Comparison of nitration and oxidation of tyrosine in advanced human carotid plaque proteins

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The importance of reactive nitrogen species in atherosclerosis remains poorly understood, despite the semi-quantitative evidence for the presence of 3-nitrotyrosine provided by immunohistochemical staining studies. At this time, there appear to be no data describing the prevalence of nitration relative to oxidation in atherosclerotic plaque proteins. The present study used 3-nitrotyrosine and dityrosine as markers of nitration and oxidation respectively to examine the relative abundance of each process. Substantial methodological improvements were required to overcome problems associated with sensitivity and artefactual production of 3-nitrotyrosine when quantified by GLC-MS. It was shown that careful selection of hydrolysis vessel, sample reduction and the use of the oxazolinone derivative provided sample stability and exquisite sensitivity. Using these methods, it

was observed that the frequency of nitration was 92 ± 15 $\mu\text{mol/mol}$ of tyrosine (0.01%). Dityrosine was present at 1.5 ± 0.14 mmol/mol of tyrosine (0.30%) using HPLC/fluorescence; thus nitration accounted for approx. 3% of the tyrosine modifications measured. Given that other modifications of tyrosine are known to occur in carotid plaque proteins, the contribution of nitration to the total pool of modified tyrosine is very small. However, the possibility of metabolic processes or chemical agents modifying 3-nitrotyrosine to secondary oxidation products remains an alternative explanation for the low levels demonstrated in this study.

Key words: 3-aminotyrosine, artefactual nitration, atherosclerosis, 3-nitrotyrosine, oxazolinone.

INTRODUCTION

The measurement of 3-nitrotyrosine (3-NO₂-Tyr) has been of interest for many years, as it is thought to represent the product of reactions involving reactive nitrogen species *in vivo* [1]. 3-NO₂-Tyr has been detected in samples of diseased tissue, including atherosclerotic plaque, Alzheimer's disease lesions and multiple sclerosis plaques [2,3]. Thus the measurement of this compound is potentially important in the understanding of nitrative mechanisms underlying many disease states. In the past, 3-NO₂-Tyr in lesion proteins has been described largely using immunological staining techniques [4], but has fallen short of quantification. In addition to this, protein oxidation products have been quantified in lesion material, but concurrent measurements of 3-NO₂-Tyr were not made [5,6]. Therefore the relative contribution of nitration to protein modification in atherosclerotic lesions remains unknown.

The measurement of 3-NO₂-Tyr has proven difficult, especially with regard to controlling the artefactual nitration of tyrosine during sample handling. Recently a method was published in which alkaline conditions were used to effect the hydrolysis of proteins [7]. The authors indicated that there is no nitration during the acidic clean-up and cold derivatization procedures, but diligence is required in the exclusion of air from the hydrolysis sample. An understanding of the shortfalls of acidic hydrolysis procedures may enable one to hydrolyse protein under the less rigorous conditions of low pH.

The labour-intensive nature of existing derivatizations [7,8], problems with the loss of large quantities of sample (> 25 mg/l) and artefactual nitration provided the impetus to investigate, in

detail, the problems associated with this assay. Among the most significant outcomes of the present investigation was the observation that the choice of hydrolysis vessel has significant effects on sample stability. In addition, this paper presents a new, facile and highly sensitive procedure for analysing 3-NO₂-Tyr by GLC-MS with negative chemical ionization (NCI), which yields significant savings in time and cost per sample. We have now assayed dityrosine and 3-NO₂-Tyr levels in carotid artery atherosclerotic plaque proteins to determine the extent to which tyrosine is nitrated rather than oxidized in human atherosclerotic lesions.

EXPERIMENTAL

Materials

[¹³C₆]Tyrosine was obtained from Cambridge Isotope Laboratories (Andover, MA, U.S.A.). Tyrosine, 3-aminotyrosine (ATyr) and all fluorinated materials were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 3-NO₂-Tyr was sourced from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). Eppendorf tubes (1.5 ml) were obtained from Interpath Services (Leda, Western Australia, Australia; cat. no. 616001), 0.5 dram borosilicate tubes were from Southern Biological Services (Nunawading, Victoria, Australia) and Sarstedt tubes were supplied directly by Sarstedt. Pyrex tubes (7 ml) were supplied by Crown Scientific (Belmont, Western Australia, Australia). High Flow C18 solid-phase extraction cartridges were obtained from Alltech (Baulkham Hills, New South Wales, Australia). All water was purified using a Milli-Q Plus purification unit, and all solvents were redistilled, HPLC grade. Samples were dried under

Abbreviations used: ATyr, 3-aminotyrosine; BHT, butylated hydroxytoluene; NCI, negative chemical ionization; 3-NO₂-Tyr, 3-nitrotyrosine.

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vacuum, with warming, using a Savant SpeedVac SVC200 on maximum heat (45 °C approx).

Subjects and atherosclerotic plaques

Category V or VI human carotid atherosclerotic plaques, as defined by Stary et al. [9,10], were obtained from patients undergoing carotid endarterectomy (with approval from the Royal Perth Hospital Ethics Committee and informed patient consent), and were stored immediately in cold PBS (pH 7.4) containing butylated hydroxytoluene (BHT; 0.02 mg/ml) and EDTA (0.75 mg/ml) on ice. These were washed in fresh, cold buffer and stored dry until analysis (−80 °C).

Protein preparation

Eight human carotid artery atherosclerotic plaque samples were homogenized as described previously [11]. Plaque homogenates were subjected to a Folch extraction by addition of 1 vol. of methanol and 2 vol. of chloroform followed by vigorous mixing using a vortex mixer. Centrifugation (2500 g, 10 min) produced a protein pellet at the interface of the two phases, which was washed with acetonitrile and dried overnight under vacuum.

Standards

[¹³C₆]Tyrosine was used as an internal standard for the quantification of tyrosine in biological samples. This was also nitrated using nitrous acid to produce [¹³C₆]nitrotyrosine for use as an internal standard in the quantification of 3-NO₂-Tyr in biological samples. Briefly, [¹³C₆]tyrosine (100 mg, 0.53 mmol) was dissolved in the minimum of aqueous KOH (3 M, < 5 ml) and diluted with water (100 ml). Acetic acid (glacial, 100 ml) was added and the solution was stirred constantly at room temperature. Sodium nitrite (2.28 g, 33 mmol) was dissolved in water (50 ml) and added to the tyrosine solution at a rate of 1 ml per h for 22 h using a syringe pump (IVAC 770). Excess nitrite was removed using a cation-exchange resin (Bio-Rad; 50W-X8 resin, 100–200 mesh, hydrogen form) and the product was eluted using ammonia (3 M). The dried eluate was taken up into methanol, frozen overnight (−20 °C) and filtered. The recovery was quantitative (HPLC-UV) using this method and the standard was stored dry (−20 °C).

Dityrosine standard was synthesized by adding ferric chloride hexahydrate (30 mg) to tyrosine (10 mg) and heating the mixture (110 °C) in water (1 ml) for 4 min. Water (1 ml) and KOH solution (3 M, 1 ml) were added and the mixture was centrifuged briefly (2500 g, 1 min). The solid thus produced was discarded and the supernatant was adjusted to pH 5.9. This was frozen overnight (−20 °C) and, upon thawing to room temperature, was centrifuged and the supernatant was subjected to reverse-phase chromatography using a Phenomenex Aqua C18 column (25 cm × 4.6 mm × 5 μm). The mobile phase was 0.1 M ammonium acetate buffer, pH 5.9, and fractions were collected at a flow rate of 1 ml/min. Detection was by UV absorbance at 280 nm. Fractions corresponding to dityrosine [17.0 min, λ_{ex} = 289 nm, λ_{em} = 410 nm; direct-infusion tandem MS (Agilent 1100 series Ion Trap instrument): (M+H)⁺ 361, (M+H−NH₃)⁺ 344, (M+H−H₂O−CO)⁺ 315, (M+H−2NH₃−CO₂)⁺ 283] were collected.

Acid hydrolysis

An assessment of BHT as an alternative preservative to phenol was achieved by adding BHT (1 %, w/v) or phenol (1 %, v/v) to

samples of 3-NO₂-Tyr (100 μl) from a stock solution (1 mg/ml) and examining sample stability under hydrolysis conditions (1 ml of 6 M HCl, 110 °C, 20 h) in borosilicate vessels. These samples were tested by HPLC-UV [12] in order to eliminate downstream processes that may affect recovery. The data suggested that hydrolysis was problematic, and so additional tests were carried out using GLC-MS. A subsequent comparison of several hydrolysis vessels was carried out in which authentic 3-NO₂-Tyr was submitted to the hydrolysis conditions indicated above in each vessel in the presence of phenol. Eppendorf tubes gave maximum recovery of 3-NO₂-Tyr, and these tubes were used for subsequent examination of phenol as a preservative. When phenol was added, 10 μl of melted phenol was added for every 1 ml of hydrochloric acid (1 %, v/v) which is similar to the amount added by others (1 %, v/w) [8].

To assess artefactual nitration of tyrosine during hydrolysis in Eppendorf tubes, sodium nitrite solution with a nitrite concentration equivalent to plasma levels (0.14 μM) [13] was used to dilute authentic tyrosine solution (1 mg/ml) 1:100 (v/v). Samples (100 μl) were treated using the above hydrolysis conditions (106 °C), reduced using dithionite, derivatized (see below) and analysed by GLC-MS with NCI. This process was repeated using sodium nitrate with a nitrate concentration equivalent to that of plasma (28 μM; this was the average of concentrations reported in several publications [14–17]). These experiments were conducted in triplicate.

[¹³C₆]Nitrotyrosine (300 pmol) and [¹³C₆]tyrosine (1 μmol) were added as internal standards to proteins (10 mg) which were then hydrolysed (106 °C, 20 h) in 1.5 ml polypropylene Eppendorf tubes using HCl (6 M, 1 ml). Samples were then dried under vacuum.

Reduction and derivatization of hydrolysates

Crude hydrolysates were dried and then reconstituted in buffer (400 μl, 0.1 % trifluoroacetic acid, pH 5, using ammonia). Aqueous sodium dithionite (0.1 ml, 10 mM), also in buffer, was added to the samples, which were mixed briefly and permitted to sit at room temperature for 30 min. Samples were then applied directly to a reverse-phase cartridge that had been prepared using methanol (2 ml), water (2 ml) and buffer (6 ml) as described previously [7]. The samples were then washed with water (1 ml), eluted using 25 % (v/v) methanol in water (2 ml) and collected into 7 ml Pyrex tubes. The eluate was partitioned, allowing one portion (5 μl) for tyrosine analysis and the remainder for 3-NO₂-Tyr or dityrosine analysis. Each was dried under vacuum.

To the dry samples in 7 ml Pyrex tubes, toluene (1 ml for tyrosine analysis; 400 μl for 3-NO₂-Tyr analysis), trifluoroacetic acid (100 μl) and trifluoroacetic acid anhydride (100 μl) were added. Samples were heated at 110 °C for 20 min. The samples were cooled to room temperature and then analysed neat (3-NO₂-Tyr) or diluted 1:5 (v/v) with toluene (tyrosine) for analysis by GLC-MS.

Alternative derivatizations were attempted using longer-chain reactants, i.e. pentafluoropropanoic acid/pentafluoropropanoic acid anhydride and heptafluorobutyric acid/heptafluorobutyric acid anhydride combinations were tested. In this case, equivalent reaction conditions were used and the reaction mixtures were tested by GLC-MS.

GLC-MS analysis of tyrosine and 3-NO₂-Tyr

All samples (1 μl) were analysed on a Hewlett-Packard 6890 gas chromatograph fitted with an HP5-MS column (30 m × 0.25 mm × 0.25 μm) and interfaced with an Agilent 5973 mass-

selective detector unless otherwise indicated. Helium carrier gas was maintained at a constant flow rate of 1 ml/min. The inlet was maintained at 250 °C. The oven was held at 85 °C for 1.5 min, then raised at 15 °C per min to a final temperature of 280 °C, which was maintained for 5 min. Using NCI and selected ion monitoring for m/z 369 and m/z 375, both ATyr and the $^{13}\text{C}_6$ -labelled internal standard were detected at a retention time of approx. 9.25 min. Similarly, concurrent monitoring of m/z 355 and m/z 361 allowed the simultaneous detection of tyrosine and the $^{13}\text{C}_6$ -labelled internal standard at a retention time of 8.55 min. Methane was used as the reagent gas for NCI of analytes.

HPLC analysis of dityrosine

Protein hydrolysates were processed using the solid-phase extraction procedure described above. The eluate was partitioned such that 5 μl was used for tyrosine analysis by GLC-MS (as described above) and the remainder was dried and reconstituted in buffer A (400 μl ; see below) for analysis by HPLC. The recovery of dityrosine for this procedure was 80%. For each plaque sample, 5 μl was injected into a Hewlett-Packard 1100 HPLC interfaced with a 1046A fluorescence detector set with λ_{ex} = 289 nm and λ_{em} = 410 nm. The samples were chromatographed using a Phenomenex C18 Aqua column (25 cm $\times$ 4.6 mm $\times$ 5 μm). The mobile phase was isocratic using 97% buffer A (0.1 M ammonium acetate, pH 5.9, using ammonia solution) and 3% methanol. Dityrosine eluted after 9.8 min, and the column was flushed with 100% methanol for 10 min after each analysis.

Standard curves and limits of detection

Internal standard ($^{13}\text{C}_6$ nitrotyrosine; 300 pmol) was added to samples of unlabelled 3-NO₂-Tyr (range 100–2000 pmol). The samples were reduced and then derivatized and analysed as described above. Results of triplicate experiments were plotted as the sample/internal-standard ion ratio against the known amount of 3-NO₂-Tyr added. The limit of detection was determined by analysing sequential dilutions of a stock solution of 3-NO₂-Tyr.

For dityrosine (λ_{ex} = 289 nm, λ_{em} = 410 nm), an external calibration curve was produced from two independent stock solutions of the synthetic standard, and samples were quantified by comparison with this standard curve. The limit of detection was determined by serial dilution of a stock solution of dityrosine.

Statistical treatment of data

Data are presented as means $\pm$ S.E.M., and comparisons between groups was carried out using Student's *t* test.

RESULTS

Acid hydrolysis

The 3-nitro and 3-amino derivatives of tyrosine were highly unstable to acid hydrolysis in borosilicate glass vials, such that 0.1 mM solutions were completely undetectable by GLC-MS. Phenol and BHT were tested as preservatives of 3-NO₂-Tyr (Figure 1A), and they exhibited different degrees of protection. Nitrogen flushing of samples and HCl had no effect on recovery, which indicated the possibility of an effect of vessel type on the stability of the sample during hydrolysis. Pyrex provided substantial relief from the problems of the borosilicate vessels, and all subsequent comparisons were carried out using Pyrex as the glass container (results not shown). Figure 1(B) demonstrates

Figure 1 Effects of preservatives and vessel type on relative recovery of 3-NO₂-Tyr during hydrolysis

(A) When 3-NO₂-Tyr (0.1 mg/ml, 100 μl) was submitted to hydrolysis in borosilicate glass vessels, the yield obtained (HPLC-UV) depended upon the preservative added (* P < 0.01 compared with control; # P < 0.01 compared with BHT). However (B), when identical samples were submitted to hydrolysis in the presence of phenol (1%, v/v) in tubes made of various materials, then different yields were obtained (* P < 0.05, GLC-MS). Since Eppendorf tubes provided maximum sample stability, they were tested with and without phenol added (C), and no difference in yield was observed (GLC-MS).

that, as a hydrolysis vessel, Eppendorf tubes proved superior to both Pyrex and Sarstedt tubes, a polypropylene alternative. Furthermore, when Eppendorf tubes were used, there was no difference in 3-NO₂-Tyr recovery whether or not phenol was added (Figure 1C). Therefore, since Eppendorf tubes had proven successful in preventing the instability of 3-NO₂-Tyr, hydrolysis was carried out in Eppendorf tubes in the absence of phenol. The performance of the Eppendorf tubes was found to be unaffected when several batch numbers were compared.

When authentic tyrosine was hydrolysed in the presence of nitrite at a concentration equivalent to that of plasma, then no nitration was observed. Similarly, no nitration was observed when nitrate was added to the hydrolysis mixture. This was only investigated in Eppendorf tubes, as discussed below.

Reduction and derivatization of protein hydrolysates

The reduction of the nitro group of 3-NO₂-Tyr to an amino group for the purposes of analysis is not new [18]; however, in this case, the reduction step was not carried out in a buffered

Figure 2 Structure and full-scan mass spectrum of the oxazolinone derivatives of (A) tyrosine and (B) ATyr

A dry sample of each amino acid was derivatized by adding toluene (up to 1 ml), trifluoroacetic acid (100 μ l) and trifluoroacetic acid anhydride (100 μ l), then heating at 110 °C for 20 min. During analysis, selected ion monitoring of m/z 355 and m/z 369 was used to monitor tyrosine and ATyr respectively. The corresponding $^{13}\text{C}_6$ -labelled internal standards were monitored at m/z 361 and m/z 375 respectively.

solution as described previously [8]. Instead, it was carried out using the same buffer as used for the solid-phase extraction procedure, and this was found to provide equivalent performance (UV-visible, GLC-MS; results not shown). It was found that an interfering peak in GLC-MS could be removed completely if the reduction was permitted to proceed for at least 30 min. The reduction, apart from conferring an approximate 650 % increase in sensitivity to the assay (results not shown), was also utilized prior to the derivatization procedure in order to avoid the detection of 3- NO_2 -Tyr produced artefactually during the procedure. It was established that 3- NO_2 -Tyr is not reduced to ATyr during derivatization; therefore, reduction prior to derivatization is a convenient means of avoiding the detection of artefacts.

Characterization of the oxazolinone derivative

The derivatization presented here creates an oxazolinone derivative whose structure is shown together with its NCI mass spectrum for both ATyr and tyrosine in Figure 2. A trifluoromethyl group is located at the 2-position of the oxazolinone ring, and a trifluoroacetyl group is present on the hydroxy and amine residues of the aromatic ring. The molecular ion for ATyr, m/z 466, is not detected at typical analytical concentrations; however, exclusive fragmentation to m/z 369, corresponding to loss of a trifluoroacetate group, is observed. The internal standard can be detected at m/z 375 due to the incorporation of ^{13}C in all six of

Figure 3 Standard curves for dityrosine and the oxazolinone derivative of ATyr

Two independent stock solutions of dityrosine were used to prepare samples of known concentration. The area under the peak due to the standard was plotted against the known mass injected. Samples containing 100–2000 pmol of 3- NO_2 -Tyr and 300 pmol of $^{13}\text{C}_6$ 3- NO_2 -Tyr were reduced, converted into the oxazolinone and analysed by GLC-MS. Using the area under the peak, the ratio of the analyte/internal standard was plotted against the known amount of authentic standard added.

the aromatic positions. In contrast, tyrosine was observed not to fragment to any great extent, instead presenting as a molecular ion at m/z 355. Again, the internal standard could be detected 6 Da higher, at m/z 361. The 111 Da difference between each of the molecular ions is consistent with the presence of a trifluoroacetamide group ($\text{CF}_3\text{CONH-}$) on the aromatic ring of tyrosine.

To increase the sensitivity of this derivative to NCI, we attempted to synthesize analogues with longer-chain perfluorocarbon groups, but these attempts failed to produce any detectable products. Therefore the formation of oxazolinones from amino acids was only possible using the simplest of the perfluoro reagents.

Standard curves and limits of detection

The standard curve for each analyte demonstrated good linearity in the range tested (Figure 3). The minimum detection level of the oxazolinone derivative of ATyr was determined as 100 amol, with a signal-to-noise ratio of approx. 90:1 (root mean square). This compared favourably with other data, where 400 amol gave a signal-to-noise ratio of 10:1 [8]. Using standard ATyr, the oxazolinone derivative demonstrated approximately two orders of magnitude greater sensitivity (results not shown) compared with the method of Crowley et al. [8]. A comparison with the method of Frost et al. [7] could not be carried out, as direct on-column injection is required.

For dityrosine, the standard curve (produced from independent stock solutions) demonstrated good linearity in the range of concentrations that did not overload the detector. For excitation at 289 nm a limit of detection of 150 fmol on the column was observed, with a signal-to-noise ratio of approx. 10:1.

Determination of 3-NO₂-Tyr and dityrosine in carotid plaque proteins

Dityrosine was present at a concentration of 1.5 ± 0.14 mmol/mol of tyrosine after correction for recovery (80%). Nitration occurred at a concentration of 92 ± 15 μ mol/mol of tyrosine. Therefore, if dityrosine is considered to account for the modification of two tyrosine residues, then these amounts equate to 0.3% and 0.01% of tyrosine residues respectively. That is, tyrosine nitration occurs at a frequency which is only 3% that of tyrosine dimerization. The coefficient of variation for this assay was found to be approx. 5% for both 3-NO₂-Tyr and dityrosine, and 4% for tyrosine.

DISCUSSION

The relative contributions of oxidation and nitration to the modification of proteins could be important for understanding the mechanisms that initiate or aggravate atherosclerosis, and perhaps other human disease states. Our results indicate that, at least in human carotid artery atherosclerotic plaques, the relative contribution of nitration is actually quite small, with only 0.01% of the tyrosine residues being nitrated. The apparent difficulty in measuring 3-NO₂-Tyr probably accounts for the lack of data concurrently comparing oxidative and nitrative processes in atherosclerotic plaque proteins.

Although it has not been specified in previous reports, there were considerable differences in measured 3-NO₂-Tyr levels when vials made of different materials were used to effect sample hydrolysis. The inclusion of substances such as benzoic acid and phenol by other investigators [8,18] reflects the need to protect samples during hydrolysis. The underlying problem with the hydrolysis procedure is indicated by the variable recovery using different preservatives. It was found that the 3-nitro- and 3-amino-derivatives of tyrosine were not stable to hydrolysis in glass, and the remedy to this problem was the use of polypropylene Eppendorf tubes. Sarstedt tubes, an alternative polypropylene vessel, performed as poorly as glass, and it is possible that displacement of oxygen by water/acid vapours from the non-airtight Eppendorf tubes plays an additional role in their suitability.

The absence of nitration observed when samples of authentic tyrosine were submitted to hydrolysis in Eppendorf tubes using physiological concentrations of nitrite and nitrate is in contrast with previous results [7]. However, it should be noted that a nitrite concentration more than 700 times greater than the physiological level was used for those previous experiments and, even with this abundance, only 1% nitration was observed. In addition, using this level of nitrite, it was observed that no nitration occurred at pH 3, 4 or 5, despite a 20 h incubation [7]. This demonstrates that acidic conditions are necessary, but not sufficient, to induce nitration of tyrosine. It is not surprising, then, that at the physiological levels of nitrite (and nitrate) used herein, no nitration was observed. It is feasible, however, that nitration occurred, but at levels below the limit of sensitivity of this assay. We have observed that acidified nitrite and nitrate solutions can give rise to brown, nitrogenous gases. This may be one mechanism behind the loss of inorganic nitrogen from acidic solutions that may explain why large excesses of nitrite are required for the nitration of tyrosine. As our experiments had shown that nitration does not occur in the presence of physiological concentrations of nitrite and nitrate, proteins were not treated (e.g. molecular-mass cut-off filters, dialysis) to remove these species; however, they were probably largely removed by the Folch extraction, since we have observed that aqueous

sodium nitrite remains soluble when added to one or more volumes of methanol.

We have also observed that glass containers are a source of reactive nitrogen species during the derivatization procedure, so it is most probable that reactive species that induce artefactual nitration during hydrolysis emanate from the glass containers, and are not due to the presence of inorganic nitrogenous species in biological samples. Reduction of the crude hydrolysate prior to the acidic derivatization used herein provides the key to avoiding the detection of artefacts produced during derivatization. This is because the focus of the analysis shifts to ATyr, avoiding detection of artefactual 3-NO₂-Tyr.

The synthesis of the oxazolinone derivative of 3-NO₂-Tyr was used because of its rapid production time and its high sensitivity to NCI. The formation of oxazolinone derivatives of amino acids using trifluoroacetic acid anhydride was first described by Weyand and Glockler in 1956 [19]. Grahl-Neilsen and Movik [20] applied the concept to the GC of amino acids when GC was in its infancy. Oxazolinone derivatives were viewed as potentially useful for amino acid identification by Ferrito et al. [21], who demonstrated that, under electron-impact conditions, the oxazolinones produced simple mass spectra. This feature is replicated for ATyr: at typical analytical concentrations, only the ($M - 97$)⁻ ion (m/z 369) is generated, which equates to loss of trifluoroacetate. This allows selected ion monitoring of a single ion, which maximizes the probability of detecting an ionized analyte molecule. The formation of the oxazolinone is a rapid, single-step and quantitative (HPLC-UV, GLC-MS with electron impact and NCI; results not shown) procedure, and assists in decreasing the sample handling time.

The oxazolinone derivative as an alternative means of the measurement of 3-NO₂-Tyr has provided substantial insight into the paucity of this latter compound in the proteins of advanced carotid artery atherosclerotic lesions, demonstrating that nitration is not a major modification of tyrosine. The finding that approx. 0.01% of tyrosine residues are nitrated is consistent with the findings of others that less than 2% of tyrosine could be nitrated in lesions [22] from a range of categories [9,10]. Furthermore, since other tyrosine modifications, such as 3-chlorotyrosine and dopa, are known to occur in carotid plaques [5,6], the amount of nitration relative to other modifications of tyrosine is likely to be below 3%. This does not preclude nitration of selected arterial wall proteins as an important initiating mechanism in atherosclerosis [23], and this remains to be fully elucidated. It also remains possible that the low levels of 3-NO₂-Tyr demonstrated in the present study could reflect further modification of 3-NO₂-Tyr by subsequent metabolic or chemical processes (e.g. an effect of hypochlorous acid [24]), and that analytical methods produce underestimates of *in vivo* nitration.

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