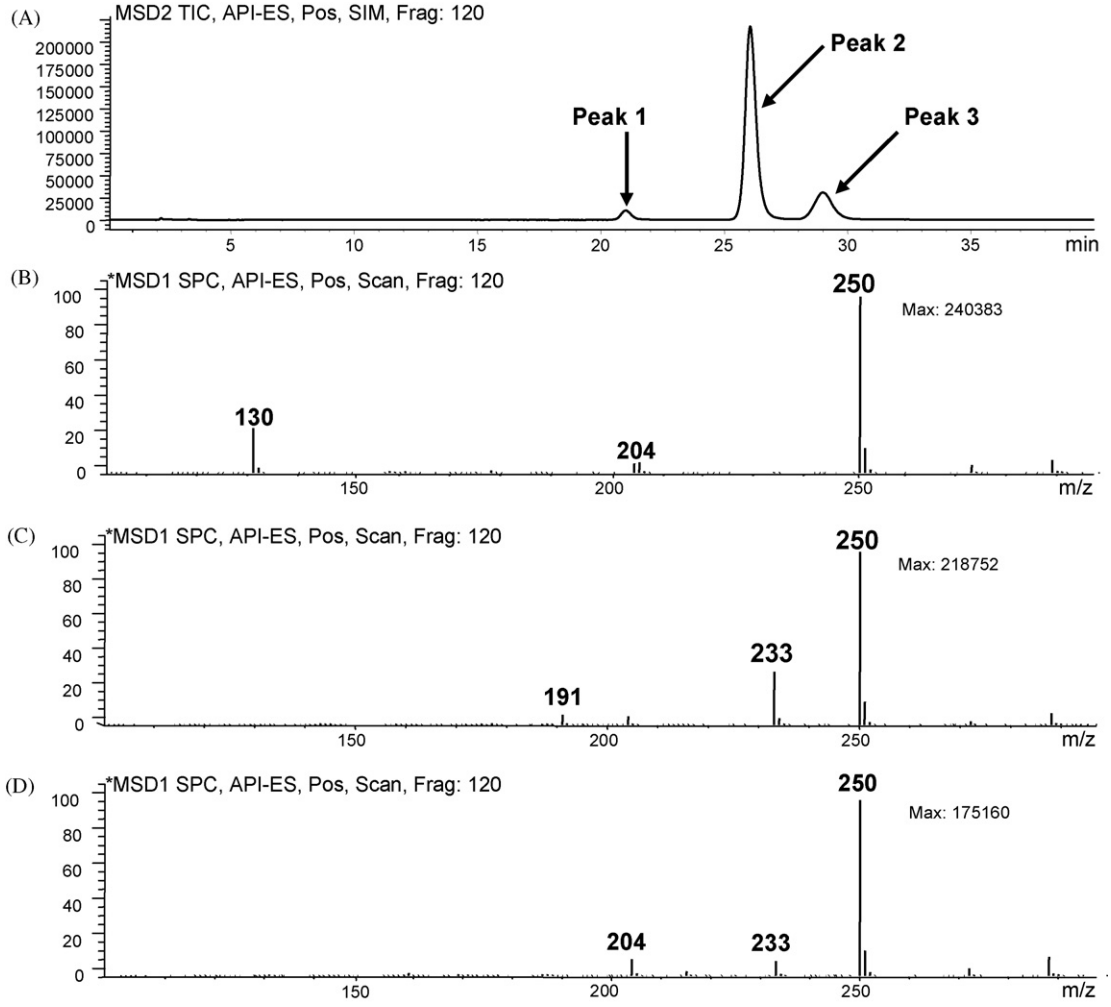

Fig. 1. SIM chromatogram and mass spectra of the products of NO_2Trp synthesis: (A) SIM chromatogram (*m/z* 250) of reaction mixture, mass spectra of the peaks, which were eluted from the C_{18} column at 21.1 min (B), 26.2 min (C) and 29.0 min (D). LC–MS conditions are described in Section 2.

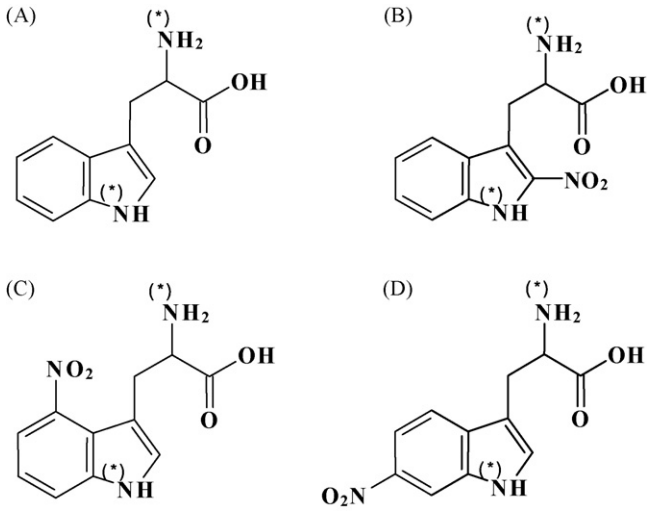

Fig. 2. Chemical structures of Trp, NO_2Trp isomers and each stable isotopically labeled compound. (A) Trp, (B) 2-NO_2Trp, (C) 4-NO_2Trp (D) 6-NO_2Trp. Asterisk (*) indicates nitrogen 15-labeled.

NO_2Trp standard solutions, respectively. Then, the signals of *m/z* 188, 130, 159 and 233 product ions versus collision energy were investigated, respectively. The optimal collision energies were 11, 10, 19 and 11 V for Trp, 2-, 4- and 6-NO_2Trp standard solutions, respectively. The ionization of the samples at the LC–MS interface is affected by the mobile phase; hence, a mobile phase containing a volatile acid or salt is used frequently. In this study, the responses were measured using 0–0.1% acetic acid in water–acetonitrile (v/v) as the mobile phase. The responses of Trp, 2-, 4- and 6-NO_2Trp were increased by the addition of acetic acid to the mobile phase. The increase in response reached a maximum and leveled off when 0.005% acetic acid was added. The LC conditions were as follows: 90% solvent A and 10% solvent B for 20 min, and 30% solvent A and 70% solvent B for 10 min. Then, the column was equilibrated with 90% solvent A and 10% solvent B for 10 min. Under these conditions, the standard retention times were 7.3 min for Trp, 9.2 min for 2-NO_2Trp, 12.9 min for 6-NO_2Trp and 14.9 min for 4-NO_2Trp. Additionally, although 6-NO_2Trp appeared concomitantly with 4-NO_2Trp in the MRM chromatogram of m/z 250 → 233, 2- and 4-NO_2Trp was individually detectable in the MRM chromatogram of m/z 250 → 130 and 250 → 159, respectively.

3.3. Validation of LC–MS/MS

The calculated instrument detection limit (IDL) of Trp, 2-, 4- and 6-NO_2Trp of the standard solutions were 1.3, 0.4, 1.6 and 0.6 nM, respectively, for LC–MS/MS detection at the ratio of the compound's signal to the background signal (S/N) of 3. In addition, the instrument quantification limit (IQL) calculated when S/N = 10 were 4.0, 1.5, 5.5 and 2.0 nM for Trp, 2-, 4- and 6-NO_2Trp, respectively. On the other hand, the limit of quantification (LOQ) in the real sample was 50, 3.0, 10 and 4.0 nM for Trp, 2-, 4- and 6-NO_2Trp, respectively. In order that Trp existed in the blank sample, it set up 50 nM from which the satisfactory recovery rate was obtained. The peak area ratio with respect to each surrogate standard was plotted, and the

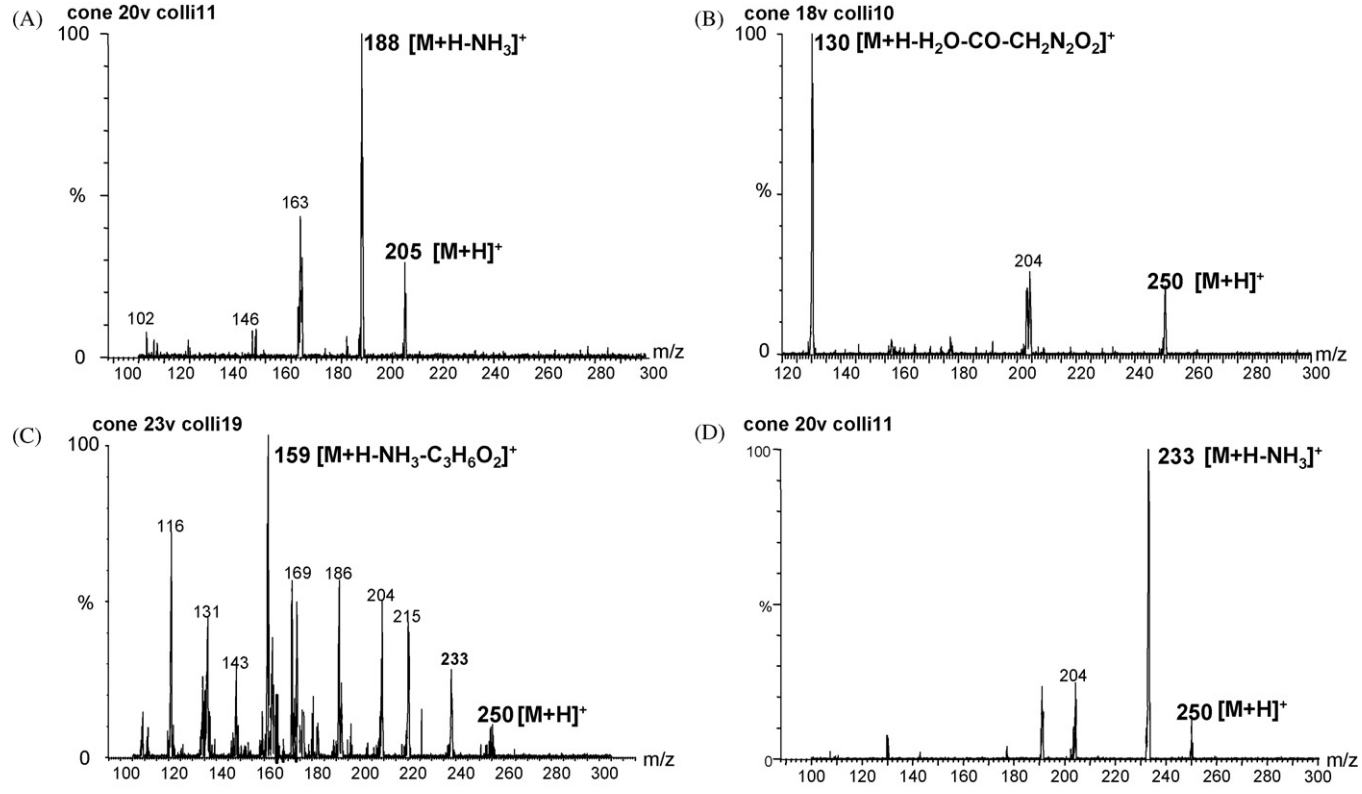

Fig. 3. Product ion spectra of (A) Trp, (B) 2-NO_2Trp, (C) 4-NO_2Trp and (D) 6-NO_2Trp.

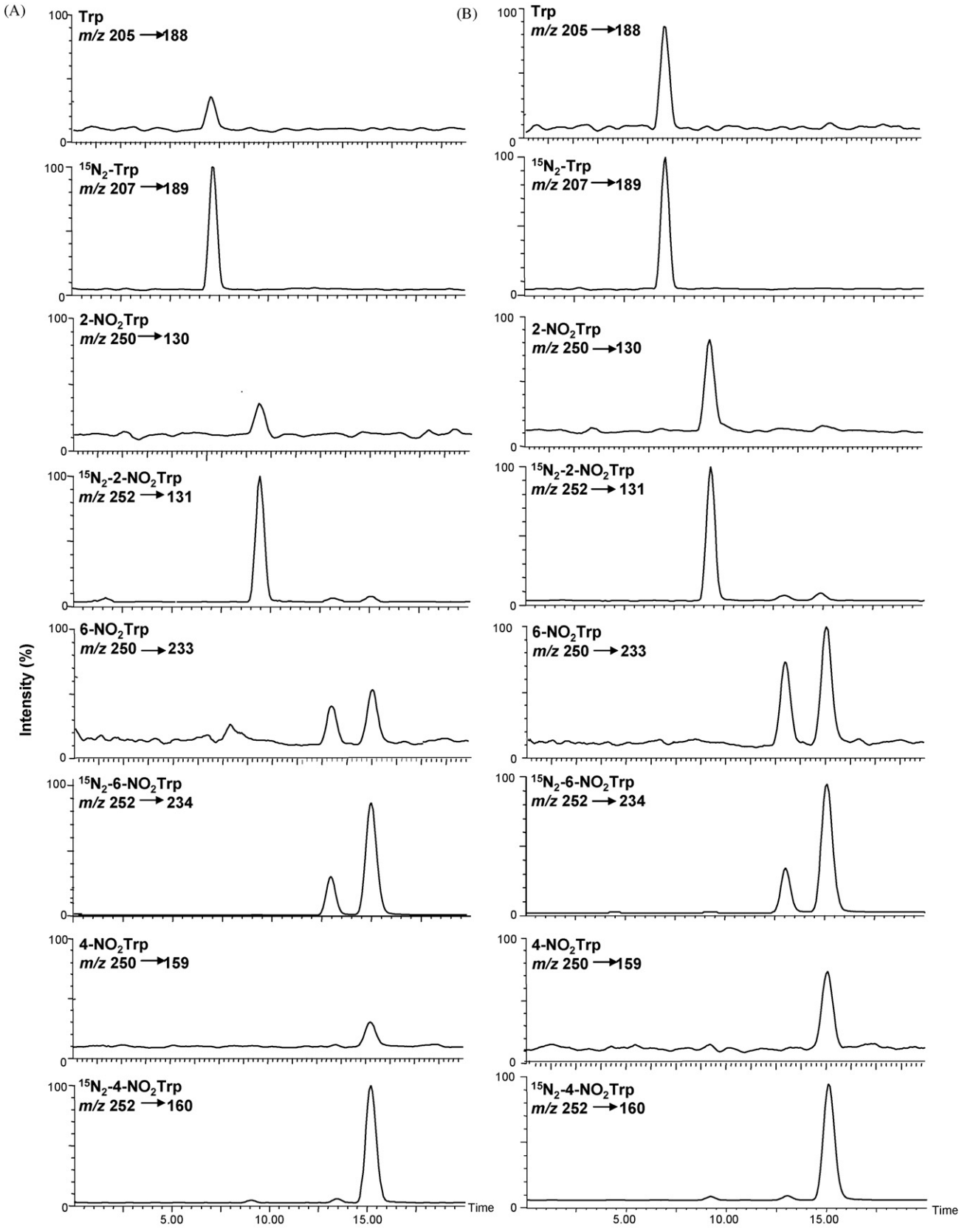

Fig. 4. MRM chromatograms of the mixture of Trp, NO_2Trp isomers and each surrogate standards at the LOD (A) and LOQ (B) levels. LC–MS/MS conditions are described in Section 2.

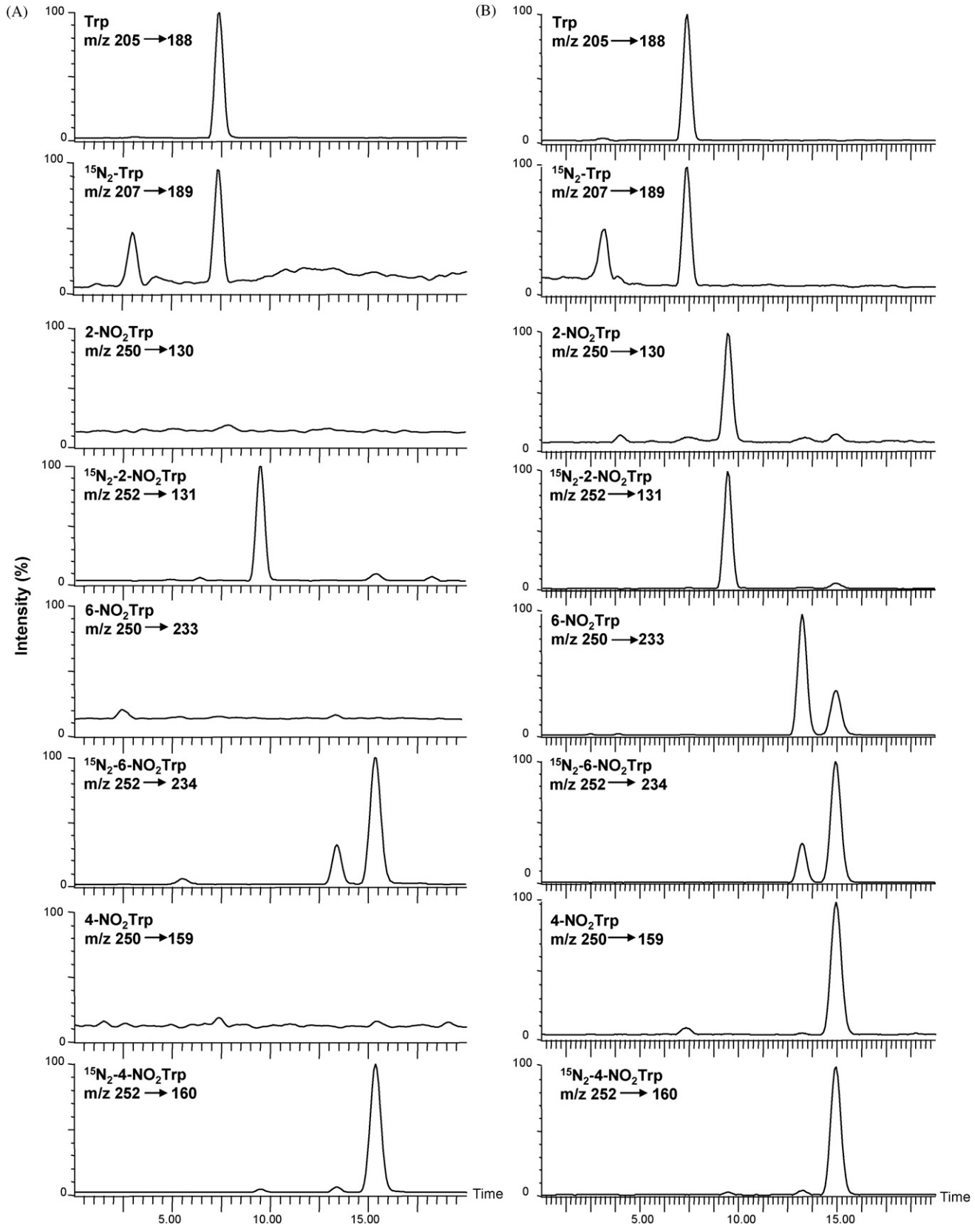

Fig. 5. MRM chromatograms of the products of BSA reacted with/without $ONOO^-$ after digestion with protease. 10 mg/ml BSA dissolved in 1.0 mM DTPA containing sodium phosphate buffer (pH 7.4) was incubated with several concentrations $ONOO^-$ (0–1000 μM) for 60 min at 37 °C. (A) Control, (B) 10 mg/ml BSA after treated with 1.0 mM $ONOO^-$. Protein digestion procedure is described in Section 2.

Table 1
Recoveries of Trp and NO_2Trp isomers in mice liver samples ($n = 5$)

	Added (nM)	Concentration	Recovery (%)	R.S.D. (%)
Trp	50	132.8 ± 4.3	100.7	7.10
	250	330.9 ± 2.7	99.5	1.00
	500	586.7 ± 12.7	100.6	2.54
	0	81.3 ± 1.3	–	–
2-NO_2Trp	5	4.9 ± 0.3	98.3	6.76
	50	50.5 ± 1.0	100.9	1.91
	500	520.8 ± 17.7	104.2	3.40
4-NO_2Trp	10	9.9 ± 0.5	100.8	4.79
	50	51.5 ± 0.7	103.1	1.35
	500	528.4 ± 14.9	105.7	2.83
6-NO_2Trp	5	5.2 ± 0.32	103.1	6.25
	50	51.0 ± 1.41	102.0	2.77
	500	529.5 ± 6.49	105.9	1.22

response was found to be linear over the calibration range, from LOQ to 1000 nM, with a correlation coefficient (r) of 0.999. The average retention times of Trp, 2-, 4- and 6-NO_2Trp standards were 7.27 min (R.S.D. = 0.49%, $n = 5$), 9.15 min (R.S.D. = 0.39%, $n = 5$), 14.97 min (R.S.D. = 0.34%, $n = 5$) and 12.94 min (R.S.D. = 0.59%, $n = 5$), respectively. Moreover, the accuracy of the R.S.D.s of the peak areas was 2.80, 2.04, 1.44 and 1.65%, respectively. MRM chromatograms of the mixture of Trp, NO_2Trp isomers and their corresponding surrogate standards in water at LOD and LOQ levels are shown in Fig. 4. Although the measurement sensitivity of 4-NO_2Trp in m/z 250 → 233 was the same as that in 250 → 159, m/z 250 → 159 was always monitored to confirm a peak.

As shown in Table 1, the recoveries from mice liver samples were 98.3–105.9% for each compound.

These results indicate that the method enables the precise determination of Trp and NO_2Trp isomers with the use of their surrogate standard, and is applicable to the detection of these compounds in animal tissue samples. Additionally, this method made it possible to measure 2-NO_2Trp and 4-NO_2Trp selectively in three isomers with the same molecular weight using the advantage of LC–MS/MS, respectively.

3.4. NO_2Trp formation in BSA by the addition of $ONOO^-$

In order to confirm whether NO_2Trp isomers are generated as a result of reacting protein with $ONOO^-$, we applied our method to the analysis of BSA treated with $ONOO^-$. Typical MRM chromatograms of the products of BSA treated with/without $ONOO^-$ are shown in Fig. 5. The NO_2Trp levels in BSA treated with $ONOO^-$ at several concentrations are shown in Fig. 6. NO_2Trp formation was increased with increasing concentration of $ONOO^-$ (0–1000 μM). By reacting with $ONOO^-$, it became clear that all three isomers of NO_2Trp were generated in BSA, and the amount generated decreased in the order of 6-, 4- and 2-NO_2Trp. Although 6-NO_2Trp is widely known in the nitrated Trp, there is almost no report about other isomers related biological function. However, in our study, it was confirmed to generate 2-, 4-NO_2Trp as well as 6-NO_2Trp in the BSA treated

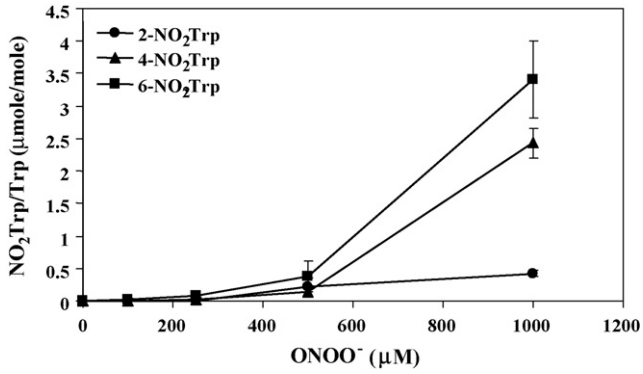

Fig. 6. Changes in NO_2Trp levels in BSA reacted with several concentrations $ONOO^-$. 10 mg/ml BSA dissolved in 1.0 mM DTPA containing sodium phosphate buffer (pH 7.4) was incubated with several concentrations $ONOO^-$ (0–1000 μM) for 60 min at 37 °C. The amount of generation of each isomer was calculated by obtaining the ratio with Trp. Values are mean ± S.D. of data for 5 samples.

with $ONOO^-$. This result indicates the need to measure all the isomers of NO_2Trp in order to evaluate Trp modification induced by RNS.

3.5. Determination of Trp and NO_2Trp in liver of mice administered APAP

It has been reported that NO_2Tyr is formed in the liver of mice administered a toxic dose of APAP [18–21], which implies that reactive oxygen and nitrogen species may play a key role in the mediation of APAP hepatotoxicity. In order to confirm whether the new method can detect NO_2Trp levels *in vivo*, it was applied to the analysis of liver protein in mice administered APAP. Typical MRM chromatograms of mouse liver protein samples are shown in Fig. 7. No peaks indicative of NO_2Trp were observed in control mice. In contrast, peaks indicative of 4- and 6-NO_2Trp were observed in APAP-treated mice. The 4- and 6-NO_2Trp/Trp ratios in liver proteins at 2, 4 and 8 h after APAP administration are summarized in Table 2. Although 2-NO_2Trp was not observed in all samples, 4-NO_2Trp and 6-NO_2Trp were detected in APAP-treated mice at 9.03–12.60 and 24.54–32.71 nmol/mol of Trp, respectively. Additionally, 6-NO_2Trp was detected in all the samples at 8 h after APAP administration.

In our previous study, NO_2Tyr was detected in both APAP treated and control mouse liver samples at a range of 58.2–81.6 μmol/mol of Tyr [23]. Furthermore, the increase of the NO_2Tyr/Tyr ratio was observed 2 h after APAP administration. The ratio reached a maximum (81.6 ± 7.0 μmol/mol of Tyr) at 4 h. On the other hand, no NO_2Trp was detected in control mice in the present experiment. Although 6-NO_2Trp was detected in all the samples 8 h after APAP administration, the time-dependent increase of the 6-NO_2Trp/Trp ratio was not observed. Additionally, since NO_2Trp/Trp (nmol/mol) ratio was 1/1000 of NO_2Tyr/Tyr (μmol/mol), these results may indicate that Tyr is more easily nitrated than Trp, and that NO_2Tyr is more suitable than NO_2Trp as a biomarker of RNS in physiological conditions. To date, no NO_2Trp has been detected in proteins from biological materials, and its existence was con-

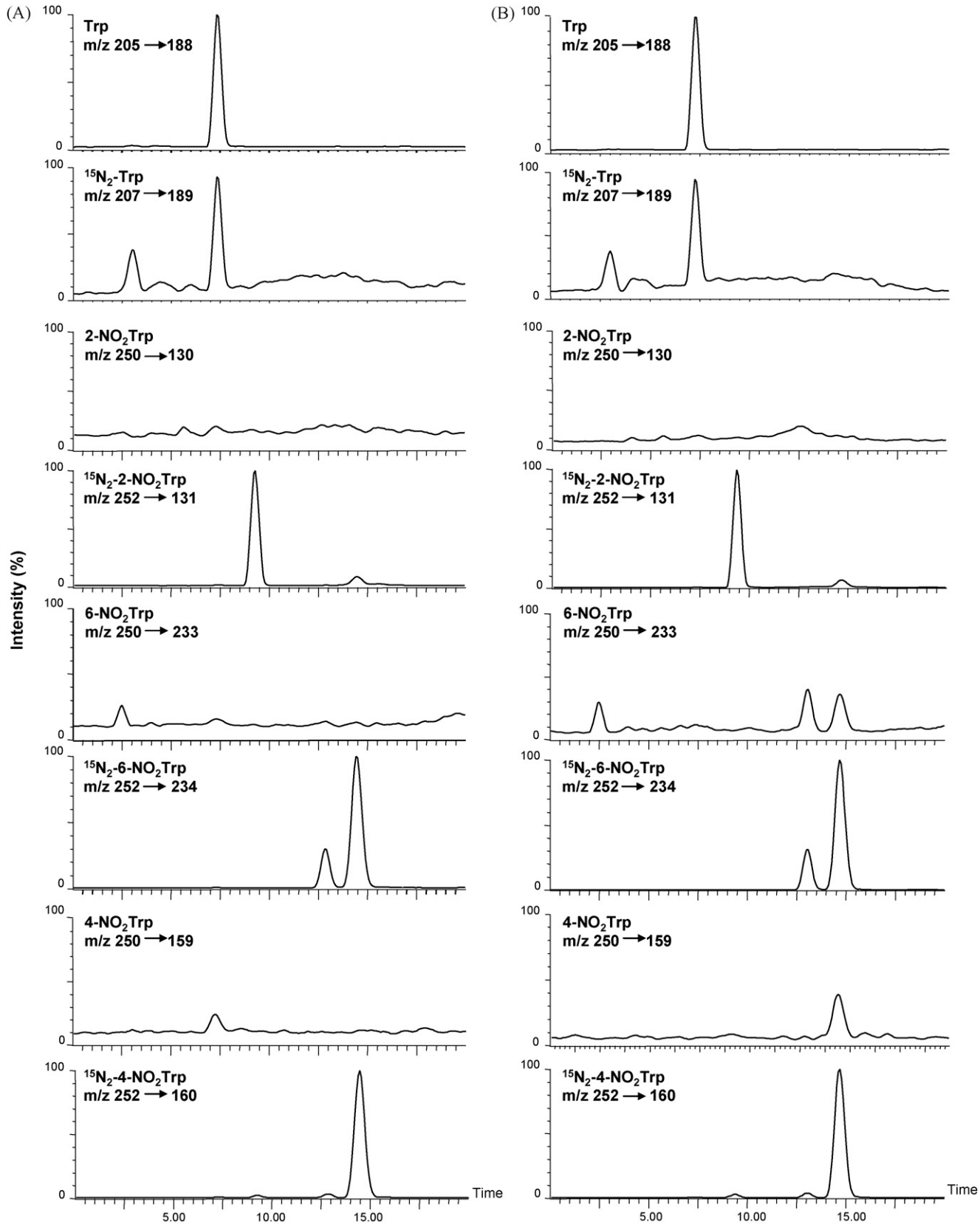

Fig. 7. MRM chromatograms of the mouse liver protein samples containing surrogate standards: (A) liver from a saline treated mouse; (B) liver from mouse administrated 300 mg/kg APAP for 8 h after treatment.

Table 2
Concentrations of 4- and 6-NO_2Trp in the mice administrated 300 mg/kg APAP for 2, 4 and 8 h after treatment

	Sample no.	4-NO_2Trp/Trp (nmol/mol)	6-NO_2Trp/Trp (nmol/mol)
Control	1	N.D.	N.D.
	2	N.D.	N.D.
	3	N.D.	N.D.
	4	N.D.	N.D.
	5	N.D.	N.D.
APAP 2 h	6	2.99	29.58
	7	N.D.	N.D.
	8	N.D.	N.D.
	9	N.D.	N.D.
	10	N.D.	N.D.
APAP 4 h	11	N.D.	N.D.
	12	3.92	26.96
	13	2.78	24.54
	14	N.D.	32.71
	15	N.D.	N.D.
APAP 8 h	16	3.45	31.36
	17	2.24	30.51
	18	N.D.	30.17
	19	N.D.	28.02
	20	N.D.	29.32

N.D.: not detected.

firmed for the first time with our method. These results indicate that NO_2Trp isomers need to be measured in order to evaluate RNS related damage. Furthermore, since we found that not only NO_2Tyr but also NO_2Trp is generated in the liver of mice administered APAP, NO_2Trp may be generated in other diseases in which NO_2Tyr generation is known, and may contribute to RNS-related several damages. Therefore, although NO_2Trp may not be suitable as a biomarker of RNS in physiological conditions, taking into consideration the influence on SOD, it is applicable as a powerful tool to clarify new mechanisms RNS related damage.

4. Conclusions

We have developed an LC–ESI-MS/MS method to detect Trp and NO_2Trp isomers. Use of the stable isotopes of Trp and NO_2Trp gave high precision of analysis. As our proposed method does not need SPE, it can be utilized for the routine analysis of *in vivo* samples. 4- and 6-NO_2Trp were successfully detected in the liver of mice administered APAP. Additionally, since our method that includes protein digestion has sufficient sensitivity and precision, it could also be used for the detection of NO_2Trp generated in other organs or enzymes. Finally, our LC–ESI-MS/MS method was able to measure Trp and NO_2Trp isomers in a small amount of tissue sample and could therefore contribute to exploring the mechanisms underlying RNS-mediated toxicity and carcinogenicity in animal experiments

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