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Simultaneous determination of 3-nitrotyrosine and tyrosine in plasma proteins of rats and assessment of artifactual tyrosine nitration

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Abstract

A sensitive and specific isotope dilution liquid chromatography–electrospray tandem mass spectrometry method was developed for the determination of the 3-nitrotyrosine residue levels in rat plasma proteins. The assay is based on the cleavage of proteins with concentrated hydrochloric acid to release both 3-nitrotyrosine and tyrosine. To control the potential artifactual nitration of tyrosine residues during the proteolysis, samples are spiked with $^{13}C_9$-labeled tyrosine and the level of $^{13}C_9$-labeled 3-nitrotyrosine is measured. The clean-up process entails hydrolysate fortification with 2,5,6-d_3-3-nitrotyrosine, followed by solid-phase extraction on octadecylsilyl (to isolate tyrosine) and aminopropylsilyl (to isolate 3-nitrotyrosine) cartridges. Tyrosine and 3-nitrotyrosine fractions are mixed in an appropriate ratio prior to the analysis. The method was applied to animals exposed to ferric nitrilotriacetate to induce oxidative stress.

Keywords: Nitration; Nitrotyrosine; Tyrosine; Proteins

1. Introduction

Reactive nitrogen and oxygen species are implicated in many disease conditions including chronic inflammation [1], atherosclerosis [2], rheumatoid arthritis [3], some neurodegenerative diseases [4] and respiratory conditions [5]. The increased levels of 3-nitrotyrosine (nitroTyr), which have been detected in the tissues affected by these diseases have been attributed to a higher formation of peroxynitrite [6,7]. Therefore, nitroTyr was proposed as a suitable chemical marker for assessing the nitration level in proteins [8], and it is generally believed that peroxynitrite is the main contributor to the formation of nitroTyr. However, it has been suggested that nitroTyr formation may be induced by other species such as nitrogen dioxide, nitrous acid or nitronium chloride [9].

To assess the nitration level of tyrosine residues in proteins, several approaches have been developed. Immunohistochemistry has been extensively used to detect nitroTyr in tissues and culture cells [10–12], but methods including HPLC with either ultra-violet [13], electrochemical [14,15] and fluorescence de-

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tection [16] have also been used to determine the nitroTyr levels. During the past 5 years, mass spectrometry approaches have been developed to ensure a higher specificity together with a suitable sensitivity [17,18]. This type of detector was coupled with either gas [19,20] or liquid chromatography [21]. Recently, it was demonstrated that the derivatization conditions required for the GC–MS detection may induce an artifactual nitration of tyrosine [22], and efforts were made to develop artifact free methods [17,23].

In the present study, we have developed a method to measure nitroTyr and investigated the potential artifactual nitration of tyrosine that may occur during proteolysis with hydrochloric acid. Our method was applied to rat plasma protein samples and measurements were performed on animals treated with ferric nitrilotriacetate (Fe-NTA), a strong oxidizing agent. The method entails protein precipitation, followed by protein hydrolysis with hydrochloric acid, solid-phase extraction of both nitroTyr and tyrosine (Tyr) and analysis by liquid chromatography with on-line tandem mass spectrometry (LC–MS–MS). Tyr and nitroTyr fractions are mixed in an appropriate ratio to enable a simultaneous mass spectrometric detection of Tyr and its nitrated derivative. $^{13}C_9$-labeled Tyr was added to proteins prior to the proteolysis and $^{13}C_9$-labeled nitroTyr was monitored to assess the contribution of artifactual nitroTyr formation. The artifactual contribution was used to correct the signal of nitroTyr, and reach the endogenous level of nitroTyr residues in proteins. The model was validated with rat plasma protein samples supplemented with sodium nitrite to generate a significant proteolysis-induced artifactual nitration of tyrosine.

2. Experimental

2.1. Chemicals and reagents

Methanol and acetonitrile gradient grade, acetic acid, ammonium acetate and formate were obtained from Merck (Darmstadt, Germany). Tyr was provided by Sigma–Aldrich (Steinheim, Switzerland) and nitroTyr as well as bovine serum albumin (BSA) were from Sigma (St. Louis, MO, USA). 2,3,5,6-d_4-L-Tyrosine (99.1% D), α,α′-d_2-L-tyrosine (98% D) and α,β,γ,1,2,3,4,5,6-$^{13}C_9$-L-tyrosine ([$^{13}C_9$]Tyr) (97–98% ^{13}C) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The preparation of labeled nitroTyr was based on the very high reactivity of tetranitromethane (Aldrich, Milwaukee, WI, USA) towards Tyr [24]. The contribution of unlabeled Tyr in the standard of [$^{13}C_9$]Tyr was measured to be 0.27%. The contribution of nitroTyr in 2,5,6-d_3-3-nitro-L-tyrosine ([d_3]nitroTyr) was 0.89%.

2.2. Quantification of proteins

The Coomassie® Plus Protein Assay Reagent Kit from Pierce (Rockford, IL, USA) was used to carry out the quantification of proteins. Standards were prepared with the BSA stock solution at 2.0 mg/ml provided in the kit. The calibration curve was prepared with five doses ranging from 62.5 to 1000 μg/ml. The samples were either 5- of 10-fold diluted prior to the reaction and the analysis. The Coomassie® Plus reagent (3 ml) was added to 100 μl of both calibrators and diluted samples. The resulting solutions were kept 5 min at room temperature. Quantification was performed on a Lambda Bio 20 UV-spectrophotometer (Perkin-Elmer, Norwalk, CT, USA) with a detection wavelength set at 595 nm.

2.3. Preparation of the rat plasma extracts

Three milliliters of cold acetone–methanol (50:50, v/v) were added to 500 μl of plasma, and the resulting suspensions were stored for 90 min at 4 °C prior to centrifugation at 3000 rpm (15 min, 4 °C) on a Mistral 2000R apparatus (Zivy, Oberwil, Switzerland). Supernatants were removed and a further 2 ml acetone–methanol (50:50, v/v) was added to the protein pellets. The proteins were re-suspended, stored for 1 h at 4 °C and again centrifuged. The supernatants were discarded and protein pellets dissolved in water prior to quantification. Plasma protein (400 μg) was fortified with 52 μg of [$^{13}C_9$]Tyr prior to evaporation to dryness under a gentle stream of nitrogen. Samples were reconstituted in 2 ml of 6 *M* HCl, kept 24 h at 110 °C and then rotary evaporated to dryness. Dry residues were spiked with [d_3]nitroTyr (1.10 ng), reconstituted in 1

ml of ammonium acetate 2 m*M* (pH 4.35) and samples were applied to preconditioned octadecylsilyl 500-mg Bond Elut-cartridges (Varian, Middelburg, The Netherlands). The cartridges were then washed with 1 ml of ammonium acetate 2 m*M* (pH 4.35) and Tyr was eluted with 1 ml of methanol–ammonium acetate 2 m*M* (pH 4.35) (5:95, v/v). Then, cartridges were washed with 1 ml of methanol–ammonium acetate 2 m*M* (pH 4.35) (10:90, v/v) and nitroTyr was eluted with 1.5 ml of methanol–ammonium acetate 2 m*M* (pH 4.35) (20:80, v/v). nitroTyr-containing fractions were submitted to a second purification stage on aminopropyl 500-mg Isolute-cartridges (Separtis, Grellingen, Switzerland). Fractions were applied onto preconditioned cartridges (with methanol–ammonium acetate 2 m*M* (pH 4.35) (20:80), and washed with 2 ml of ammonium formate 2 m*M* (pH 3.55). Elution of nitroTyr was achieved by applying 1.5 ml of water–acetic acid (25:15, v/v). Fractions were evaporated to dryness and Tyr-containing dry residues were reconstituted in 600 μl of water. A 2-μl aliquot of the extracts was added to the nitroTyr dry residues. The volumes were adjusted to 30 μl with water at which point they were ready for injection in the LC–MS–MS system. The clean-up process is shown schematically in Fig. 1.

2.4. Detection by liquid chromatography coupled with tandem mass spectrometry

The HPLC system consisted of a HP series 1100 (Hewlett-Packard, Palo-Alto, CA, USA) equipped with a XTerra MS C_{18} (150×1.0 mm I.D., 3.5 μm) microbore column (Waters, Milford, MA, USA) with an in-column flow-rate set at 50 μl/min. Gradient (solvent A: water pH 2.7 adjusted with formic acid; solvent B: methanol–water, pH 2.7, 80:20, v/v): 0–5 min: A/B (100:0, v/v); 5–19 min: linear ramp to A/B (37:63, v/v); 19–20 min: linear ramp to A/B (0:100, v/v) (during 4 min). Typical retention times: nitroTyr, 17.0 min; Tyr, 6.3 min. Injection volume, 10 μl. The detection was performed by positive electrospray ionization tandem mass spectrometry on a Finnigan MAT TSQ 7000 equipped with the API 2 interface (San Jose, CA, USA). Spray voltage was set at 1.1 kV and the capillary temperature at 360 °C. The sheath gas pressure was 90 p.s.i. The acquisition was carried out in the selected reaction monitoring mode and two characteristic transitions were monitored for each compound in order to improve the selectivity. nitroTyr: *m/z* 227→181 (collision energy 20 eV) and *m/z* 259→181 (collision energy 24 eV); [d_3]nitroTyr: *m/z* 230→184 and *m/z* 262→184; α,β,γ,1,2,3,4,5,6-$^{13}C_9$-3-nitro-L-tyrosine ([$^{13}C_9$]nitr-

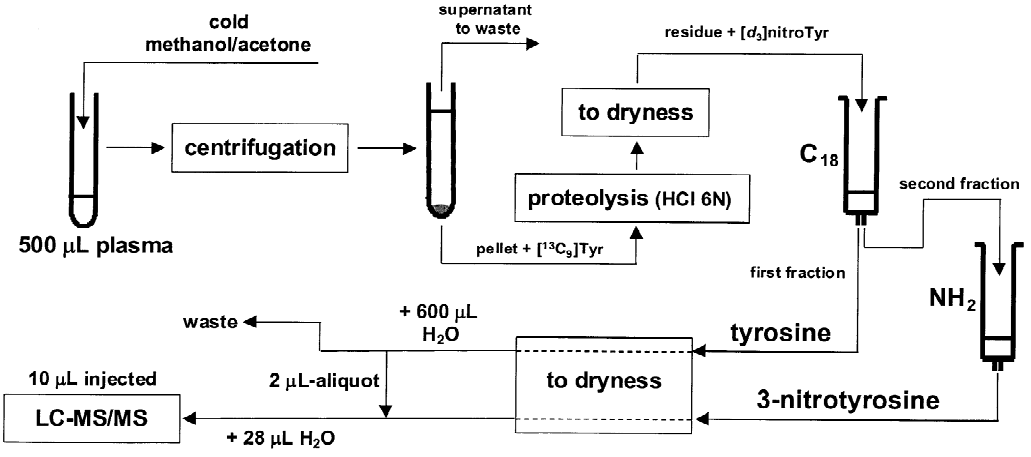

Fig. 1. Main steps of the cleanup process. Protein pellet is dissolved in water and only 400 μg of protein is used for the proteolysis (C_{18}: octadecylsilyl stationary phase cartridge; NH_2: aminopropylsilyl stationary phase cartridge; [$^{13}C_9$]Tyr: α,β,γ,1,2,3,4,5,6-$^{13}C_9$-L-tyrosine; [d_3]nitroTyr: 2,5,6-d_3-3-nitro-L-tyrosine).

oTyr): *m/z* 236→189 and *m/z* 268→189; Tyr: *m/z* 182→165 (collision energy 22 eV) and *m/z* 182→136 (collision energy 28 eV); [$^{13}C_9$]Tyr: *m/z* 191→174 and *m/z* 191→144. Peak width was set at 1.0 Da with a 500-ms scan time per transition.

2.5. Quantification of 3-nitro-L-tyrosine and L-tyrosine in extracts and evaluation of the results

The calibration curve for nitroTyr consisted of five points within the range (on column) of 30–1100 pg (theoretical range: 225–8250 pg/mg of protein), and each calibration standard solution was fortified with 1.10 ng of [d_3]nitroTyr and 52 μg of [$^{13}C_9$]Tyr. [d_3]nitroTyr was used as an internal standard in order to accurately quantify nitroTyr and [$^{13}C_9$]nitroTyr. The Tyr content was determined with a calibration curve ranging from 6–195 ng (theoretical range: 13.6–443 μg per mg of protein) using [$^{13}C_9$]Tyr as an internal standard (52 μg spiked). Signals of transitions *m/z* 182→165, *m/z* 191→174, *m/z* 227→165, *m/z* 236→189 and *m/z* 230→184 were used to quantify Tyr, [$^{13}C_9$]Tyr, nitroTyr, [$^{13}C_9$]nitroTyr and [d_3]nitroTyr, respectively. Samples were prepared in quadruplicate to estimate the precision of the method. A linear regression model was used, and the equation of the curve was used to back calculate the concentration of the standards. The accuracy of the back calculated concentration (accuracy = 100 × (back calculated value − expected value)/expected value) was used to control the linearity of the response. The limit of detection (LOD) was estimated (by extrapolation) for nitroTyr only, and was defined on the basis of a peak showing a signal-to-noise ratio of 3:1.

2.6. Animals

Measurements of protein-bound nitroTyr were performed in plasma proteins of male Fischer-344 rats (Iffa-Credo, L'Arbresle, France) weighing between 150 and 200 g. Animals were acclimatized 24 h in wood shaved cages and provided with Nafag 890 food and water ad libitum prior to the treatment. A single i.p. injection of Fe-NTA (15 mg/kg) in 50 m*M* $NaHCO_3$ was given to two rats (Fe-trd 1 and 2), whereas two control animals (ctrl 1 and 2) only received the saline solution. After 4 h, animals were sacrificed under anesthesia (pentobarbital 60 mg/kg i.p.) to collect blood. Heparinized blood samples were immediately centrifuged, and plasma samples were stored at −80 °C until further analysis.

3. Results

3.1. Tandem mass spectrometry-fragmentation patterns of 3-nitro-L-tyrosine and L-tyrosine

3.1.1. 3-Nitro-L-tyrosine

Tandem mass spectrometry experiments were carried out on either nitroTyr, [d_3]nitroTyr, α,α′-d_2-3-nitro-L-tyrosine ([d_2]nitroTyr) or [$^{13}C_9$]nitroTyr (see structures in Fig. 2), which provided valuable information on the fragmentation pathway of nitroTyr (Table 1). A full scan spectrum of nitroTyr exhibited a prominent ion at *m/z* 227 corresponding to the protonated molecule. The assignment of this species was confirmed by the analysis of [d_3]-, [d_2]- and [$^{13}C_9$]nitroTyr which gave rise to ions at *m/z* 230, 229 and 236, respectively. In addition, the spectrum of nitroTyr showed an additional ion at *m/z* 259 when recorded in the presence of methanol (not observed with acetonitrile) which was assigned to a protonated methanol adduct of nitroTyr. Indeed, the mass difference between ions at *m/z* 259 and 227 ($\Delta M = 32$ Da) corresponds to the molecular mass of methanol and this difference was also observed in the case of [d_3]nitroTyr (ions *m/z* 262 and 230) and [$^{13}C_9$]nitroTyr (ions *m/z* 268 and 236).

The 15-eV collision energy spectrum of nitroTyr, depicted in Fig. 3, exhibited fragment ions at *m/z* 210 and 181, i.e., mass losses of $\Delta M = -17$ and −46 Da, respectively. The loss of 17 amu can be rationalized in terms of the elimination of an ammonia molecule from the *pseudo*-molecular ion $[M+H]^+$. This assignment is supported by examination of the product ion spectrum of nitroTyr in D_2O (parent ion of *m/z* 232) which exhibited a fragment ion at *m/z* 212, consistent with the loss of perdeuterated ammonia (20 Da). Examination of the spectra of the isotopically labeled analogs was particularly helpful in the elucidation of the structure of the fragment ion at *m/z* 181 (loss of 46 Da) from the parent ion $[M+H]^+$. It has been previously suggested that this

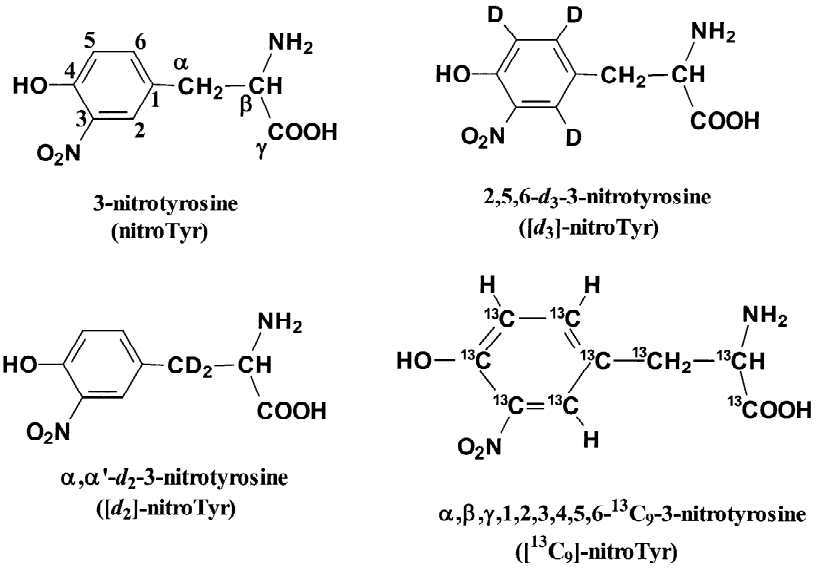

Fig. 2. Chemical structures of 3-nitrotyrosine (numbering of carbon atoms is indicated) and trideuterated or carbon-13-labeled derivatives.

fragment is formed by loss of NO_2 [21]; however, this assignment is not supported by the product ion spectra of nitroTyr in D_2O and [$^{13}C_9$]nitroTyr. Specifically, the product ion spectrum of nitroTyr in D_2O shows a transition of m/z 232→184 (loss of 48 amu, DCOOD) while the spectrum of [$^{13}C_9$]nitroTyr shows a transition m/z 236→189 (loss of 47 u, H^{13}COOH). Thus, these data unequivocally demonstrate that the fragmentation is due to the loss of formic acid (HCOOH).

A minor peak at m/z 168 present in the 15-eV CID spectrum of nitroTyr (shifted to m/z 171 in the spectrum of [d_3]nitroTyr), was found to increase in relative abundance when the spectra were acquired at higher collision energies. The 3-Da upshift in the spectrum of [d_3]nitroTyr indicates that the intact aromatic ring is contained in the m/z 168 fragment. In the case of [d_2]nitroTyr, the ion was shifted to m/z 169, suggesting the loss of one of the benzylic hydrogens in the process of formation of this fragment. When the nitroTyr spectrum was acquired from a D_2O matrix, the fragment was shifted upwards by 2 Da to m/z 170. Since it was previously established that m/z 168 contained the intact aromatic ring, it is reasonable to assume that one exchangeable proton can be accounted for by the hydroxyl substituent at position 4. Therefore, the second exchangeable proton can readily by assigned

Table 1
Assignment of nitroTyr high mass fragment ions and upmass shifts observed in fragment ion spectra of deuterated and carbon-13-labeled nitroTyr derivatives

nitroTyr ion (Th)	Assignment	Ion upmass shift (Th)			
		nitroTyr in D_2O	[d_3]nitroTyr	[d_2]nitroTyr	[$^{13}C_9$]nitroTyr
227	M+H	+5	+3	+2	+9
210	M+H–NH_3	+2	+3	+2	+9
181	M+H–HCOOH	+3	+3	+2	+8
168	210–CH_2CO	+2	+3	+1	+7

[d_3]NitroTyr, 2,5,6-d_3-3-nitro-L-tyrosine; [d_2]nitroTyr, α,α′-d_2-3-nitro-L-tyrosine; [$^{13}C_9$]nitroTyr, α,β,γ,1,2,3,4,5,6-$^{13}C_9$-3-nitro-L-tyrosine.

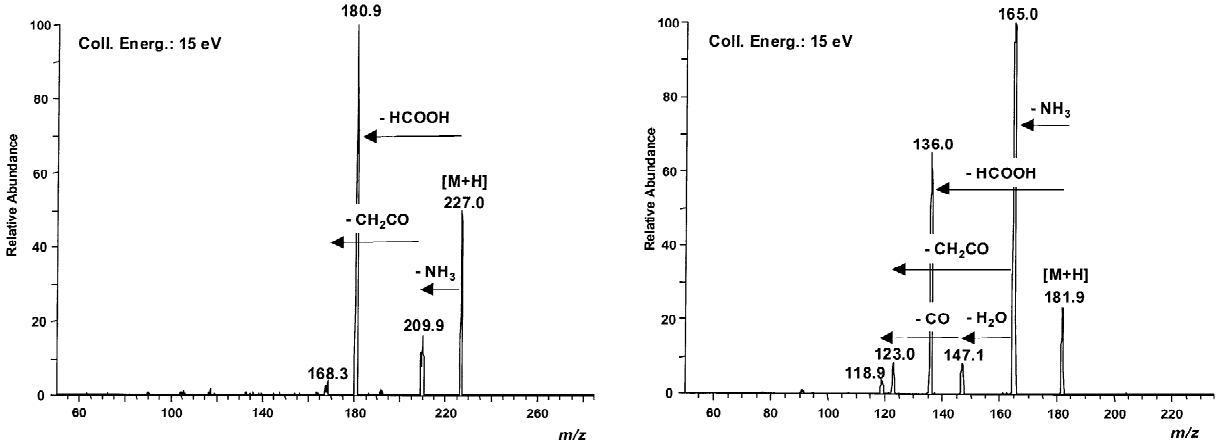

Fig. 3. Fragment ion spectra of 3-nitro-L-tyrosine (left) and L-tyrosine (right).

to the one associated with the carboxylic group. Thus, the fragment ion at m/z 168 was rationalized in terms of a proton migration from carbon C-α to C-β accompanied by a concerted four-member ring rearrangement involving atoms C-α, C-β, COOH. This leads to the neutral loss of a CH_2CO moiety (42 Da). This postulated mechanism is consistent with the observation of the occurrence of the ion at m/z 175 in the fragment ion spectrum of [$^{13}C_9$]nitroTyr. Indeed, the upmass shift (with respect to nitroTyr) of this ion was $\Delta M = +7$ Da whereas $\Delta M = +9$ Da was observed on the *pseudo*-molecular ion of [$^{13}C_9$]nitroTyr, indicating that two carbon-13 atoms have been lost during this process (loss of $^{13}CH_2^{13}CO$). A scheme outlining the sequence of these fragmentations is presented in Fig. 4.

3.1.2. L-Tyrosine

The collision-induced dissociation of Tyr ([M+H]$^+$ = 182) ([d_2]Tyr, [M+H]$^+$ = 184, respectively) generated fragment ions at m/z 165 and 136 (m/z 167 and 138, respectively) which, similarly to nitroTyr, were assigned to the elimination of ammonia and formic acid. In addition, an ion at m/z 123 was

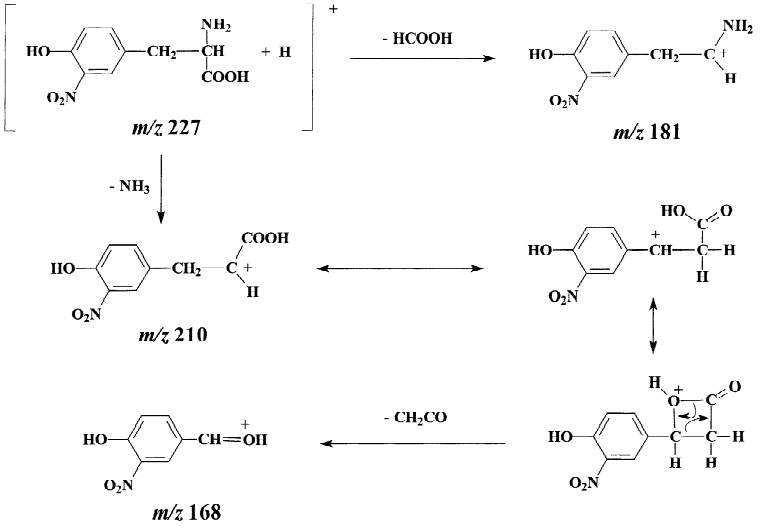

Fig. 4. Postulated fragmentation pathway of *pseudo*-molecular [M+H]$^+$ ion of 3-nitro-L-tyrosine by collision induced dissociation.

observed in the fragment ion spectrum of Tyr which was assigned to the neutral loss of CH_2CO from the carbocation at m/z 165 (see Section 3.1.1). Interestingly, this ion showed an upmass shift of 1 amu (m/z 124) when the fragment ion spectrum was recorded with [d_2]Tyr instead of Tyr. This is in accordance with our proposed mechanism for the elimination of CH_2CO. Indeed, in the case of [d_2]Tyr, the migration of a deuterium atom from position α to position β would lead to the loss of CHDCO ($\Delta M = -43$ Da) corresponding to the formation of an ion at m/z 124. In addition, ions at m/z 147 and 119 observed in the fragment ion spectrum of Tyr were assigned to water elimination from the carbocation at m/z 165 and carbon monoxide elimination from the ion at m/z 147, respectively.

3.2. Features and performance of the method

3.2.1. Liquid chromatography–mass spectrometry conditions

The detection of a compound in any biological matrices at trace level is a challenging task, and reliability of the measurements is achieved when both sensitivity (to improve precision) and specificity (to improve accuracy) are obtained. From a practical point of view, many endogenous compounds may be co-extracted with the analyte generating a severe increase of the background level and/or a distortion of the peak shape. To avoid these undesirable effects, a second solid-phase extraction on aminopropyl cartridge was performed for nitroTyr and the detection was carried out in the multiple reaction monitoring mode which ensured specificity as well as selectivity. The specificity was further improved by recording the signals of two different transitions per analyte: one transition was used for the quantification and the second one to confirm the identity of the analyte in the extract. The quantification transitions were m/z 227→181, m/z 230→184 and m/z 236→189 for nitroTyr, [d_3]nitroTyr and [$^{13}C_9$]nitroTyr, respectively, whereas the identity transitions were m/z 259→181, m/z 262→184 and m/z 268→189. For either nitroTyr, [d_3]nitroTyr or [$^{13}C_9$]nitroTyr the signal ratio of the ''quantification transition''/''identity transition'' was calculated in the plasma extracts and compared to the values obtained in the standards of the calibration curve. It was observed that the ratios in the samples were not statistically different from the standards ($\alpha = 0.05$), indicating that the detection by two-transition tandem mass spectrometry is highly specific. The electrospray source was optimized to obtain the highest signal with nitroTyr which had to be detected at the pg level, and the same conditions were utilized for the detection of Tyr. The level of Tyr and [$^{13}C_9$]Tyr in extracts was high enough to ensure an intense signal for the transitions m/z 182→165 and m/z 191→174, respectively, without further specific optimization of their responses.

Another objective of the development of the method was to optimize the ionization yield in the electrospray source. To reach this goal, the pH of the mobile phase was optimized. We found that the nitroTyr signal was increased when the pH of the aqueous solution was below 3.0, and the signal response was increased by a factor of 3.5-fold when the pH was reduced from 5.8 to 2.7. Analyses performed at pH values lower than 2.7 resulted in a severe ion suppression and were accompanied by a steep increase of the current (above 30 mA). In view of these observations, the pH of the aqueous component of the chromatographic gradient in mobile phases was adjusted to 2.7, and separations were done with an XTerra C_{18} column, which is reported to withstand acidic aqueous solutions to pH 1.0, and ensure high resolving power.

3.2.2. Proteolysis conditions and its cleavage efficacy

A standard solution of BSA was used to assess the completeness of the protein hydrolysis. BSA was treated under the same conditions as plasma proteins (6 *M* HCl at 110 °C over 24 h), and the composition of the amino acids in the hydrolysate was determined on an Analyzer System 6300 from Beckman (Fullerton, CA, USA). The measured amounts of amino acid were compared to the expected values [25,26]. For most (13) of the 20 detected amino acids, the relative expected value (calculated amount versus expected one) ranged from 90 to 103%, indicating that acidic hydrolysis conditions were suitable for a complete release of the amino acids. The completeness of the cleavage was confirmed by the relatively good relative expected values of slowly released amino acids such as Val, Ile and Leu (95, 89 and

97%, respectively) [27]. The instabilities of Ser, Cys, Met and Trp [28] were demonstrated (relative expected value below 90%), and the conversion of Asn and Gln (not detected) into Asp and Glu (relative expected value at 128%), respectively, was observed [28]. In addition, a stability test performed on Tyr and nitroTyr demonstrated only slight degradation under these proteolysis conditions, and only 5–10% of these standards were lost after a 24 h treatment at 110 °C in 6 *M* HCl. Moreover, there was no significant difference ($\alpha = 0.05$) between degradation rates of Tyr and nitroTyr. These results indicate that the amount of nitroTyr can be expressed with respect to Tyr without the need for any correction due to the relative degradation of the analytes.

3.2.3. Performance of the assay

An excellent linearity of the response was observed for both nitroTyr and Tyr (correlation coefficient above 0.999). This is confirmed by the accuracies of the back calculated concentrations of the standard calibration solutions which never exceeded ±10%. These values ranged from −10.2 to 4.1% for Tyr and from −6.3 to 3.0% for nitroTyr in the range 100–1100 pg. Only the 30 pg of nitroTyr concentration exhibited a lower accuracy measured at 26%. The proteolysis of 400 μg (i.e., 133 μg equivalent injected) of rat plasma protein provided chromatograms with signal-to-noise ratios of nitroTyr (transition m/z 227→181) in the range 8–13, indicating that this amount of plasma proteins approaches the minimum amount required to obtain a nitroTyr peak of sufficient intensity to ensure a precise integration of the signal (Fig. 5). Given these values, the LOD can be estimated at 2.2 pg of nitroTyr per μg of Tyr (using 400 μg of plasma proteins). The recoveries were not calculated, however the percentage of expected value (100×signal in samples/signal in standards) was calculated by using the area of the internal standard signal. The loss of signal response due to both ion suppression effect and loss of analyte during the sample cleanup procedure are included in the calculation of the percentage of expected value. For [d_3]nitroTyr, the value was 21.6±0.6%, whereas 30.8±0.1% was found for [$^{13}C_9$]Tyr. The inter-sample variation of the internal standard signal was excellent for Tyr (C.V. = 3.4%) whereas the C.V. was 26% for nitroTyr. The latter value may be attributed to a stronger matrix effect on the ionization yield of nitroTyr than on Tyr. Indeed, the low amounts of nitroTyr (detected at the pg level) may be very sensitive to any variation of coeluting endogenous products. In contrast, the intra-sample variation of nitroTyr was very good, ranging from 2.7 to 4.0%. In the case of Tyr, the intra-sample variation was 0.4–0.6%.

4. Discussion

4.1. Determination of the artifactual nitration of L-tyrosine during the proteolysis step

Protein cleavage under strong hydrochloric acid conditions (6 *M*, 110 °C, 24 h) may induce an artifactual nitration of Tyr leading to an over-estimation of the nitroTyr signal. Particularly, Tyr residues are known to be the main target in protein nitration processes [24]. Any trace of either nitrate or nitrite in the sample may have a severe effect on the artifactual formation and analysis of nitroTyr. Proteins contain a huge excess of Tyr compared to the amount of nitroTyr and therefore, the artifactual nitration of low levels of Tyr may generate a significant contribution of artifactual nitroTyr in the chromatographic signal. To control the artifactual contribution of nitroTyr in the signal, rat plasma protein samples were fortified with [$^{13}C_9$]Tyr prior to the proteolysis stage, and the level of [$^{13}C_9$]nitroTyr in samples was compared to the one in reference samples. To accurately measure the level of [$^{13}C_9$]nitroTyr in the standard of [$^{13}C_9$]Tyr, a mixture containing 52 μg of [$^{13}C_9$]Tyr and 1.10 ng of [d_3]nitroTyr was dissolved in 30 μl of water, and 10 μl was injected in the LC–MS–MS system for analysis (reference samples). This provided a reference value of 28.0±0.7 pg of [$^{13}C_9$]nitroTyr per μg of [$^{13}C_9$]Tyr. In parallel, the nitroTyr and [$^{13}C_9$]nitroTyr levels were measured in a plasma sample supplemented with sodium nitrite at 13.5 μg per mg of protein ($n = 4$), and 87±9 pg of nitroTyr per μg of Tyr and 86±4 pg of [$^{13}C_9$]nitroTyr per μg of [$^{13}C_9$]Tyr were found. The 3-fold increase of the [$^{13}C_9$]nitroTyr level with respect to the reference value unambiguously demonstrates that an artifactual

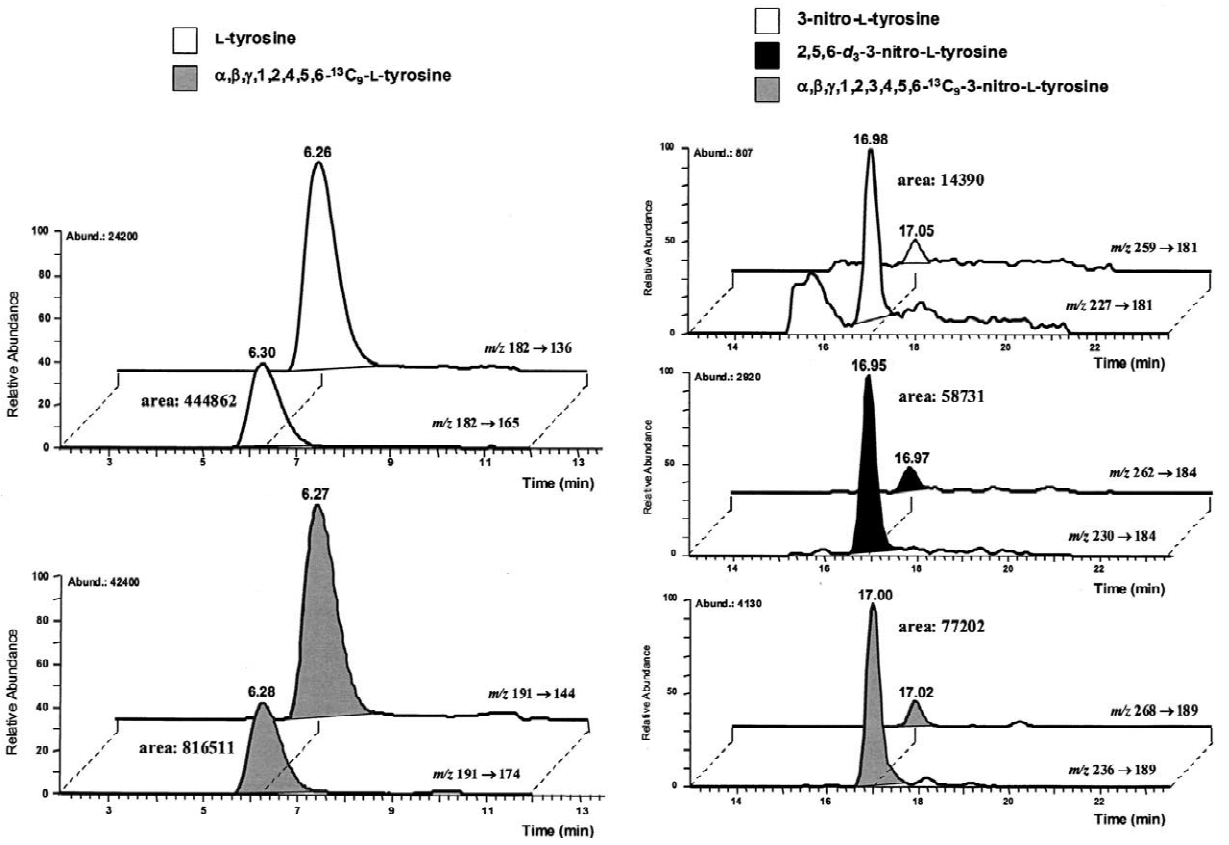

Fig. 5. Chromatographic profile of a rat plasma protein (135 μg injected) sample containing 69.0 μg of Tyr per mg of protein and 10.4 pg of nitroTyr per μg of Tyr (correction applied). The plasma was fortified with 51.8 μg of [$^{13}C_9$]Tyr and the hydrolysate with 1.10 ng of [d_3]nitroTyr.

nitration of Tyr (corresponding to a level of 58±4 pg of [$^{13}C_9$]nitroTyr per μg of [$^{13}C_9$]Tyr) has occurred during the proteolysis. The same measurements performed in the plasma sample (without addition of sodium nitrite) provided levels at 28.2±1.2 pg of nitroTyr per μg of Tyr and 26.5±0.1 pg of [$^{13}C_9$]nitroTyr per μg of [$^{13}C_9$]Tyr, indicating that no artifact could be detected. Indeed, 26.5±0.1 pg of [$^{13}C_9$]nitroTyr per μg of [$^{13}C_9$]Tyr is not statistically different from the reference value ($\alpha = 0.05$). When the nitroTyr level measured in the nitrite-supplemented plasma is corrected for the contribution of the artifactual nitroTyr, the resulting level is 29±10 pg of nitroTyr per μg of Tyr. This value does not show any statistical difference ($\alpha = 0.05$) with the level detected in the native sample. Altogether, this finding demonstrates that the control of the artifactual formation of [$^{13}C_9$]nitroTyr level may not only help to detect a possible artifact but also may correct a sample with an artifact-induced trend in order to reach the endogenous level of nitroTyr. One should point out that the precision of the correction is highly improved when the amounts of [$^{13}C_9$]nitroTyr (in both reference and samples) and nitroTyr are also determined with a good precision.

4.2. Measurement of plasma protein-bound 3-nitro-L-tyrosine in control and Fe-NTA-treated rats

The measurement of nitroTyr in rat plasma protein samples showed that a slight artifactual nitration of Tyr has occurred during the proteolysis. In each sample, the [$^{13}C_9$]nitroTyr level was above the reference value, and the difference was statistically

Table 2
Measurements (mean±SD, $n=4$) of nitroTyr, [$^{13}C_9$]nitroTyr and Tyr in plasma proteins of Fe-NTA-treated rats (15 mg/kg), and evaluation of the contribution of the artifactual nitration of Tyr over the hydrochloric acid-mediated proteolysis (6 *M*, 110 °C, 24 h)

	Tyr (μg/mg protein)	nitroTyr (pg/μg Tyr)	[$^{13}C_9$]nitroTyr (pg/μg [$^{13}C_9$]Tyr)	Artifactual [$^{13}C_9$]nitroTyr (pg/μg [$^{13}C_9$]Tyr)	Corrected nitroTyr (pg/μg Tyr)
Reference[a]	–	–	28.0±0.7	–	–
Control:					
rat ctrl 1	65.9±2.3	9.1±0.5	30.6±0.5	2.6±0.9	6.5±1.0
rat ctrl 2	71.2±1.1	7.8±0.3	30.8±1.2	2.8±1.4	5.0±1.4
Treated:					
rat Fe-trd 1	74.8±1.0	16.1±1.2	34.2±1.1	6.2±1.3	9.9±1.7
rat Fe-trd 2	67.8±1.6	12.3±0.8	30.8±1.1	2.8±1.3	9.5±1.5

[a] The [$^{13}C_9$]nitroTyr level in the standard of [$^{13}C_9$]Tyr was measured with five calibrators fortified with [$^{13}C_9$]Tyr.

significant ($P<0.05$) (Table 2). Therefore, a correction was applied to the nitroTyr values to obtain the endogenous amount of nitroTyr residues within plasma proteins. The control samples provided 5.8±1.1 pg of nitroTyr per μg of Tyr, whereas 9.7±0.3 pg per μg of Tyr were found in Fe-NTA-treated rat samples, indicating a significant increase in the animals submitted to a Fe-NTA-mediated oxidative stress ($P<0.05$). This observation suggests that nitroTyr may be a useful oxidation marker of proteins to assess the cytotoxic action of Fe-NTA. The effect of Fe-NTA on biomolecules has already been reported and it was shown that either DNA or Fe-NTA-induced 4-hydroxy-2-nonenal were able to covalently bond to proteins [29,30]. Some recent measurements of basal level of nitroTyr residues in rat plasma proteins, obtained by liquid chromatography coupled with a dual-mode electrochemical detector, provided values at 57±23 mol of nitroTyr per 10^6 mol of Tyr and 23±7 mol of nitroTyr per 10^6 mol of Tyr, depending on the diet [14]. In the absence of artifactual nitration, these levels suggest that the amounts of nitroTyr present in plasma proteins may be influenced by diet, which may help to explain the lower levels of nitroTyr we obtained: 4.6±0.9 mol of nitroTyr per 10^6 mol of Tyr (5.8±1.1 pg of nitroTyr per μg of Tyr). In contrast to these findings, Shigenaga et al. [1] estimated a basal level of nitroTyr residues in rat plasma proteins at 0.37±0.32 mol of nitroTyr per 10^6 mol of Tyr. This method was using an electrochemical detection, following the reduction of nitroTyr to 3-aminotyrosine and acetylation. The conversion of nitroTyr into *N*-acetyl-3-aminotyrosine resulted in a significant increase of the sensitivity of the response; however, 3-aminotyrosine is unstable and is degraded with a half-life of around 3 h (personal observation, TD). Indeed, it is well established that 3-aminotyrosine may auto-oxidize back to 3-nitrotyrosine [31], and the particular reactivity of *ortho*-aminophenol derivatives (because of the proximity of the hydroxyl and amino groups) may generate undesirable degradation of 3-aminotyrosine [32]. Particularly, the use of any acid chloride or anhydride may lead to a cyclisation of the amino and hydroxyl groups generating a benzoxazole derivative. This process has been observed when *ortho*-aminophenol was treated with acetic anhydride to produce the 2-methylbenzoxazole derivative [33]. These chemical reactivities of 3-aminotyrosine may explain, at least in part, some of the discrepancies in nitroTyr estimates using these two different methods.

5. Conclusion

A novel method for the determination of the nitroTyr and Tyr residues in rat plasma proteins was developed. The steps for the sample preparation involved protein cleavage in concentrated hydrochloric acid, and use of solid-phase extractions on octadecysilyl cartridges to isolate Tyr and aminopropylsilyl cartridges to isolate nitroTyr. Fractions of interest were mixed in an appropriate ratio to simultaneously detect nitroTyr and Tyr by liquid chromatography–electrospray tandem mass spectrometry.

Carbon-13-labeled Tyr was supplemented in plasma to evaluate the formation of carbon-13-labeled nitro-Tyr (artifactual nitration of tyrosine) and trideuterated nitroTyr was added into hydrolysates to accurately measure nitroTyr in the extract. The LOD was estimated at 2.2 pg of nitroTyr per μg of Tyr. The comparison of the nitroTyr level in Fe-NTA-treated rat samples (9.7±0.3 pg per μg of Tyr) with the control animals (5.8±1.1 pg per μg of Tyr) demonstrated a significant 67% increase in the treated animals.

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