Amino Acids (2003) 25: 351–361
DOI 10.1007/s00726-003-0022-z

Amino Acids
Printed in Austria

A review of approaches to the analysis of 3-nitrotyrosine

Review Article

M. W. Duncan

Biochemical Mass Spectrometry, University of Colorado Health Sciences Center, Denver, Colorado, U.S.A.

Received October 28, 2002
Accepted May 8, 2003
Published online November 7, 2003; © Springer-Verlag 2003

Summary. Our understanding of *in vivo* tyrosine nitration has been confounded by problems associated with the analytical approaches that have been employed to quantify 3-nitroyrosine (3-NT). Trace analysis is a demanding task under the best of circumstances, but 3-NT offers some special concerns. This review examines some of these concerns and discusses approaches to ensuring that carefully validated analytical data are generated.

Keywords: 3-Nitrotyrosine – Trace analysis – Artifactual formation – Assay validation – Mass spectrometry

Introduction

The superoxide anion ($O_2^{\bullet -}$) can react with nitric oxide ($NO^{\bullet}$) to produce the powerful oxidant peroxynitrite ($ONOO^-$) (Beckman et al., 1990).

$$O_2^{\bullet -} + NO^{\bullet}\ (\text{nitric oxide}) \longrightarrow ONOO^-\ (\text{peroxynitrite})$$

It is believed that peroxynitrite is produced by inflammatory cells to defend against cancer cells or infection caused by parasites, viruses, bacteria and cancerous cells, but at the same time it can be damaging to host cells and tissues. Nitration of the amino acid tyrosine, to produce 3-nitrotyrosine, is reportedly a reliable index of damage left by peroxynitrite (see Fig. 1). Peroxynitrite-mediated damage has been implicated in a range of disease pathologies, and consistent with this, elevated levels of 3-nitrotyrosine have been reported in atherosclerosis (Beckman et al., 1994; Cromheeke et al., 1999), multiple sclerosis (Cross et al., 1998; Oleszak et al., 1998; van der Veen et al., 1997), Alzheimer's disease (Hensley et al., 1998; Smith et al., 1997; Teunissen et al., 2002; Tohgi et al., 1999), Parkinson's and animals models of the disease (Ferrante et al., 1999; Giasson et al., 2000; Good et al., 1998; Good et al., 1996; Pennathur et al., 1999), cystic fibrosis (Balint et al., 2001; Morrissey et al., 2002), asthma (Hanazawa et al., 2000), other lung diseases (Haddad et al., 1994; Hansen et al., 2000; Kharitonov and Barnes, 2002), myocardial dysfunction (Cesselli et al., 2001; Feng et al., 2001; Ferdinandy et al., 2000; Mihm et al., 2001; Zweier et al., 2001), stroke (Forster et al., 1999), trauma (Liu et al., 2000; Nag et al., 2001), organ rejection (Akizuki et al., 2000; Albrecht et al., 2002; De Andrade et al., 2000; Ravalli et al., 1998; Sakurai et al., 1999; Szabolcs et al., 1996; Szabolcs et al., 1998; Yamaguchi et al., 1999), amyotrophic lateral sclerosis (Abe et al., 1995), inflamed human colonic epithelium (Singer et al., 1996), chronic hepatitis (Cuzzocrea et al., 1998; Garcia-Monzon et al., 2000), cirrhosis (Cuzzocrea et al., 1998), experimental osteoarthritis (Hashimoto et al., 1999; Pelletier et al., 1999), diabetes (Ceriello et al., 2001) and a string of other disorders. While peroxynitrite also damages lipids, DNA and other cellular constituents, most investigators have concentrated on protein damage for both analytical and biological reasons. In addition to 3-nitrotyrosine serving as a marker of exposure to peroxynitrite, tyrosine nitration itself may impair cell function.

The role of peroxynitrite in biological systems continues to be intensively examined, but establishing its significance is more complicated than first anticipated. This complexity arises for two main reasons. First, 3-nitrotyrosine was originally believed to be a specific marker of peroxynitrite, but recent data do not support this assumption (Hurst, 2002). It is now evident that nitration can occur *in vivo* independently of peroxynitrite exposure, *e.g.*, *via* hypochlorous acid and peroxidase enzymes (Gaut

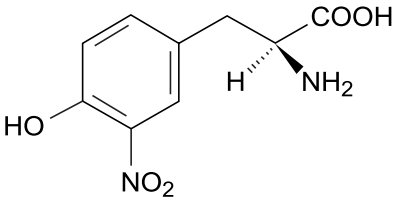

Fig. 1. 3-Nitrotyrosine, MW 226.2 and molecular formula: $C_9H_{10}N_2O_5$

et al., 2002b). Second, additional confusion has been generated because sub-optimal analytical methods have been applied to the quantification of trace levels of 3-nitrotyrosine in biological tissues and fluids and the importance of these analytical data in defining a role for peroxynitrite cannot be over-emphasized.

Several years ago Halliwell suggested:

> "... an under-addressed problem is the reliability of assays used to detect and measure 3-nitrotyrosine in tissues and body fluids: immunostaining results vary between laboratories and simple HPLC methods are susceptible to artifacts. Exposure of biological material to low pH (*e.g.*, during acidic hydrolysis to liberate nitrotyrosine from proteins) or to H_2O_2 might cause artifactual generation of nitrotyrosine from NO_2^- in the samples. This may be the origin of some of the very large values for tissue nitrotyrosine levels quoted in the literature." (Halliwell et al., 1999)

That was certainly the case in 1999, and in reality, it has changed little since then. Our perception of the importance of 3-nitrotyrosine in biological systems has been formulated, in large part, based on the analytical data and some of these are of questionable merit. It is only recently that the analytical task has gained the attention to detail that it rightly deserves.

In this chapter, the analytical issues associated with the quantification of 3-nitrotyrosine are discussed with an emphasis on fitness-for-purpose. An important underlying principle in this chapter is that 3-nitrotyrosine analysis presents special challenges and these exist independent of the specific hardware that might be employed. To put this another way, without consideration of these fundamental issues, it is possible to generate erroneous data, even with the most sophisticated of hardware.

Quantitative analysis in a biological setting: definitions and general considerations

Introduction

The detection, identification and quantification of a target compound, especially at low concentrations, are some of the most challenging tasks the scientist undertakes. The goal of analysis should therefore be to develop a method that is both reliable and appropriate to the task (*i.e.*, fit for the purpose), not the least because the analytical data frequently underpin the biological argument, and if there are errors in the analysis, our understanding of the system and its function is flawed. While we tend to place equal merit on all published quantitative data, in reality, analytical work varies enormously in quality. Accuracy, precision and the limit of quantification vary based on the method employed, and the skill and attention to detail of the investigators. These issues are nowhere more important or evident than in the literature on 3-nitrotyrosine determinations. This analyte presents special challenges because of the low levels present *in vivo* and the potential for artifactual formation.

Assay validation is the process by which the ability of an assay to achieve its purpose is assessed, *i.e.*, to quantify analyte concentrations with a degree of accuracy and precision fit for the intended purpose. The process of validation certifies that the analytical method performs as intended, and ensures that no quantitative data are compromised by errors in procedure (Green, 1996). The validation also defines the performance criteria that will be used to accept or reject analytical data generated in subsequent applications of the assay, as well as the conditions necessary to ensure analyte stability during sample storage and work-up. While validation is tedious and places severe restraints on the analyst, this process is essential if meaningful data are to be generated. A serious criticism that can be directed at much of the 3-nitrotyrosine work undertaken so far is that validation has not been undertaken, or if it has been performed, it has been compromised.

Definitions

Some key parameters are critical to the discussion that follows, and for this reason, they are defined below. Their relevance in the context of 3-nitrotyrosine determinations is discussed in the sections that follow.

The limit of detection (LOD) is the lowest concentration of analyte that can be detected under the stated experimental conditions. No levels below the limit of detection can be reported or quantified. The term "*sensitivity*" is often used in a vague or poorly defined manner in place of this term. *Sensitivity* is the lowest concentration (or amount) that can be distinguished from background noise. Traditional measuring devices link concentration with such properties as absorption of ultraviolet light (spectroscopic techniques), absorption of visible light (colorimetric techniques), or current (electrical

techniques). For most instrumental methods there is a linear response to some physical stimulus with respect to the amount of that analyte introduced over an amount or concentration range and therefore: $R = mC + S_{bl}$ where C = concentration, R = response, m = *sensitivity* and S_{bl} = blank signal.

The lower limit of quantification (LLOQ or LLQ) is the smallest amount or lowest concentration that can be quantified with the accuracy and precision required by the assay.

Precision is a measure of the ability to reproducibly return the same value for a sample that is repeatedly analyzed. The precision decreases as the analyte concentration decreases, ultimately reaching unacceptable values at the point at which the measured signal approaches the limit of quantification.

Accuracy measures the closeness with which an individual measurement approaches the true value. Because all actual measurements are estimates of the real value, only by producing replicate measurements of the concentration of a given sample can the true value be approached. Accuracy can be determined when samples containing known concentrations of analyte are measured. These quality control (QC) samples are used to mimic the way unknowns will be treated and as a means of assessing the adequacy of storage for preserving sample integrity.

The *matrix* is the collection of all chemical species among which the analyte is found.

Stability is an index of the propensity for the chemical integrity of the analyte to change, for example during freezing/thawing, and during sample work-up and analysis.

Selectivity is the ability to measure the response arising from one analyte independent of any other sample component(s).

Linearity is the range over which test results are proportional to the concentration of the analyte.

Approaches to 3-nitrotyrosine analysis

General introduction

The detection and quantification of 3-nitrotyrosine have employed a variety of methods including immunoassay, HPLC with a variety of detection methods, and gas or liquid chromatography coupled to either single stage or tandem mass spectrometry. Each individual assay and the data it generates need to be assessed mindful of the criteria introduced above. Assays applied to the analysis of 3-nitrotyrosine in biological samples must offer a low limit of detection, accuracy and precision, and how well these objectives are met is defined by the validation parameters. When these are not reported, the ability of the assay to yield appropriate values for the analyte concentration cannot be objectively assessed. This becomes particularly significant when the assay is operated near the limit of detection and especially when quantitative comparisons are made. In this region, false positive and false negative identifications are common and quantitative determinations can display very large errors. As discussed later, the additional issue of artifactual formation further complicates the situation and has the potential to invalidate all data.

Some of the reported analytical strategies are discussed below with particular attention focused on assay validity.

Antibody-based quantitative methods

A surprisingly large proportion of the data available on 3-nitrotyrosine in tissues and fluids has been derived from antibody-based methods. For example, immunohistochemical staining methods (Viera et al., 1999) have been used to demonstrate increased levels of 3-nitrotyrosine in lung tissue from patients with cystic fibrosis (Morrissey et al., 2002); inflamed human colonic epithelium (Singer et al., 1996); in the lower motor neuron of amyotrophic lateral sclerosis patients (Abe et al., 1995; Beal et al., 1997), chronic hepatitis (Cuzzocrea et al., 1998; Garcia-Monzon et al., 2000), cirrhosis (Cuzzocrea et al., 1998), experimental osteoarthritis (Hashimoto et al., 1999; Pelletier et al., 1999), Parkinson's disease (Good et al., 1998), Alzheimer's disease (Smith et al., 1997) and in atherosclerotic plaques (Cromheeke et al., 1999). By employing ELISA assays, others have reported increased 3-nitrotyrosine formation in diabetic plasma (Ceriello et al., 2001). Other ELISA assays (Oldreive et al., 2001; ter Steege et al., 1998) and solid phase immunoradiochemical methods to measure 3-nitrotyrosine have been developed (Banks et al., 1998). In all these studies, in the absence of rigorous assay validation, the differential appearance of immunoreactive proteins (*i.e.*, ''nitrated proteins'') in disease or treatment samples provides the investigators with some confidence in the selectivity of the assay. However, in much of this work, the reliably of the method is difficult to assess and key parameters, such as LOD, LLOQ and linearity, are not defined. In addition, the ability of the method to specifically measure only nitrated substrates, even in a complex matrix, is often not addressed. Because it is dependent on a wide range of parameters, including the nature of the antibody, sample type, amount, other components present and time, selectivity must be determined in each setting. When assays that are not rigorously validated are applied to real-world

problems, a statistically significant change in levels cannot be attributed to the target analyte(s) without ambiguity. While changes may be evident, the source of the change is questionable. Further, if no apparent change is evident, this may be because a lack of specificity has obscured it. In short, when the selectivity of the assay is poor, invalid data result. Methods that do not carefully address selectivity, accuracy and precision in a range of control samples should be considered semi-quantitative and the results interpreted accordingly. Khan and colleagues have acknowledged the limitations of their own competitive ELISA approach and accordingly described it as semi-quantitative (Khan et al., 1998).

Methods based on gas chromatography with thermal energy analysis

Early analytical reports of 3-nitrotyrosine in tissue and blood proteins were performed by gas chromatography (GC) with a thermal energy analyzer (GC-TEA). It was found that free and protein-bound tyrosine residues easily react with nitrating/nitrosating agents to yield 3-nitrotyrosine (Ohshima et al., 1990). Later, a method for 3-nitrotyrosine analysis in human plasma was developed and tested in cigarette smokers and healthy control subjects. Here, 3-nitrotyrosine was determined with a sequential HPLC-GC-TEA technique. This approach offered a limit of detection of 0.02 ng/injection and was employed to demonstrate that 3-nitrotyrosine was elevated in the plasma of smokers: ($n = 11$; mean $\pm$ SD: 1.60 ± 1.24 ng/mg protein) vs. nonsmokers (present in only 2 of 20 subjects at levels of 1.10 and 1.20 ng/mg protein) (Petruzzelli et al., 1997). While an important approach, TEA detection does not offer the selectivity and sensitivity necessary to measure 3-nitrotyrosine levels in basal samples.

High-performance liquid chromatography (HPLC) with electrochemical detection

HPLC is a powerful separation technique, but it must be combined with a detection method for quantitative analysis. Both UV (Crow and Ischiropoulos, 1996) and fluorescence detection (*i.e.*, following pre-column derivatization with 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole) (Kamisaki et al., 1996) have been employed to measure 3-nitrotyrosine, but these approaches prove too insensitive for the quantification of the low levels found in most fluids or tissues. Consequently, HPLC coupled with electrochemical detection (HPLC-ECD) has been most commonly adopted to measure 3-nitrotyrosine in biological samples (Crow, 1999; Ishida et al., 2002; Shigenaga, 1999; Shigenaga et al., 1997). This combination offers good separation and high sensitivity. For example, Shigenaga elected to reduce 3-nitrotyrosine to 3-aminotyrosine and then employed HPLC-ECD analysis (Shigenaga et al., 1997). Although this strategy involves chemical modification of the analyte, this markedly improved the LLOD. One downfall, however, is that 3-aminotyrosine is reportedly produced *via* endogenous tyrosine amination.

Ohshima and colleagues developed an alternative approach that involves reversed-phase HPLC separation, subsequent reduction by a platinum column to 3-aminotyrosine, and then electrochemical detection. This method is reportedly simple, selective, and sensitive (detection limit, 0.1 pmol per 20 μL injection) (Ohshima et al., 1999). Others have used HPLC with array ECD and applied this to the analysis of 3-nitrotyrosine in brain tissue of Alzheimer's patients (Hensley et al., 1997; Hensley et al., 1998). However, analysis by LC-ECD can lead to erroneous results even in the hands of experienced scientists. Halliwell and colleagues identified a peak with a retention time similar to 3-nitrotyrosine in brain tissue from patients with Parkinson's disease, Huntington's chorea, multiple system atrophy, and Alzheimer's disease, but not in control tissue (Kaur et al., 1998). Careful review of their data, and a series of well-conceived studies, demonstrated that this peak was not 3-nitrotyrosine. Appropriately, these investigators suggested that analyses performed without careful controls and attention to detail could confound.

Mass spectrometry-based assays

Mass spectrometry is less readily accessible, requires skilled operators and the hardware is expensive; nevertheless, it is a powerful analytical approach that offers some unique benefits when applied to the analysis of 3-nitrotyrosine. The most important of these are the selectivity available with selected mass (or selected reaction) detection, the sensitivity of the mass spectrometer as a detector, and the ability to perform complex studies that take full advantage of stable-isotope labeled analogs of the compound of interest. In view of the complexity inherent in the analysis of 3-nitrotyrosine, and the confounding results evident in the literature, several groups have adopted mass spectrometry. There is good evidence that the most carefully constructed of these approaches are beginning, for the first time, to give us a realistic

assessment of the levels of 3-nitrotyrosine, both free and protein bound.

Mass spectrometry is most frequently coupled with one of two commonly adopted chromatographic options, GC or HPLC. These two options, and the methods based on them, are discussed separately.

GC-MS and GC-MS-MS. Gas chromatography coupled to mass spectrometry is frequently available to investigators in a research environment and it offers some important advantages. When operated in the electron capture negative ion (NIEC) mode, this is a uniquely sensitive analytical combination. The major limitation of GC-MS, however, is that the analyte of interest has to be converted to the gas phase prior to separation and ionization. For polar analytes, even of low mass, this often requires excessive temperatures and involves significant analyte degradation. To circumvent this, a wide range of chemical derivatization strategies have been developed and commercialized that impart volatility. Amongst the most powerful of these are the perfluorinated reagents that markedly enhance the electron-capturing properties *and* significantly reduce the boiling point of the sample. Therefore, assays based on GC-MS often make use of these reagents to gain two advantages: increased volatility and an improved limit of detection through the generation of electron-capturing products.

Several groups have developed GC-MS assays for 3-nitrotyrosine determinations (Crowley et al., 1998; Frost et al., 2000; Gaut et al., 2002a; Jiang and Balazy, 1998; Yi et al., 2000). Most recently, Gaut and colleagues described a specific, sensitive and quantitative method for detecting 3-nitrotyrosine, together with chlorotyrosine and bromotyrosine (Gaut et al., 2002a). These investigators employed stable isotope labeled standards for precise quantification and to assess artifact generation. In keeping with the findings of others (Frost et al., 2000), Gaut et al. found it necessary to use on-column injection to optimize peak shape and retain sensitivity with repeated analysis. On-column injection is the method by which a sample is directly injected manually into the analytical column from a fine-bore syringe. This approach minimizes problems arising from the interactions between the analyte and the glass injection liner of the GC.

Of the other reported methods, one is of special note. Tsikas and colleagues (Schwedhelm et al., 1999) utilized the exceptional sensitivity of NIEC in an assay of 3-nitrotyrosine that has been rigorously validated. Their approach employed gas chromatography-tandem mass spectrometry (GC-MS-MS) for the accurate and precise quantification of free 3-nitrotyrosine in human plasma in the basal state. They reported a mean concentration of 2.8 nM (n = 11; range 1.4–4.2 nM) for free 3-nitrotyrosine in plasma. To this point, this represented the lowest concentration reported for free 3-nitrotyrosine in the plasma of healthy humans and this value is consistent with determinations made by others (Yi et al., 2000). To avoid artifactual formation of 3-nitrotyrosine during work-up, Tsikas and colleagues employed HPLC separation, prior to derivatization, to resolve 3-nitrotyrosine from nitrate, nitrite and tyrosine. Further, these investigators optimized precision and accuracy by incorporating stable isotope labeled internal standards (*i.e.*, isotopomers) and by operating in the selected reaction-monitoring mode. The key analytical parameters were all carefully determined. For example, by utilizing 3-nitro-[$^{14}C_9$]-tyrosine, the overall recovery was determined as $50 \pm 5\%$. The LOD was 4 amol, the LLOQ 125 pM, accuracy $\geq 80\%$ and precision $\geq 94\%$ (Schwedhelm et al., 1999). There is one important limitation of this method, however, and that is that the complexity of the process severely limits the throughput of the analysis, and consequently, limits the type of studies that can be performed. Nevertheless, it is far preferable to have limited good quality data, than it is to be flooded with complex and confounding results. This work is therefore an excellent example of the attention to detail required to generate data fit for the purpose of assessing the biological role of reactive nitrogen species and 3-nitrotyrosine.

LC-MS methods

LC-MS (and LC-MS-MS) offer advantages over GC-based methods, not the least of which is that it is no longer necessary to modify the analyte to impart volatility. Because chemical manipulation can be eliminated, sample handling, the potential for side-reactions, losses and contamination are also minimized.

We have developed an accurate LC/MS/MS assay for the simultaneous determination of tyrosine and 3-nitrotyrosine in physiological samples (Yi et al., 2000). We adopted LC-MS-MS only after considerable effort directed at GC-based methods. Our GC-MS work was persistently compromised because of the propensity of reagents to catalyze the nitration of tyrosine by traces of nitrate present in samples. We investigated milder conditions for derivatization, as well as other derivative types, but could never be confident that the problem was completely eliminated. The LC-MS-MS assay we developed allows simultaneous quantification of both tyrosine and 3-nitrotyrosine, and has been optimized so that the

3-nitrotyrosine peak shape, and that of [$^{13}C_6$]-labeled 3-nitrotyrosine, were not distorted, even in the presence of a huge excess of tyrosine. 3-Nitrotyrosine standards were prepared up to a concentration of 100 pg/μL and the resulting calibration curves were linear across the range 0.5 and 100 pg/μL (2.2–44.2 fmoles/μL; $r^2 = 0.999$). The detection limit (at a S/N = 5) for standard samples was 0.5 pg/μL (2.2 fmoles/μL or 10 fmoles on column) and *ca.* 1 pg/μL (4.4 fmoles/μL) for 3-nitrotyrosine in biological samples.

Kaur and Halliwell (Kaur and Halliwell, 1994) report 3-nitrotyrosine levels up to 272 pg/μL (1.2 pmoles/μL) when employing a relatively insensitive HPLC analysis with a detection limit of 50 pg/μL (221 fmoles/μL). Based on our detection limit of 1 pg/μL (4.4 fmoles/μL), levels of this magnitude would be easily detected in our system. However, we could not detect 3-nitrotyrosine in any of the synovial fluid samples. Therefore, we predict that 3-nitrotyrosine levels in human synovial fluid are at least 100-fold lower than those previously reported. We subsequently spiked each sample – the technique of standard addition – with 0, 1, and 5 pg/μL of 3-nitrotyrosine and then re-analyzed them: these analyses unambiguously confirmed that levels of 3-nitrotyrosine in the synovial fluid samples tested were less than 1 pg/μL (4.4 fmoles/μL). The chromatograms generated from a single subject with active rheumatoid arthritis (RA), both before and after spiking with 3-nitrotyrosine demonstrate that levels as low as 1 pg/μL could be detected and quantified in synovial fluid samples. (See Fig. 2.)

Similarly, previous studies (Crowley et al., 1998; Leeuwenburgh et al., 1997) report the presence of high levels (*ca.* 150 μmole/mole tyrosine) of protein-bound 3-nitrotyrosine in rat heart tissue, but when we analyzed tissue from 6 rat hearts by LC-MS-MS, no detectable 3-nitrotyrosine was found. Because these same samples contained *ca.* 10 μg tyrosine and, based on the detection limit of 1 pg/μL for the LC-MS-MS assay, we estimate the amount of 3-nitrotyrosine to be less than 200 pg (*i.e.*, <15 μmole/mole tyrosine). (See Table 1.)

Subsequent to our own study, several other LC-MS assays for 3-nitrotyrosine have been reported (Althaus et al., 2000; Brennan et al., 2002; Delatour et al., 2002a; Delatour et al., 2002b). The most recent of these build on the work already undertaken by others and incorporate careful controls to avoid artifact formation. While

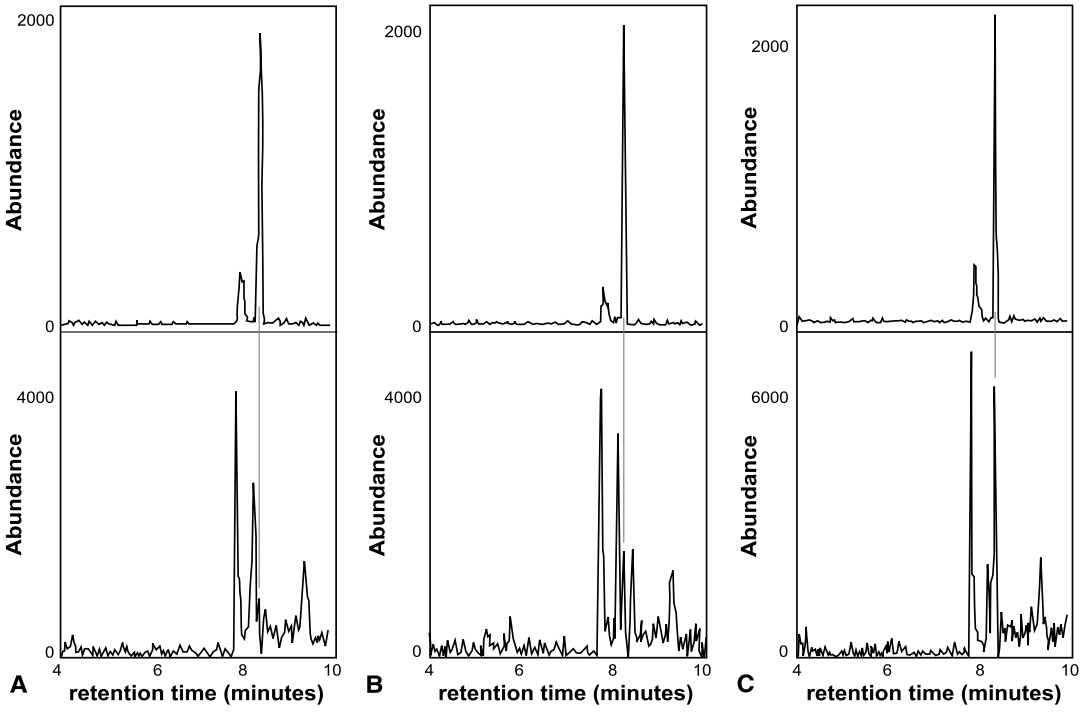

Fig. 2. LC/MS chromatogram for the determination of free 3-nitrotyrosine in synovial fluid of an RA patient before (**A**) and after the addition of 1 pg/mL (**B**) or 5 pg/mL (**C**) 3-nitrotyrosine. Upper mass trace shows precursor-product reaction 233 → 187 (internal standard), lower trace shows 227 → 181 (3-nitrotyrosine). From: Yi, 2000 #1392

Table 1. Levels of 3-nitrotyrosine in different sample matrices as reported by several groups. (Units as reported in the original publication)

Sample matrix	LC/MS/MS	Reported values
Human Plasma	<4.4 nmol/L	31.6 ± 6 nmol/L (Kamisaki et al., 1996); 2.8 ± 0.84 nmol/L (Schwedhelm et al., 1999)
Rheumatoid Arthritis Synovial Fluid	<4.4 nmol/L	0–1.2 μmol/L (Kaur and Halliwell, 1994)
Rat Cardiac Tissue	<15 μmol/mol Tyr	110–154 μmol/mol (Crowley et al., 1998; Leeuwenburgh et al., 1998)
Basal Rat Plasma	<0.44–1.5 nM	(Delatour et al., 2002a)

based on LC-MS-MS rather than GC-MS-MS, these studies still require elaborate sample work-up to ensure good accuracy and precision. In one of these studies, the investigators elected to convert 3-nitrotyrosine to its *n*-butyl ester prior to analysis (Delatour et al., 2002a). This approach enhanced sensitivity (LOD = 0.07 pmoles on-column, LLOQ = 0.23 pmoles on-column) and aided in the removal of other components from the sample matrix.

Only in the last year or so are we beginning to see reports of assays that take full advantage of the sensitivity, selectivity and flexibility of LC-MS-MS. Because these more recent studies have paid particular attention to the issues relating to fitness for purpose, we are starting to generate a better appreciation of 3-nitrotyrosine levels in biological tissues and fluids. This trend will undoubtedly continue, but we have had setbacks because of a lack of attention to the essential details of trace analysis.

Unfortunately, the potential of LC-MS(-MS) to contribute to our understanding of reactive nitrogen species and 3-nitrotyrosine is under-estimated and at times misunderstood. For example, Gaut and colleagues used both GC-MS (ECNI) and LC-MS-MS for their determinations of 3-nitrotyrosine (discussed previously) (Gaut et al., 2002a) and concluded that GC-MS (ECNI) is 100-fold more sensitive for analyzing all three analytes (*i.e.*, 3-nitrotyrosine, chlorotyrosine and bromotyrosine). This conclusion is not justified and erroneously contributes to ongoing misconceptions. In the hands of these investigators, and with the instruments available to them, they were unable to reach the same LOD by LC-MS-MS that they could by GC-MS. However, this is not surprising because the ion trap instrument employed to generate the LC-MS-MS data is not well suited to quantitative work. The failings of their LC-MS assay are, in large part, a consequence of the system they employed. By using a more appropriate system – ideally a triple quadrupole system – the achievable LOD (and LLOQ) would improve significantly.

It is also important to recognize that there are ongoing advances in instrument performance that relate to source design, analyzer performance and ion detection. Major changes in source design have markedly improved the sensitivity of LC-MS systems over just the last few years. It is therefore likely that performance on a LC-MS system purchased today will be markedly better than a system that is just a few years old. While there are ongoing improvements in GC-MS, this configuration is in a very different phase of product development. Bench top GC-MS systems have been available for over 20 years and on-going developments refine, rather than dramatically enhance performance. By contrast, over recent years the consecutive LC-MS(-MS) systems from the major instrument manufacturers exhibit significant performance enhancements. In addition, such factors as volume introduced, the specifics of the work-up process, together with familiarity with the hardware, can all markedly distort a comparison of this type. Suffice it to say, the available evidence is consistent with the view that assays with comparable performance can be developed with either approach. However, because MS hardware is expensive, most of us are not in a position to regularly upgrade our equipment to ensure that performance is always characteristic of the state-of-the-technology.

Sample handling issues: extraction, derivatization and artifact formation

Finally, it is worth emphasizing that the performance of any analytical method is only as good as the process *in toto*; it is not just the analytical hardware that is important. Some of the other considerations in the analysis of 3-nitrotyrosine are discussed in this section. It is noteworthy that adequate sensitivity is available with either HPLC-ECD, GC-MS(-MS) or LC-MS(-MS), but as discussed below, options based on mass spectrometry offer some significant advantages.

Protein Hydrolysis. 3-Nitrotyrosine exists in two separate forms: as the free amino acid and as a constituent amino acid within a protein backbone. Analytical studies have measured both forms, and the methods are essentially the same, although, additional steps must be

incorporated when targeting the protein-bound amino acid. These steps, involving protein isolation and proteolysis, complicate the analysis, and can induce artifactual formation. Nitration of tyrosine under acidic conditions and in the presence of only trace quantities of nitrite is well documented. Shigenaga et al. drew attention to the potential generation of artifactual 3-nitrotyrosine arising from HCl-catalyzed nitration of tyrosine, either by nitrite and nitrate ions, during hydrolysis (Shigenaga et al., 1997). Similarly, Ohshima et al. demonstrated nitration of tyrosine residues in protein by nitrite under acidic and neutral conditions (Ohshima et al., 1999).

Several approaches to circumventing artifact formation during protein hydrolysis have been reported. We adopted a gas phase HCl hydrolysis and demonstrated that no artifacts arose during this step (Yi et al., 2000). Addition of 1% (w\v) phenol to act as a scavenger for nitration has also been tried. This reduces but does not eliminate the problem (Shigenaga et al., 1997). Others have employed enzymatic digestion of proteins to determine the nitration ratio (ratio of 3-nitrotyrosine to tyrosine) (Shigenaga et al., 1997), but here proteolysis is rarely complete and autolysis of the protease itself can contribute to the levels of tyrosine and 3-nitrotyrosine (Yi et al., 2000). Still others have employed alkaline hydrolysis (Frost et al., 2000).

Derivatization. Derivatization procedures are only an absolute requirement for GC-MS analysis and it has been demonstrated that in this setting they can lead to the artifactual nitration of tyrosine when performed under acidic conditions (Yi et al., 2000). However, derivatization has been incorporated into both HPLC-ECD (Shigenaga et al., 1997) and LC-MS-MS (Delatour et al., 2002a) assays to achieve other specific objectives, *i.e*., to facilitate extraction or to enhance ionization. For example, Shigenaga and colleagues employed several separate steps in their analysis to convert 3-nitrotyrosine to N-acetyl-3-aminotyrosine (*i.e*., *via* acetylation, O-deacetylation, and dithionite reduction) to aid in extraction and detection (Shigenaga et al., 1997). Complex steps of this type are not uncommon when measuring trace levels of a compound in a complex matrix.

Selection of Internal Standards – benefits of mass specific detection. Good precision requires the incorporation of internal standards with HPLC-ECD, GC-MS(-MS) and LC-MS(-MS). It is in this area that the unique benefits of mass spectrometry come to the fore. Mass spectrometry allows the investigator to incorporate isotopomers (stable isotope analogs) to serve as the standard. Because these differ from the analyte in physical, but not chemical properties, they behave in a manner identical to the analyte during extraction, derivatization and ionization. However, in the final stage of the analysis – ion detection – signals unique for the analyte and standard can be measured. In this manner, the standard serves as a perfect mimic of the analyte, thereby allowing optimal precision. HPLC-ECD analyses require that a structural analogue or homologue serve as the internal standard, but this can be a serious compromise. First, such a standard may display chemical properties distinct from those of the analyte; and second, the analyst is restricted to selecting an internal standard that has a retention time distinct from all other components of the sample. When working at or close to the LLOQ, isotopomers can offer improved accuracy and precision, and for this reason alone mass spectrometry offers an important advantage.

Measuring Artifact Formation – an additional benefit of mass selective detection. As discussed throughout this review, artifact formation can confound 3-nitrotyrosine determinations, but this complication can be difficult to identify and even more difficult to quantify. However, isotopomers can serve as reliable markers of chemical changes during sample handling and provide a solution to this problem. For example, the chemical properties of [$^{13}C_6$]-tyrosine are identical to those of native tyrosine and therefore both substrates (*i.e*., tyrosine and [$^{13}C_6$]-tyrosine) undergo transformation (*i.e*., to 3-nitrotyrosine and [$^{13}C_6$]-3-nitrotyrosine) with the same propensity. With a mass-specific detector both species can be monitored, and the extent of conversion of synthetic [$^{13}C_6$]-tyrosine to [$^{13}C_6$]-3-nitrotyrosine serves as a precise indicator of *ex vivo* artifactual 3-nitrotyrosine formation. Some groups have utilized exactly this approach to assess the performance of their analytical methods (Yi et al., 2000).

Conclusions

Quantitative analysis is frequently a challenge, but the problems posed by 3-nitrotyrosine are particularly significant. While it is often possible to cut corners and make compromises in the development and application of an assay, this has proven a dangerous practice with 3-nitrotyrosine. Some of the high levels and dramatic changes observed in early work may not prove real. It is therefore prudent to critically review previous studies, particularly the analytical aspects of the work, and where justified, replicate those of special significance. Our understanding of the biological significance of reactive nitrogen species will only advance when we ensure that validated analytical data form the foundations of our understanding.

Acknowledgement

I thank my colleagues Drs. David Thompson and Joseph Zirrolli for their expert review and constructive revision of this manuscript.

References

Abe K, Pan LH, Watanabe M, Kato T, Itoyama Y (1995) Induction of nitrotyrosine-like immunoreactivity in the lower motor neuron of amyotrophic lateral sclerosis. Neurosci Lett 199: 152–154

Akizuki E, Akaike T, Okamoto S, Fujii S, Yamaguchi Y, Ogawa M, Maeda H (2000) Role of nitric oxide and superoxide in acute cardiac allograft rejection in rats. Proc Soc Exp Biol Med 225: 151–159

Albrecht EW, Stegeman CA, Tiebosch AT, Tegzess AM, van Goor H (2002) Expression of inducible and endothelial nitric oxide synthases, formation of peroxynitrite and reactive oxygen species in human chronic renal transplant failure. Am J Transplant 2: 448–453

Althaus JS, Schmidt KR, Fountain ST, Tseng MT, Carroll RT, Galatsis P, Hall ED (2000) LC-MS/MS detection of peroxynitrite-derived 3-nitrotyrosine in rat microvessels. Free Radic Biol Med 29: 1085–1095

Balint B, Kharitonov SA, Hanazawa T, Donnelly LE, Shah PL, Hodson ME, Barnes PJ (2001) Increased nitrotyrosine in exhaled breath condensate in cystic fibrosis. Eur Respir J 17: 1201–1207

Banks BA, Ischiropoulos H, McClelland M, Ballard PL, Ballard RA (1998) Plasma 3-nitrotyrosine is elevated in premature infants who develop bronchopulmonary dysplasia. Pediatrics 101: 870–874

Beal MF, Ferrante RJ, Browne SE, Matthews RT, Kowall NW, Brown RH Jr (1997) Increased 3-nitrotyrosine in both sporadic and familial amyotrophic lateral sclerosis. Ann Neurol 42: 644–654

Beckman JS, Ye YZ, Anderson PG, Chen J, Accavitti MA, Tarpey MM, White CR (1994) Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. Biol Chem Hoppe Seyler 375: 81–88

Brennan ML, Wu W, Fu X, Shen Z, Song W, Frost H, Vadseth C, Narine L, Lenkiewicz E, Borchers MT, Lusis AJ, Lee JJ, Lee NA, Abu-Soud HM, Ischiropoulos H, Hazen SL (2002) A tale of two controversies: defining both the role of peroxidases in nitrotyrosine formation *in vivo* using eosinophil peroxidase and myeloperoxidase-deficient mice, and the nature of peroxidase-generated reactive nitrogen species. J Biol Chem 277: 17415–17427

Ceriello A, Mercuri F, Quagliaro L, Assaloni R, Motz E, Tonutti L, Taboga C (2001) Detection of nitrotyrosine in the diabetic plasma: evidence of oxidative stress. Diabetologia 44: 834–838

Cesselli D, Jakoniuk I, Barlucchi L, Beltrami AP, Hintze TH, Nadal-Ginard B, Kajstura J, Leri A, Anversa P (2001) Oxidative stress-mediated cardiac cell death is a major determinant of ventricular dysfunction and failure in dog dilated cardiomyopathy. Circ Res 89: 279–286

Cromheeke KM, Kockx MM, De Meyer GR, Bosmans JM, Bult H, Beelaerts WJ, Vrints CJ, Herman AG (1999) Inducible nitric oxide synthase colocalizes with signs of lipid oxidation/peroxidation in human atherosclerotic plaques. Cardiovasc Res 43: 744–754

Cross AH, Manning PT, Keeling RM, Schmidt RE, Misko TP (1998) Peroxynitrite formation within the central nervous system in active multiple sclerosis. J Neuroimmunol 88: 45–56

Crow JP (1999) Measurement and significance of free and protein-bound 3-nitrotyrosine, 3-chlorotyrosine, and free 3-nitro-4-hydroxyphenylacetic acid in biologic samples: a high-performance liquid chromatography method using electrochemical detection. Methods Enzymol 301: 151–160

Crow JP, Ischiropoulos H (1996) Detection and quantitation of nitrotyrosine residues in proteins: *in vivo* marker of peroxynitrite. Methods Enzymol 269: 185–194

Crowley JR, Yarasheski K, Leeuwenburgh C, Turk J, Heinecke JW (1998) Isotope dilution mass spectrometric quantification of 3-nitrotyrosine in proteins and tissues is facilitated by reduction to 3-aminotyrosine. Anal Biochem 259: 127–135

Cuzzocrea S, Zingarelli B, Villari D, Caputi AP, Longo G (1998) Evidence for *in vivo* peroxynitrite production in human chronic hepatitis. Life Sci 63: PL25–PL30

De Andrade JA, Crow JP, Viera L, Bruce Alexander C, Randall Young K, McGiffin DC, Zorn GL, Zhu S, Matalon S, Jackson RM (2000) Protein nitration, metabolites of reactive nitrogen species, and inflammation in lung allografts. Am J Respir Crit Care Med 161: 2035–2042

Delatour T, Guy PA, Stadler RH, Turesky RJ (2002a) 3-Nitrotyrosine butyl ester: a novel derivative to assess tyrosine nitration in rat plasma by liquid chromatography-tandem mass spectrometry detection. Anal Biochem 302: 10–18

Delatour T, Richoz J, Vouros P, Turesky R (2002b) Simultaneous determination of 3-nitrotyrosine and tyrosine in plasma proteins of rats and assessment of artifactual tyrosine nitration. J Chromatogr B Analyt Technol Biomed Life Sci 779: 189

Feng Q, Lu X, Jones DL, Shen J, Arnold JM (2001) Increased inducible nitric oxide synthase expression contributes to myocardial dysfunction and higher mortality after myocardial infarction in mice. Circulation 104: 700–704

Ferdinandy P, Danial H, Ambrus I, Rothery RA, Schulz R (2000) Peroxynitrite is a major contributor to cytokine-induced myocardial contractile failure. Circ Res 87: 241–247

Ferrante RJ, Hantraye P, Brouillet E, Beal MF (1999) Increased nitrotyrosine immunoreactivity in substantia nigra neurons in MPTP treated baboons is blocked by inhibition of neuronal nitric oxide synthase. Brain Res 823: 177–182

Forster C, Clark HB, Ross ME, Iadecola C (1999) Inducible nitric oxide synthase expression in human cerebral infarcts. Acta Neuropathol (Berl) 97: 215–220

Frost MT, Halliwell B, Moore KP (2000) Analysis of free and protein-bound nitrotyrosine in human plasma by a gas chromatography/mass spectrometry method that avoids nitration artifacts. Biochem J 345 Pt 3: 453–458

Garcia-Monzon C, Majano PL, Zubia I, Sanz P, Apolinario A, Moreno-Otero R (2000) Intrahepatic accumulation of nitrotyrosine in chronic viral hepatitis is associated with histological severity of liver disease. J Hepatol 32: 331–338

Gaut JP, Byun J, Tran HD, Heinecke JW (2002a) Artifact-free quantification of free 3-chlorotyrosine, 3-bromotyrosine, and 3-nitrotyrosine in human plasma by electron capture-negative chemical ionization gas chromatography mass spectrometry and liquid chromatography-electrospray ionization tandem mass spectrometry. Anal Biochem 300: 252–259

Gaut JP, Byun J, Tran HD, Lauber WM, Carroll JA, Hotchkiss RS, Belaaouaj A, Heinecke JW (2002b) Myeloperoxidase produces nitrating oxidants *in vivo*. J Clin Invest 109: 1311–1319

Giasson BI, Duda JE, Murray IV, Chen Q, Souza JM, Hurtig HI, Ischiropoulos H, Trojanowski JQ, Lee VM (2000) Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. Science 290: 985–989

Good PF, Hsu A, Werner P, Perl DP, Olanow CW (1998) Protein nitration in Parkinson's disease. J Neuropathol Exp Neurol 57: 338–342

Good PF, Werner P, Hsu A, Olanow CW, Perl DP (1996) Evidence of neuronal oxidative damage in Alzheimer's disease. Am J Pathol 149: 21–28

Green JM (1996) A practical guide to analytical method validation. Anal Chem 68: 305A–309A

Haddad IY, Pataki G, Hu P, Galliani C, Beckman JS, Matalon S (1994) Quantitation of nitrotyrosine levels in lung sections of patients and animals with acute lung injury. J Clin Invest 94: 2407–2413

Halliwell B, Zhao K, Whiteman M (1999) Nitric oxide and peroxynitrite. The ugly, the uglier and the not so good: a personal view of recent controversies. Free Radic Res 31: 651–669

Hanazawa T, Kharitonov SA, Barnes PJ (2000) Increased nitrotyrosine in exhaled breath condensate of patients with asthma. Am J Respir Crit Care Med 162: 1273–1276

Hansen PR, Holm AM, Svendsen UG, Olsen PS, Andersen CB (2000) Apoptosis and formation of peroxynitrite in the lungs of patients with obliterative bronchiolitis. J Heart Lung Transplant 19: 160–166

Hashimoto S, Takahashi K, Ochs RL, Coutts RD, Amiel D, Lotz M (1999) Nitric oxide production and apoptosis in cells of the meniscus during experimental osteoarthritis. Arthritis Rheum 42: 2123–2131

Hensley K, Maidt ML, Pye QN, Stewart CA, Wack M, Tabatabaie T, Floyd RA (1997) Quantitation of protein-bound 3-nitrotyrosine and 3,4-dihydroxyphenylalanine by high-performance liquid chromatography with electrochemical array detection [published erratum appears in Anal Biochem 1998 Feb 1; 256(1): 148]. Anal Biochem 251: 187–195

Hensley K, Maidt ML, Yu Z, Sang H, Markesbery WR, Floyd RA (1998) Electrochemical analysis of protein nitrotyrosine and dityrosine in the Alzheimer brain indicates region-specific accumulation. J Neurosci 18: 8126–8132

Hurst JK (2002) Whence nitrotyrosine? J Clin Invest 109: 1287–1289

Ishida N, Hasegawa T, Mukai K, Watanabe M, Nishino H (2002) Determination of nitrotyrosine by HPLC-ECD and its application. J Vet Med Sci 64: 401–404

Jiang H, Balazy M (1998) Detection of 3-nitrotyrosine in human platelets exposed to peroxynitrite by a new gas chromatography/mass spectrometry assay. Nitric Oxide 2: 350–359

Kamisaki Y, Wada K, Nakamoto K, Kishimoto Y, Kitano M, Itoh T (1996) Sensitive determination of nitrotyrosine in human plasma by isocratic high-performance liquid chromatography. J Chromatogr B Biomed Appl 685: 343–347

Kaur H, Halliwell B (1994) Evidence for nitric oxide-mediated oxidative damage in chronic inflammation. Nitrotyrosine in serum and synovial fluid from rheumatoid patients. FEBS Lett 350: 9–12

Kaur H, Lyras L, Jenner P, Halliwell B (1998) Artefacts in HPLC detection of 3-nitrotyrosine in human brain tissue. J Neurochem 70: 2220–2223

Khan J, Brennand DM, Bradley N, Gao B, Bruckdorfer R, Jacobs M, Brennan DM (1998) 3-Nitrotyrosine in the proteins of human plasma determined by an ELISA method [published erratum appears in Biochem J 1998 Jun 15; 332 (Pt 3): 808]. Biochem J 330: 795–801

Kharitonov SA, Barnes PJ (2002) Biomarkers of some pulmonary diseases in exhaled breath. Biomarkers 7: 1–32

Leeuwenburgh C, Hardy MM, Hazen SL, Wagner P, Oh IS, Steinbrecher UP, Heinecke JW (1997) Reactive nitrogen intermediates promote low density lipoprotein oxidation in human atherosclerotic intima. J Biol Chem 272: 1433–1436

Leeuwenburgh C, Hansen P, Shaish A, Holloszy JO, Heinecke JW (1998) Markers of protein oxidation by hydroxyl radical and reactive nitrogen species in tissues of aging rats. Am J Physiol 274: R453–R461

Liu D, Ling X, Wen J, Liu J (2000) The role of reactive nitrogen species in secondary spinal cord injury: formation of nitric oxide, peroxynitrite, and nitrated protein. J Neurochem 75: 2144–2154

Mihm MJ, Coyle CM, Schanbacher BL, Weinstein DM, Bauer JA (2001) Peroxynitrite induced nitration and inactivation of myofibrillar creatine kinase in experimental heart failure. Cardiovasc Res 49: 798–807

Morrissey B, Schilling K, Weil J, Silkoff P, Rodman D (2002) Nitric oxide and protein nitration in the cystic fibrosis airway. Arch Biochem Biophys 406: 33

Nag S, Picard P, Stewart DJ (2001) Expression of nitric oxide synthases and nitrotyrosine during blood-brain barrier breakdown and repair after cold injury. Lab Invest 81: 41–49

Ohshima H, Celan I, Chazotte L, Pignatelli B, Mower HF (1999) Analysis of 3-nitrotyrosine in biological fluids and protein hydrolyzates by high-performance liquid chromatography using a postseparation, on-line reduction column and electrochemical detection: results with various nitrating agents. Nitric Oxide 3: 132–141

Ohshima H, Friesen M, Brouet I, Bartsch H (1990) Nitrotyrosine as a new marker for endogenous nitrosation and nitration of proteins. Food Chem Toxicol 28: 647–652

Oldreive C, Bradley N, Bruckdorfer R, Rice-Evans C (2001) Lack of influence of dietary nitrate/nitrite on plasma nitrotyrosine levels measured using a competitive inhibition of binding ELISA assay. Free Radic Res 35: 377–386

Oleszak EL, Zaczynska E, Bhattacharjee M, Butunoi C, Legido A, Katsetos CD (1998) Inducible nitric oxide synthase and nitrotyrosine are found in monocytes/macrophages and/or astrocytes in acute, but not in chronic, multiple sclerosis. Clin Diagn Lab Immunol 5: 438–445

Pelletier JP, Lascau-Coman V, Jovanovic D, Fernandes JC, Manning P, Connor JR, Currie MG, Martel-Pelletier J (1999) Selective inhibition of inducible nitric oxide synthase in experimental osteoarthritis is associated with reduction in tissue levels of catabolic factors. J Rheumatol 26: 2002–2014

Pennathur S, Jackson-Lewis V, Przedborski S, Heinecke JW (1999) Mass spectrometric quantification of 3-nitrotyrosine, ortho-tyrosine, and o,o′-dityrosine in brain tissue of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice, a model of oxidative stress in Parkinson's disease. J Biol Chem 274: 34621–34628

Petruzzelli S, Puntoni R, Mimotti P, Pulera N, Baliva F, Fornai E, Giuntini C (1997) Plasma 3-nitrotyrosine in cigarette smokers. Am J Respir Crit Care Med 156: 1902–1907

Ravalli S, Albala A, Ming M, Szabolcs M, Barbone A, Michler RE, Cannon PJ (1998) Inducible nitric oxide synthase expression in smooth muscle cells and macrophages of human transplant coronary artery disease. Circulation 97: 2338–2345

Sakurai M, Fukuyama N, Iguchi A, Akimoto H, Ohmi M, Yokoyama H, Nakazawa H, Tabayashi K (1999) Quantitative analysis of cardiac 3-L-nitrotyrosine during acute allograft rejection in an experimental heart transplantation. Transplantation 68: 1818–1822

Schwedhelm E, Tsikas D, Gutzki FM, Frolich JC (1999) Gas chromatographic-tandem mass spectrometric quantification of free 3-nitrotyrosine in human plasma at the basal state. Anal Biochem 276: 195–203

Shigenaga MK (1999) Quantitation of protein-bound 3-nitrotyrosine by high-performance liquid chromatography with electrochemical detection. Methods Enzymol 301: 27–40

Shigenaga MK, Lee HH, Blount BC, Christen S, Shigeno ET, Yip H, Ames BN (1997) Inflammation and NO(X)-induced nitration: assay for 3-nitrotyrosine by HPLC with electrochemical detection. Proc Natl Acad Sci USA 94: 3211–3216

Singer II, Kawka DW, Scott S, Weidner JR, Mumford RA, Riehl TE, Stenson WF (1996) Expression of inducible nitric oxide synthase and nitrotyrosine in colonic epithelium in inflammatory bowel disease. Gastroenterology 111: 871–885

Smith MA, Richey HP, Sayre LM, Beckman JS, Perry G (1997) Widespread peroxynitrite-mediated damage in Alzheimer's disease. J Neurosci 17: 2653–2657

Szabolcs M, Michler RE, Yang X, Aji W, Roy D, Athan E, Sciacca RR, Minanov OP, Cannon PJ (1996) Apoptosis of cardiac myocytes during cardiac allograft rejection. Relation to induction of nitric oxide synthase. Circulation 94: 1665–1673

Szabolcs MJ, Ravalli S, Minanov O, Sciacca RR, Michler RE, Cannon PJ (1998) Apoptosis and increased expression of inducible nitric oxide synthase in human allograft rejection. Transplantation 65: 804–812

ter Steege JC, Koster-Kamphuis L, van Straaten EA, Forget PP, Buurman WA (1998) Nitrotyrosine in plasma of celiac disease patients as detected by a new sandwich ELISA. Free Radic Biol Med 25: 953–963

Teunissen CE, de Vente J, Steinbusch HW, De Bruijn C (2002) Biochemical markers related to Alzheimer's dementia in serum and cerebrospinal fluid. Neurobiol Aging 23: 485–508

Tohgi H, Abe T, Yamazaki K, Murata T, Ishizaki E, Isobe C (1999) Alterations of 3-nitrotyrosine concentration in the cerebrospinal fluid during aging and in patients with Alzheimer's disease. Neurosci Lett 269: 52–54

van der Veen RC, Hinton DR, Incardonna F, Hofman FM (1997) Extensive peroxynitrite activity during progressive stages of central nervous system inflammation. J Neuroimmunol 77: 1–7

Viera L, Ye YZ, Estevez AG, Beckman JS (1999) Immunohistochemical methods to detect nitrotyrosine. Methods Enzymol 301: 373–381

Yamaguchi Y, Okabe K, Matsumura F, Akizuki E, Matsuda T, Ohshiro H, Liang J, Yamada S, Mori K, Ogawa M (1999) Peroxynitrite formation during rat hepatic allograft rejection. Hepatology 29: 777–784

Yi D-H, Ingelse BA, Duncan MW, Smythe GA (2000) Quantification of 3-nitrotyrosine in biological tissues and fluids: generating valid results by eliminating artifactual formation. J Am Soc Mass Spectrom 11: 578–586

Zweier JL, Fertmann J, Wei G (2001) Nitric oxide and peroxynitrite in postischemic myocardium. Antioxid Redox Signal 3: 11–22

Authors' address: Mark W. Duncan, Box C238, Biochemical Mass Spectrometry Facility, University of Colorado Health Sciences Center, 4200 E. 9th Ave, Denver, CO 80262, U.S.A.,
Fax: (303) 315-0274, E-mail: mark.duncan@uchsc.edu