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Determination of nitrotyrosine and tyrosine by high-performance liquid chromatography with tandem mass spectrometry and immunohistochemical analysis in livers of mice administered acetaminophen

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Abstract

Nitrotyrosine (NTYR) is used as a biomarker of nitrative pathology caused by peroxynitrite ($ONOO^-$) formation. NTYR measurement in biological materials usually employs such methodologies as immunohistochemistry staining, high-performance liquid chromatography and gas chromatography. In this study, we developed a method for the determination of tyrosine (TYR) and NTYR, using liquid chromatography with tandem mass spectrometry (LC–MS/MS). In order to confirm the applicability of our method to an in vivo system, we measured protein-bound NTYR levels using by LC–MS/MS method and immunohistochemical analysis in liver of B6C3F1 mice at 2 h, 4 h 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 set up for multiple reaction monitoring (MRM), which monitored the transitions 182.2 > 136.2, 227.1 > 181.2, 191.3 > 144.4 and 236.3 > 189.5, for TYR, NTYR, [$^{13}C_9$]-TYR and [$^{13}C_9$]-NTYR, respectively. The average recoveries from mice liver protein samples spiked with 25 μM TYR and 100 nM NTYR were 94.4% and 95.6%, respectively, with correction using the added surrogate standards. The limits of quantification were 100 nM for TYR and 0.5 nM for NTYR. NTYR was detected all liver samples of mice by the proposed LC–MS/MS method. The concentration range of NTYR per milligram protein in samples was 0.17–0.3 pmol/mg protein. And the level reached a maximum at 4 h. These data were well correlated with the result obtained by an immunohistochemical reaction with anti-NTYR antibody. The LC–MS/MS method was able to determine protein-bound NTYR in a small amount of tissue sample, and is therefore expected to be a very powerful tool for evaluating $ONOO^-$ generation in an in vivo system.
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Keywords: Nitrotyrosine; Peroxynitrite; LC–MS/MS; Acetaminophen; Mouse

1. Introduction

Nitric oxide (NO) is a free radical that is generated endogenously by a variety of mammalian cells, including the vascular endothelium, neuron smooth muscle, macrophage, neutrophil, platelet and pulmonary epithelium of bronchiolar cell [1]. It has also been identified as a neurotransmitter or a neuromodulator in the neuronal system [2] and a cytotoxic factor in the immune system [3]. In addition, NO is believed to be related to tissue damage, such as ischemic/reperfusion tissue damage [4], and excitatory neuronal death [5]. It has been shown that, in an oxygenated environment, NO may directly react with the superoxide anion (O_2^-), producing peroxynitrite ($ONOO^-$) at an almost diffusion-limited rate (i.e., $6.7 \times 10^9\,l\,mol^{-1}\,s^{-1}$) [6]. Since the electrophilic nitronium species is able to react with phenolic compounds, the site-specific nitration of protein-bound tyrosine (TYR) may produce 3-nitro-L-tyrosine (NTYR), a stable end product that is frequently employed as a diagnostic marker of $ONOO^-$ [7]. Moreover, since it is known that low levels of NTYR exist in animal tissues, a highly sensitive and accurate

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analytical method is necessary to estimate the variation in NTYR levels.

Up to now, a number of analytical methods have been developed to determine NTYR levels in biological fluids and tissues, including immunohistochemical analysis [8–10] and high-performance liquid chromatography (HPLC) with ultraviolet (UV) [11,12] or electrochemical detection (ECD) [13–16]. However, Kaur et al. [17] pointed out that the use of LC–ECD or LC–UV for the detection of NTYR in human brain lesions provided erroneous data due to the presence of an unknown artifact whose detection properties are similar to those of authentic NTYR. To this end, analytical techniques for detecting NTYR based on gas chromatography–mass spectrometry (GC–MS) [18–20], liquid chromatography coupled to MS (LC–MS) and tandem mass spectrometry (LC–MS/MS) [21,22], have been developed over the past several years. However, in an animal experiment, detection of NTYR by immunohistochemical analysis is widely used, since those LC or GC methods are complicated and are not convenience. Although immunohistochemical analysis can pinpoint the place where NTYR exists, it is difficult to perform a fixed quantity. Considering that a simple, direct, high precision and convenient method was required to analyze tissue samples in vivo because of their small amount and crude state, further improvement of the LC–MS/MS is requisite.

In this study, we developed an LC–MS/MS method in which the separation system and the sample preparation method were modified from those of the conventional LC–MS/MS method. In particular, a suitable method for preparing tissue samples without the artifact formation [21,23] was used, which involved enzymatic protein digestion to detect NTYR in tissue protein. In order to compare LC–MS/MS method with immunohistochemical analysis, the detection of protein-bound NTYR levels in the liver of acetaminophen (APAP)-treated mice, an NO-mediated hepatotoxicity animal model [8–10,24], was performed by both method.

2. Materials and methods

2.1. Chemicals and reagents

TYR, trichloroacetic acid (TCA), hydrochloric acid (HCl), nitric acid, acetic acid and HPLC-grade acetonitrile were obtained from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). NTYR, protease and APAP were purchased from Sigma (St. Louis, MO). L-[$^{13}C_9$]-tyrosine ([$^{13}C_9$]-TYR) was acquired from Cambridge Isotope Laboratories (Andover, MA). Purified water was obtained with Milli-Q gradient A 10 equipped with an EDS polisher (Millipore, Bedford, MA).

2.2. Synthesis and purification of internal standards

In order to synthesize L-3-nitro-[$^{13}C_9$]-tyrosine ([$^{13}C_9$]-NTYR) as the internal standard, 18.1 mg of [$^{13}C_9$]-TYR was dissolved in 2 ml of HCl, 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 separated by HPLC (Shimadzu: LC-10ADvp pump, SIL-HTC autosampler, SPD-10Avp UV detector, Kyoto, Japan). Fifteen microliters of the reaction mixture was injected directly onto a preparative reversed-phase C_{18} column (L-Column, 4.6 mm × 150 mm, 5 μm, Chemicals Evaluation and Research Institute, Tokyo, Japan). [$^{13}C_9$]-NTYR was eluted at 13 min (maximal UV absorbance at 270 nm) with 5% methanol in 0.05% acetic acid (flow rate of 1 ml/min) and was collected by a Shimadzu fraction collector (FRC-10A). The fraction obtained was neutralized with aqueous NaOH, dried in vacuo and weighed. The product identity was confirmed by HPLC with MS (Shimadzu LCMS-2010A).

2.3. Standard solutions

Stock solutions (1.0 mM) of TYR and NTYR were prepared in 10 mM HCl, respectively. Working solutions for calibration (0.1–1000 μM for TYR and 0.5–1000 nM NTYR) were prepared by the addition of an adequate amount of surrogate standard (aqueous solution) and diluting with purified water to appropriate concentrations.

2.4. LC–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, USA). The mass spectrometer was operated using an electrospray ionization 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 II (150 mm × 2.0 mm, 5 μm) maintained at 40 °C. Solvent A was 0.01% acetic acid and solvent B was acetonitrile. The column was equilibrated with a mixture of 97% solvent A and 3% solvent B. The compounds were eluted with a continuous gradient of solvents A and B at a flow rate of 0.2 ml/min.

In the assay for NTYR, the precursor ion ($M+1$) had a mass of 227.1 *m/z*, and the selected product ion had a mass of 181.1 *m/z*. The detection at 181.1 *m/z* was more sensitive but less selective than the detection at the *m/z* of the other product ions. Correspondingly for [$^{13}C_9$]-NTYR, the precursor ion had a mass of 236.3 *m/z* and the selected product ion had a mass of 190.5 *m/z*. 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 590 l/h. All samples were diluted at the ratio of 1 to 100 before assay.

In the assay for TYR, the precursor ion had a mass of 182.2 *m/z* and the selected product ion had a mass of 136.2 *m/z*. For [$^{13}C_9$]-TYR, the precursor ion had a mass of 191.3 *m/z* and the product ion had a mass of 144.1 *m/z*. The cone voltage used was 15 V and the collision energy was 12 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 590 l/h. Because the TYR concentration was higher than the NTYR concentra-

tion, all samples were diluted at the ratio of 1 to 10,000 before assay.

2.5. Recovery

The recovery was evaluated by calculating the mean of the response at each concentration. The spiked concentrations (low and high doses) of NTYR and TYR were determined from the concentrations of NTYR and TYR in the liver protein of control mice, which were determined by LC–MS/MS. A standard sample was spiked into control mouse sample at 100 nM and 200 nM for NTYR, and at 25 μM and 50 μM for TYR, respectively.

2.6. 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 (five mice per cage) with hardwood chips for 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/12-h 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 1-week acclimation period.

2.7. Animal treatment

The protocols for this study were approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences, Japan. At the age of 6 weeks, a total of 15 mice were given APAP at a dose of 300 mg/kg by i.p. injection and sacrificed 2 h, 4 h and 8 h after the APAP treatment. Five mice that received saline were used as control. The left lobule of liver samples was fixed in buffered formalin and then routinely processed for embedding in paraffin and sectioning for hematoxylin and eosin (H&E) stain or immunohistochemical for NTYR. The remaining samples were immediately frozen in liquid nitrogen and stored at −80 °C until measurements of NTYR and TYR their protein contents.

2.8. Sample preparation

Tissue digestion was performed according to the method of Hensley et al. [16]. 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 and quantified for total protein. 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 ml of digestion buffer, dispersed in 1 ml of digestion buffer containing 2 mg of protease, 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.

2.9. Immunohistochemical analysis of NTYR

The slides were incubated in 0.6% H_2O_2 for 30 min at room temperature to block endogenous peroxidase activity. The sections were then blocked with 1% goat serum in PBS for 30 min. After washing with PBS, the sections were incubated overnight with anti-NT rabbit polyclonal IgG (BD Transduction Laboratories, San Diego, CA). The final concentration of the antibody was 1 mg/ml in 1% bovine serum albumin, in PBS. The next day, the sections were washed with PBS, and a Vector Universal Elite ABC kit (PK-6101) was used according to the manufacturer's instructions. The slides were counterstained with hematoxylin then dehydrated with ethanol and xylene before permanent mounting and microscopic evaluation.

3. Results and discussion

3.1. Optimal conditions for LC–MS/MS

The chemical structures of TYR, NTYR, [$^{13}C_9$]-TYR and [$^{13}C_9$]-NTYR are shown in Fig. 1. The mass spectrometer equipped with an electrospray ionization source using a crossflow counter electrode and ran in the positive ion mode (ESI^+) was set up for MRM of the transitions 182.2 > 136.2, 227.1 > 181.1, 191.3 > 144.1 and 236.3 > 190.5, for TYR, NTYR, [$^{13}C_9$]-TYR and [$^{13}C_9$]-NTYR, respectively.

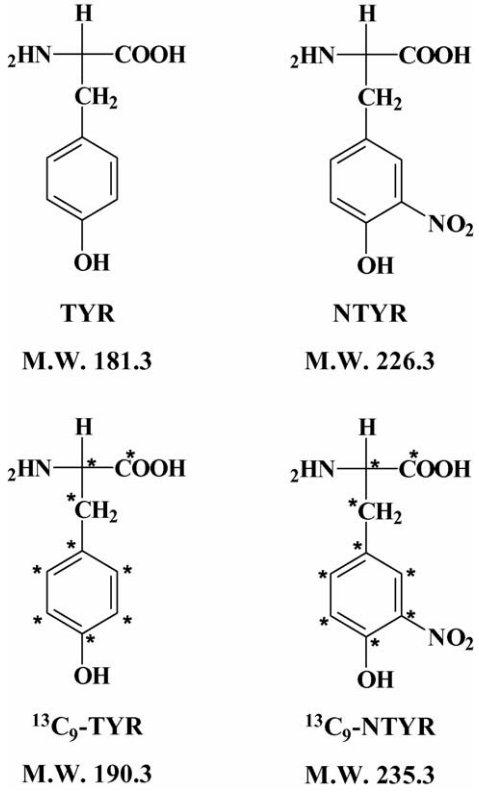

Fig. 1. Chemical structures of NTYR, TYR, [$^{13}C_9$]-NTYR and [$^{13}C_9$]-TYR. Asterisk (*) indicates carbon 13-labeled.

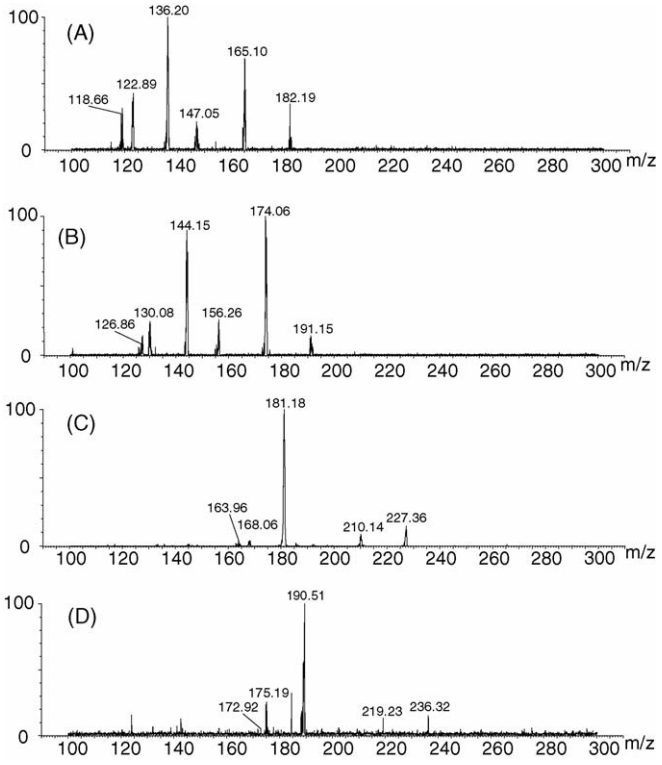

Fig. 2. Product ion spectra of: (A) TYR, (B) [$^{13}C_9$]-TYR, (C) NTYR and (D) [$^{13}C_9$]-NTYR.

Fig. 2 shows the product ion spectra for TYR, NTYR, [$^{13}C_9$]-TYR and [$^{13}C_9$]-NTYR.

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 NTYR and TYR, the signals of *m/z* 182.2 and 227.1 precursor ions versus cone voltage were investigated, respectively. The optimal cone voltages were 15 V and 20 V in the positive ion mode for TYR and NTYR standard solutions, respectively. Then, the signals of *m/z* 136.2 and 181.1 product ions versus collision energy were investigated, respectively. The optimal collision energies were 12 eV and 11 eV for TYR and NTYR standard solutions, respectively. The ionization of the samples at the LC–MS/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 TYR and NTYR were increased by the addition of acetic acid to the mobile phase. The increase in response reached a maximum and leveled off thereafter when 0.01% acetic acid was added. The gradient conditions were as follows: 97–30% solvent A and 3–70% solvent B for 9 min, 30% solvent A and 70% solvent B for 3 min, and 30–97% solvent A and 70–3% solvent B for 3 min. Then, the column was equilibrated with 97% solvent A and 3% solvent B for 5 min. Under these conditions, the typical standard retention times were 3.02 min for TYR and 5.75 min for NTYR.

Table 1
Validation of LC–MS/MS analysis

	LOD[a] (nM)	LOQ[b] (nM)	R.S.D.$_{PA}$ (%)	Correlation coefficient	R.S.D.$_{RT}$ (%)
Tyrosine	30	100	2.80	0.999 (0.1–50 μM)	0.19
Nitrotyrosine	0.1	0.5	1.44	1.000 (0.5–1000 nM)	0.20

[a] LOD: limit of detection (S/N = 3).
[b] LOQ: limit of quantitation (S/N = 10).

3.2. Validation of LC–MS/MS analysis

As shown in Table 1, The calculated limits of detection (LODs) of TYR and NTYR of the standard solutions were 30 nM and 0.1 nM, respectively, for LC–MS/MS detection with the ratio of the compound's signal to the background signal (S/N) of 3. In addition, the limits of quantification (LOQs) calculated when S/N = 10 were 100 nM and 0.5 nM for TYR and NTYR, respectively. The peak area ratio with respect to each surrogate standard was plotted, and the response was found to be linear over the calibration range with a correlation coefficient (r) of 0.999. The average retention times of TYR and NTYR standards were 3.02 min (R.S.D. = 0.2%, n = 5) and 5.75 min (R.S.D. = 0.4%, n = 5), respectively. Moreover, the accuracy of the R.S.D.s of the peak areas was 2.8% and 1.4%, respectively.

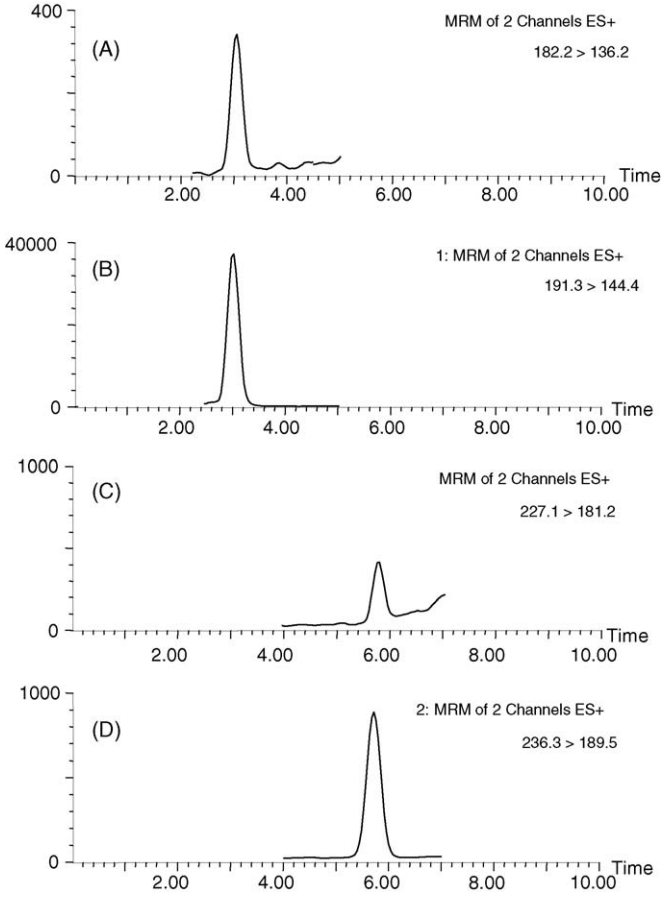

Fig. 3. MRM chromatograms of standards of NTYR, TYR and surrogate standards at the LOQ levels: (A) 100 nM TYR, (B) 5 μM [$^{13}C_9$]-TYR, (C) 0.5 nM NTYR and (D) 5 nM [$^{13}C_9$]-NTYR.

Table 2
Recoveries of TYR and NTYR in digested mice liver samples ($n = 5$)

	Added (μM)	Concentration (μM)	Recovery (%)	R.S.D. (%)
TYR	25	28.1 ± 0.7	95.6	4.97
	50	50.2 ± 2.4	92.1	2.88
	0	4.2 ± 0.2		
	Added (nM)	Concentration (nM)		
NTYR	100	109.0 ± 0.7	94.4	0.70
	200	213.6 ± 9.8	98.9	5.28
	0	14.6 ± 0.7		

MRM chromatograms of the standards of TYR and NTYR and the surrogate standards in water at the LOQ levels are shown in Fig. 3.

As shown in Table 2, the recoveries (value ± R.S.D.%, $n = 5$) were 95.6 ± 4.9% and 92.1 ± 2.9% (25 μM and 50 μM, respectively) for TYR, and 94.4 ± 0.7% and 98.9 ± 5.3% (100 nM and 200 nM, respectively) for NTYR. The results indicate that the method enables the precise determination of TYR and NTYR, and is applicable to the detection of these compounds in animal tissue samples.

The utility of the measurement of NTYR with LC–MS/MS was previously reported by Althaus et al. [21] and Thierry et al. [22]. The sensitivity of our proposed method is similar to those of the previously reported methods. However, our method achieved higher precision and greater convenience of analysis with the use of a surrogate standard, and without having to use such methods as solid phase extraction. Furthermore, we applied enzymatic protein digestion in the preparation of tissue samples.

3.3. Determination of NTYR and TYR in liver of mice administered APAP

It has been reported that NTYR is formed in the liver of mice administered a toxic dose of APAP [8–10,23], 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 has the ability to detect changes in NTYR levels in vivo, it was applied to the analysis of liver protein in mice administered APAP. Typical chromatograms of liver protein samples in control mice are shown in Fig. 4. The TYR and NTYR levels in liver proteins of mice administered APAP at 2 h, 4 h and 8 h after treatment are summarized in Table 3. The concentrations of TYR and NTYR per milligram protein in each sample were 2.94–3.91 μmol/mg protein and 0.17–0.30 pmol/mg protein, respectively. The NTYR/TYR ratio was then calculated by dividing the concentration of NTYR in pmol/mg protein by the concentration of TYR in μmol/mg protein. As a result, the basal level of the NTYR/TYR ratio in mouse liver was 58.2 ± 5.3 μmol/mol of TYR, which was almost equal to the reported basal level of the NTYR/TYR ratio in rat kidney as determined by LC–MS/MS (51.1 ± 0.6 μmol/mol of TYR) [22].

Whereas there was no significant difference in the TYR concentration per milligram protein between the APAP treatment group and the control, the NTYR concentration per milligram protein in the APAP treatment group was increased significantly at all the time points examined compared with the control. However, taking into consideration that NTYR is formed by the reaction of TYR with $ONOO^-$, the NTYR/TYR ratio would express more precisely the changes of the NTYR levels in the tissues. In this sense, it is of significance that both NTYR and TYR levels in the tissues were able to be detected by the proposed method. The increase of the NTYR/TYR ratio per milligram protein was observed 2 h after APAP administration. The ratio reached a maximum (81.6 ± 7.0 μmol/mol of TYR) at 4 h, after which it showed a slight decrease. Our results are consistent with

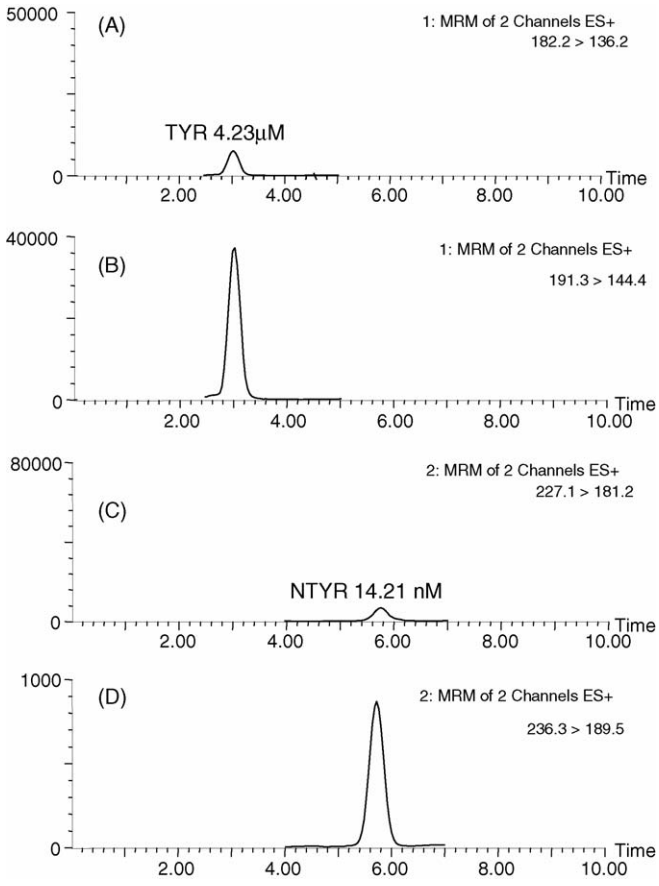

Fig. 4. MRM chromatograms of a mouse liver protein sample containing TYR (A), 5 μM [$^{13}C_9$]-TYR (B), NTYR (C) and 5 nM [$^{13}C_9$]-NTYR (D).

Table 3
Concentrations of TYR and NTYR in mice liver samples

Group	TYR (μmol/mg protein)	NTYR[a] (pmol/mg protein)	NTYR/TYR (μmol/mol)
Blank (protease only)	2.53 ± 0.14	ND	ND
Control	3.53 ± 0.20	0.20 ± 0.02	58.2 ± 5.28
APAP 300 mg/kg (i.p.)			
2 h	3.63 ± 0.08	0.26 ± 0.02*	71.7 ± 4.28*
4 h	3.43 ± 0.17	0.28 ± 0.02*	81.6 ± 6.95*
8 h	3.31 ± 0.39	0.25 ± 0.02*	75.5 ± 10.9*

[a] ND indicates NTYR concentrations lower than 0.5 nM.
* Significantly different ($P < 0.01$) from the control group.

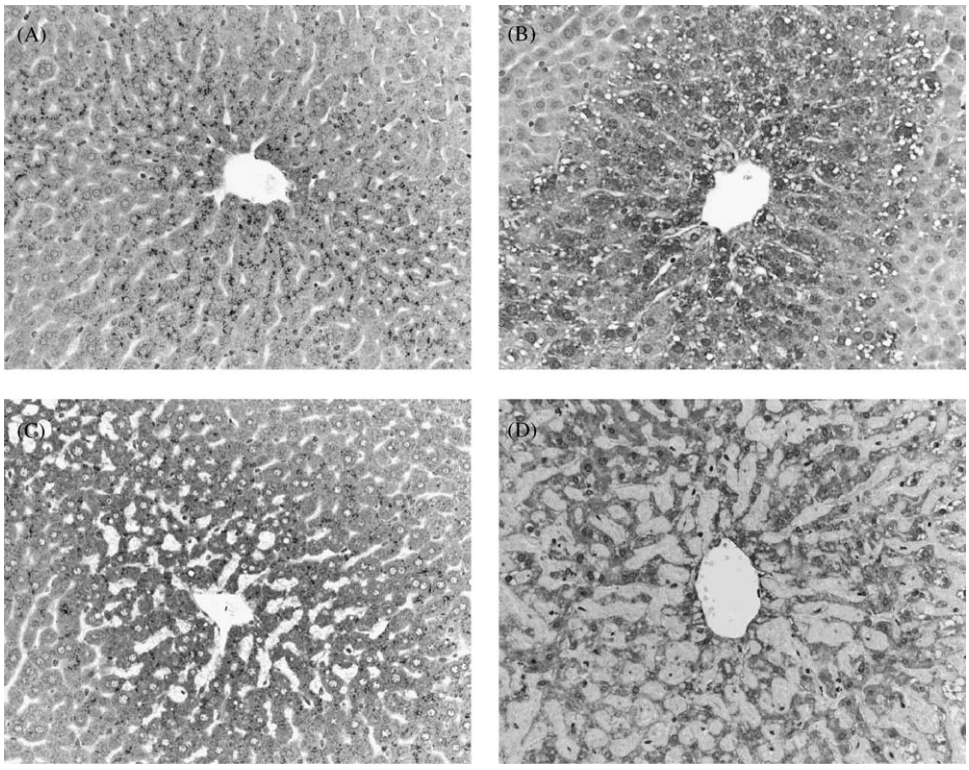

Fig. 5. Immunohistochemical staining of liver sections for NTYR protein adducts in controls and 2 h, 4 h and 8 h after 300 mg/kg of APAP. (A) Liver from a saline-treated mouse. Liver from a mouse treated with APAP (300 mg/kg) at 2 h (B), 4 h (C) and 8 h (D). Original magnification, ×360.

the previous finding that NTYR levels in the liver of mice were increased 4 h after APAP treatment using the enzyme-linked immunosorbent assay [9].

3.4. Histopathology and immunohistochemical analysis of NTYR

Histopathological findings related with APAP treatment and immunohistochemical analysis of NTYR are summarized in Table 4. In control animals, hepatocytes were weakly stained centri-lobullary for NTYR (Fig. 5A). In contrast, in APAP-treated mice, centri-lobular hepatocytes were strongly stained at 2 h after the treatment (Fig. 5B). Cytoplasmic vacuolation in centri-lobular hepatocyte was evident at this time. Both the intensity and the extent reached a maximum at 4 h, and were decreased thereafter (Fig. 5C and D). Extensive centri-lobular hepatocyte necrosis at 8 h after the treatment allows us to speculate that the slight decrease in the intensity of NTYR staining might result from hepatocyte damage. In any event, the quantitative analysis of NTYR levels using our method accurately traced optical changes in the immunohistochemical reaction with NTYR.

Table 4
Histopathological findings in hepatocyte of the mice administrated 300 mg/kg APAP for 2 h, 4 h and 8 h after treatment

Site/lesion	Group			
	Control	2 h	4 h	8 h
Centri-lobular area				
Vacuolation	−	++	+	±
Necrosis	−	−	+	+++
NTYR positive cells	±	++	+++	±
Periportal area				
Vacuolation	−	−	+	++
Necrosis	−	−	−	±
NTYR positive cells	−	−	−	+

Symbols: −, not observed; ±, slight; +, mild; ++, moderate; +++, severe.

4. Conclusions

In the present study, we developed the LC–MS/MS method in which the separation system and the sample preparation method were modified from those of the conventional LC–MS/MS method. These improvements made it possible to simplify the sample preparation, consequently this method being optimized for routine analysis of in vivo samples. As a result of applying our method to the detection of TYR and NTYR levels in the liver of mice administered APAP, the increase of NTYR levels was successfully detected. Upon comparing the results of immunohistochemical analysis with those of LC–MS/MS, we found that the amount of NTYR measured by LC–MS/MS was well correlated with that by immunohistochemical analysis.

Finally, precise quantification of NTYR in a tissue sample was possible by our LC–MS/MS method. On the other hand, histopathology and immunohistochemical analysis can know the localization of NTYR, and the cause of changes in NTYR level. The use of both our LC–MS/MS method and immunohistochemical analysis could help us explore the mechanisms

underlying NO-mediated toxicity and carcinogenicity in animal experiment.

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