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Review

Chromatographic and mass spectrometric methods for quantitative determination of 3-nitrotyrosine in biological samples and their application to human samples☆

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Abstract

The permanent modification of soluble and protein-associated tyrosine by nitration results in the formation of 3-nitrotyrosine, which can be used as a marker of "nitro-oxidative" damage to proteins. Based on the analysis of patient materials, over 40 different diseases and/or conditions have been linked to increased nitration of tyrosine. They include many cardiovascular diseases, conditions associated with immunological reactions and neurological diseases. In this article we review the existing chromatographic and mass spectrometric methods for quantitative measurements of 3-nitrotyrosine in different human biological samples including plasma, either from the free amino acid pool or from hydrolyzed proteins from different matrices.

Keywords: Reviews; HPLC; Mass spectrometry; Plasma; Urine; Cerebrospinal fluid

Contents

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1. Introduction

Nitric oxide ($NO^{\bullet}$) is produced by nitric oxide synthases (NOS), which catalyze the conversion of L-arginine to L-citrulline and $NO^{\bullet}$. Three NOS isoforms exist: neuronal NOS (nNOS or NOS1), endothelial NOS (eNOS or NOS3) and an inducible form of NOS (iNOS or NOS2). The produced $NO^{\bullet}$ has important physiological roles in many different tissues and cell types [1]. Under normal conditions $NO^{\bullet}$ is an importat messenger molecule, e.g. in the nervous system and in the cardiovascular system [2,3]. Further, in certain situations large amounts of $NO^{\bullet}$ can be produced by iNOS as an immune response in enough quantities to generate nitrogen-containing reactive compounds [4]. Another immuno response is generation of superoxide ($O_2^{\bullet -}$) by NADPH oxidase and other enzymes including NOS (Fig. 1), and high concentrations may be maintained through enzymatic competition by $NO^{\bullet}$ for available superoxide dismutases. Both $O_2^{\bullet -}$ and $NO^{\bullet}$ have an unpaired electron, favouring generation of peroxynitrite, $ONOO^{-}$, a compound that readily reacts with different biomolecules [5].

The reaction between nitrogen-containing reactive compounds or radicals, including peroxynitrite and nitrogen dioxide (NO_2), and soluble tyrosine or tyrosyl residues in proteins results in a number of tyrosine modifications, of which tyrosine nitration is one of the most investigated [6]. Formally, tyrosine nitration is the permanent addition of a nitronium group ($^{+}NO_2$) at the *ortho*-position resulting in free or protein-associated 3-nitrotyrosine (3-NT) (Fig. 1), and should be distinguished from nitrosylation, which is the addition of a nitroso (^{+}NO) group to the hydroxyl group of tyrosine. Measurement of 3-NT in biological samples is of pivotal interest for several reasons. Firstly, the nitrative modification of tyrosine can be used as an indicator of the "nitro-oxidative" stress in a sample representative of a particular biological system. The species causing tyrosine nitration usually have very short half-lifes, and measuring them directly in the biological system is extremely difficult. On the other hand, 3-NT is a very stable and suitable analyte and may serve as a biomarker for these processes [5,6]. Secondly, the 3-NT is a relatively large and bulky amino acid, and tyrosine nitration may greatly change the chemical and biological properties of soluble tyrosine and tyrosine-containing proteins [7–9]. Measurements of 3-NT are therefore of interest in protein chemistry. Even though quantitative methods are almost exclusively restricted to the analysis of free 3-NT, the 3-NT content of proteins can also be determined after enzymatic or chemical hydrolysis of the proteins into the amino acids.

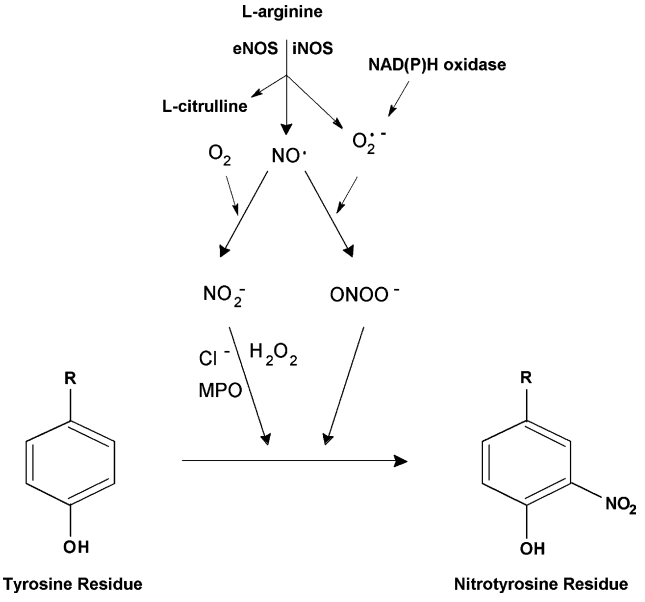

Fig. 1. Possible pathways for nitric oxide ($NO^{\bullet}$) and superoxide ($O_2^{\bullet -}$) mediated nitration of tyrosine. MPO: myeloperoxidase; $ONOO^-$: peroxynitrite. Residue R is $CH_2CH(NH_2)COOH$ in soluble tyrosine and 3-nitrotyrosine, and $CH_2CH(NH_2)CONH$-protein in proteins.

Formation of 3-NT in proteins can lead to altered protein conformation, solubility, susceptibility to aggregation and increased protein degradation [10,11]. Tyrosine nitration could also interfere with phosphorylation signaling since the nitro group is located near the phosphorylation site of tyrosine [12]. Initially, 3-NT was thought to be a unique modification of tyrosine by peroxynitrite, but more recent investigations have indicated a role for enzymes such as myeloperoxidase (MPO) [13] and eosinophil peroxidase [14] in the generation of 3-NT through the oxidation of nitrite to nitryl chloride or the formation of nitrogen dioxide (Fig. 1).

Many diseases have been identified as being associated with the increased nitration of proteins. On the basis of elevated 3-NT concentrations in patient materials, at least 40 different conditions have been suggested to be associated with elevated "nitro-oxidative" stress. To these conditions belong many cardiovascular diseases, such as myocardial inflammation [15], heart failure [16] and arteriosclerosis [17]. In addition, diseases associated with immunological reactions appear to be connected to a very high degree with the increased formation of tyrosine-nitrated proteins; they include asthma [18], systemic sclerosis [19], renal transplantation complications [20], inflammatory bowel disease [21], rheumatoid arthritis [22] and septic shock [23]. Neurological diseases are represented by amyotrophic lateral sclerosis [24], Alzheimer's disease [25], Parkinson's disease [26], multiple sclerosis [27], dementia with Lewy bodies [28] and meningitis [29], for example.

Since 3-NT was suggested as a biomarker of oxidative stress, a substantial effort has been made to develop analytical methods that can be used in biological matrices. The first approaches to be used included different immunological methods. A large part of all studies of 3-NT in biological samples have been performed using antibody-based methods as immunohistochemistry or Western blot [30]. Unfortunately, many of these assays have not been validated and therefore their accuracy, precision and limit of quantification (LOQ) are ill-defined. Several researchers have investigated the opportunity to analyze 3-NT in biological samples with chromatographic methods. The reported concentrations vary considerably and the establishment of reliable chromatographic methods has proven difficult for several reasons. The main reason is that an extremely sensitive and selective method is needed as the concentrations of 3-NT can be expected to be very low in biological samples. Even in pathological conditions the concentrations are below one nitrated tyrosine

of 10,000 tyrosines, which means that the concentrations are in the lower nM-range in most biological matrices. Most other compounds existing in the sample will therefore be present in concentrations that are several times higher. Another potential factor of interference is the handling of the samples which can cause artifactual nitration of tyrosine by nitrite and nitrate. In particular acidification, alone or in combination with an increased temperature, has been shown to cause the extensive generation of artifactual 3-NT in various biological matrices. This is probably due to the ubiquity of tyrosine, nitrate and nitrite in most biological matrices, both from endogenous and exogenous sources [30,31]. The claim for a sensitive, specific and artifact-free method will be justified and discussed in detail later in the review.

The purpose of this article is to review the analytical methods available for the quantification of 3-NT in human biological samples, with special emphasis given to the recent advances in mass spectrometry-based techniques, notably GC–MS/MS and LC–MS/MS. The application of these methods to quantify 3-NT in various matrices from humans is also reviewed. The use of mass spectrometry in proteomic approaches [32–35], which address rather qualitative aspects, will not be discussed in this review.

2. Methods of analysis

2.1. Liquid chromatographic methods using ultraviolet, fluorescence and electrochemical detection

The standard technique for analyzing amino acids at most laboratories is HPLC. For practical reasons it would be a logical strategy to extend these methods to the analysis of 3-NT. The most simple chromatographic method for 3-NT uses isocratic reversed phase HPLC and UV absorbance detection at 274 nm as reported first by Kaur and Halliwell [36]. This HPLC system uses an acidic mobile phase (pH 3) and has an LOD value of 0.2 μM. Variants of this method have been used in several studies to determine the 3-NT content in proteins [37,38] or the concentration of free 3-NT in biological matrices, such as human and rat plasma and rat urine [39–43]. The main drawback to this HPLC method is lack of selectivity and sensitivity. Furthermore, no validation data, apart from the LOD, have been reported for this HPLC-UV method. The LOD for 3-NT in HPLC methods has been lowered to 6 nM (100 fmol) by derivatizing 3-NT with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) and using fluorescence detection [44,45]. HPLC methods using electrochemical detection (ECD) have the potential to be more selective than HPLC-UV or HPLC-fluorescence methods. A number of methods, which are essentially variants of oxidative ECD with one electrode or an array of electrodes, have been reported. In oxidative ECD methods, a voltage at 700–1000 mV is usually employed to determine 3-NT content in proteins [46–48]. Use of ECD with an array of channels appears to lead to an improvement in selectivity, as information from several channels can be used for the interpretation. Two methods have used the CoulArray detector with eight channels applying voltages between 0 and +900 in steps of 100–150 V for the quantification of free 3-NT and 3-NT content in proteins [49,50]. By using redox ECD a higher degree of selectivity and sensitivity can be obtained, as the analyte is first reduced, using a voltage in the range of −800 to −1000 mV in the first cell, and then oxidized, using a voltage in the range of 250 to 1000 mV in the second cell. Redox ECD methods have been used to quantify free 3-NT and 3-NT content in proteins [51–53]. A similar, two-step method using photolysis instead of reduction in the first step, followed by oxidative ECD, has been reported to be both a selective and a sensitive way of detecting 3-NT [54]. One group has reported that oxidative ECD, in combination with a derivatization step, can be used for the quantification of 3-NT in proteins after enzymatic hydrolysis. To obtain higher selectivity and sensitivity, the analyte was acetylated and the nitro group reduced before HPLC analysis [55–58].

2.2. Liquid chromatographic-mass spectrometric methods

As mentioned above, one of the analytical challenges in the HPLC analysis of 3-NT in biological samples is to obtain the necessary selectivity. One straight-forward way to achieve this is to couple HPLC with a mass spectrometer. This combines the flexibility of the LC separation with the robust and unambiguous identification of the MS instrument, especially when used in the MS/MS mode. In a triple-stage quadrupole MS instrument operating in the MS/MS mode a specific ion is selected by the first quadrupole and fragmented in the second quadrupole to produce product ions, whereas the third quadrupole is used to select a characteristic product ion for detection. Analogous scanning and fragmentation techniques can also be performed in ion-trap instruments.

Electrospray ionization (ESI) of authentic 3-NT in the positive ion mode usually produces one intense protonated molecule, i.e. $[M+1]^+$, with a mass-to-charge (m/z) ratio of 227 [59]. Subjection of this precursor ion to collision-induced fragmentation usually yields an intense product ion at m/z 181 and other less intense ions such as at m/z 133. The ion at m/z 181 was first suggested to be due to the loss of the nitro group (46 Da) from 3-NT [60]. However, the fragmentation pattern obtained from stable-isotope labelled 3-NT suggests that the product ion at m/z 181 is rather a result of the loss of HCOOH (46 Da) from 3-NT [61].

Regularly, derivatization of analytes is not required in LC–MS, unlike in GC–MS. This is of particular importance in the analysis of 3-NT, because omission of a derivatization step may prevent artifactual formation of 3-NT. Most of the reported LC–MS/MS methods have LOD values in the order of 3–22 fmol of 3-NT. The first reported LC–MS/MS methods were performed on triple-stage quadrupole instruments using ESI. Prior to analysis solid-phase extraction (SPE) on RP materials was used for sample clean-up and amino acid concentration. The reported LOQ values in the biological samples were 10 fmol [60] for a method applied on an amino acid pool generated from rat micro vessels and 20 fmol on column (4.4 nM) for free 3-NT in human plasma [31]. The LOD and LOQ are inadequate to allow for the quantitative determination of free 3-NT in most biological samples, especially in human plasma. A similar technique for sample

extraction and separation in combination with an ion-trap instrument provided an LOD value of 1.6 nM (3.2 fmol) for free 3-NT in human plasma [62]. In a LC–MS/MS method, derivatization of 3-NT to its butyl ester, and SPE on aminopropyl material lowered the LOQ value of this methodology to 1.4 nM (0.23 pmol on column) for free 3-NT in rat plasma and the LOD for the method was 0.07 pmol on column [63]. One way to improve the selectivity and sensitivity seems to be to use Hypercarb HPLC columns in triple-stage quadrupole LC–ESI-MS/MS [64]. The Hypercarb material consists of porous spherical carbon particles, and the separation is based on the stronger retention for polar compounds. This technique was used to analyze a number of protein modifications in biological samples including 3-NT formation. A 12-kDa cut-off filter was used for sample extraction. The reported LOD for 3-NT was 0.2 nM and the method was applied to both an amino acid pool from human plasma proteins and for free 3-NT in human plasma [64]. Another triple-stage quadrupole LC–EPI-MS/MS technique used a Zorbax stable bond phenyl microbore column. The use of this column together with a concentration step resulted in an LOQ value of 0.02 nM for 3-NT in human breath condensate [65].

2.3. Gas chromatographic–mass spectrometric methods

Analytical methods using GC–MS are very useful to analyze small molecules with molecular masses of up to about 1000 Da. One requirement of the GC–MS methodology is that the analyte is volatile and thermally stable. Derivatization can improve both the volatility and thermal stability. Analysis of amino acids including 3-NT by GC–MS absolutely requires derivatization. It is not only the GC performance that can benefit from derivatization. By using proper, fluorine-containing derivatization agents and ionization conditions such as negative ion electron capture (NIEC), the signal-to-noise ratio (S/N) can be increased by several times over. All the GC–MS methods reviewed in the present article utilize such derivatization agents and electron-capture NIEC.

One of the first analytical GC–MS methods reported for 3-NT converted 3-NT into the *n*-propyl heptafluorobutyryl derivative. 3-NT was quantitated by selected-ion monitoring (SIM) of the ions at *m/z* 464 for the 3-NT and *m/z* 470 for 3-nitro-[$^{13}C_6$]-tyrosine which was used as the internal standard [66]. The LOD of this method was reported to be 1 nM (1 fmol injected). This GC–MS method was used to identify and quantify 3-NT in human plasma proteins after HCl-catalyzed hydrolysis and SPE extraction [66]. This method has also been used to analyze 3-NT in proteins from mouse brain [67] and rat heart and liver tissue [68]. The major drawback of this method is that 3-NT is not satisfactorily separated from tyrosine, nitrite and nitrate prior to GC–MS analysis or derivatization. The acidic conditions prevailing during the derivatization step in this method favor artifactual nitration of tyrosine. However, since this issue was never addressed in these publications, the possible influence on measurements cannot be determined.

One way to circumvent tyrosine nitration during sample processing is to reduce the nitro group of 3-NT to an amino group prior to the derivatization process. Analysis of 3-NT as 3-amino-tyrosine provides a mean to distinguish between 3-NT artifactually generated during the derivatization and endogenous 3-NT. In addition, conversion of 3-NT into 3-amino-tyrosine generates a second amino group which is accessible to derivatization by amino-group-specific agents. In one method 3-NT was measured as an *n*-propyl-heptafluorobutyryl derivative of 3-amino-tyrosine using 3-nitro-[$^{13}C_6$]-tyrosine as the internal standard and SPE on anion exchange material [69]. SIM of the ions at *m/z* 762 for 3-amino-tyrosine from 3-NT and of *m/z* 768 for 3-amino-[$^{13}C_6$]-tyrosine from reduced 3-nitro-[$^{13}C_6$]-tyrosine yielded an LOD value of 400 amol for 3-NT. The method has been used to quantify 3-NT in human LDL proteins [70] and in proteins from bronchial aspirates [71], each after HCl-catalyzed hydrolysis. The *n*-propyl ester-*N*,*N*,*O*-trisheptafluorobutyryl derivative has the advantage of containing 21 fluorine atoms which facilitate ionization, but it is unstable and hydrolyzes quickly in the presence of water. One consequence is decreased sensitivity of the method. Additionally, hydrolysis products containing HF can damage the GC column over time [72].

This problem has been solved by replacing the *O*-heptafluorobutyryl group by a methyl group. In addition, instead of the *n*-propyl ester the methyl ester of 3-amino-tyrosine group was prepared. The final derivative was di-*O*-methyl-di-*N*-heptafluorobutyryl derivative [73] (Fig. 2). This derivative (referred to as the *Söderling* derivative) showed chromatographic stability and ionization yielding one intense ion at *m/z* 576 for the 3-NT and the corresponding ion at *m/z* 582 for 3-nitro-[$^{13}C_6$]-tyrosine. Collision-induced dissociation of these ions produced mass spectra with intense product ions at *m/z* 545 and *m/z* 551, respectively (Fig. 3). Using these ion pairs in the selected reaction monitoring (SRM), we determined the LOD value of the method to be 30 amol of 3-NT (injected), whereas the LOQ values amounted to 0.3 nM of free 3-NT in human plasma [73]. This GC–MS/MS method was used to analyze free 3-NT in human plasma after extraction using 5 kDa cut-off filters and in human plasma proteins using enzyme-catalyzed hydrolysis and subsequent deproteination with 5 kDa cut-off filters. The conversion of 3-NT into 3-amino-tyrosine prior the derivatization was shown to be an efficient way to circumvent falsely high 3-NT concentrations from nitration of tyrosine during the derivatization process. This method has also been used to quantify free 3-NT in human cerebrospinal fluid [74] and 3-NT in exhaled breath condensate [75].

An alternative way to solve the problem associated with nitration of tyrosine during derivatization and other chromatographic steps is to separate 3-NT from tyrosine, nitrite and nitrate prior the derivatization. Schwedhelm et al. [76] suggested and applied HPLC for this purpose. This strategy was reported to effectively protect from artifactual nitration of tyrosine. Furthermore, a very high S/N ratio could be achieved by this method as numerous compounds from plasma were removed by HPLC. The 3-NT derivative used by this group is a variant of the *n*-propyl-heptafluorobutyryl derivative used previously by Leeuwenburgh et al. [66], with the exception that the phenolic hydroxyl group was converted into its trimethylsilyl ether derivative and that *N*-pentafluorobutyryl derivatives instead of

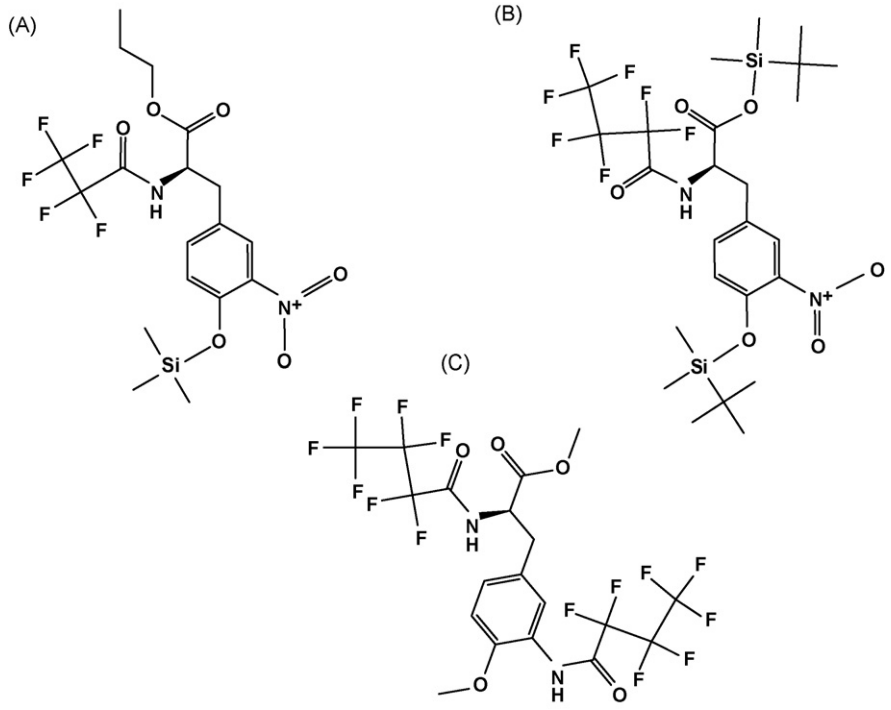

Fig. 2. Comparison of three different derivatives of 3-NT used in three different GC–MS methods: (A) an *N*-heptafluorobutyryl derivative and referred to as the *Schwedhelm* derivative; (B) an *N*-heptafluorobutyryl-*O*,*O*-di(*t*-butyldimethylsilyl) derivative referred to as the *Frost* derivative; (C) a di-*O*-methyl-di-*N*-heptafluorobutyryl derivative referred to as the *Söderling* derivative.

N-heptafluorobutyryl derivatives were prepared (referred to as the *Schwedhelm* derivative, Fig. 2). As the internal standard, 3-nitro-L-[$^{2}H_3$] tyrosine was used. Quantitative determination by GC–MS/MS in NIEC mode was performed by SRM of the product ions at *m/z* 382 and *m/z* 379, which were generated from the corresponding precursors at *m/z* 399 and *m/z* 396. The LOD of this method was reported to be 4 amol (injected amount), whereas the LOQ value was determined to be 0.125 nM of 3-nitro-L-[$^{2}H_3$] tyrosine added to human plasma. The method was used to quantify free 3-NT in human plasma utilizing 20-kDa cut-off filtering for generation of plasma ultrafiltrate and a SPE step to isolate the analytes from the respective HPLC fraction. This method has also been used to quantify 3-NT in serum albumin from human plasma after enzyme-catalyzed hydrolysis [77,78] and after modification free 3-NT in human urine [79]. Another group has also used HPLC for concentration and isolation of amino acids in protein hydrolysates prior to derivatization of 3-NT and the internal standard 3-nitro-[$^{13}C_6$]-tyrosine by pentafluorobenzyl bromide into their tri-pentafluorobenzyl derivatives [80]. The only validation data presented was LOD. Quantification of 3-NT in proteins from human platelets after HCl-hydrolysis was carried out by GC–MS in the NIEC mode and SIM of the carboxylate anions at *m/z* 585 for 3-NT and *m/z* 591 for 3-nitro-[$^{13}C_6$]-tyrosine.

One attempt to circumvent the nitration of tyrosine during sample derivatization has involved the use of milder derivatization conditions. Frost et al. [81] used ethyl heptafluorobutyrate and *N*-(*t*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (TBDMS) as the derivatization reagents to produce the *N*-heptafluorobutyryl-*O*,*O*-di(*t*-butyldimethylsilyl) derivatives (referred to as the *Frost* derivative, Fig. 2). Quantitative determination by GC–MS in the NIEC mode was performed by SIM of the ion at *m/z* 518 for 3-NT and *m/z* 527 for the internal standard 3-nitro-[$^{13}C_9$]-tyrosine. For sample extraction 30-kDa cut-off filters and SPE were used. Proteins were hydrolyzed under alkaline conditions (4 M NaOH), a procedure that should avoid nitration of tyrosine. The LOD value of this method was determined to be 1 pg (4.4 fmol, injected) of 3-NT. This method has been used to measure free 3-NT and 3-NT in proteins from human plasma [62,82,83], the 3-NT content in human HDL proteins [84], in rat plasma and liver proteins [85], and in mouse lung and plasma proteins [86].

In addition to the above mentioned derivatives for GC–MS and GC–MS/MS analyses other less frequently used derivatives have also been reported [9,47]. Thus, Yi et al. [31] prepared the trifluoroacyl/trifluoroethyl ester derivative, which allowed the detection of 1 fmol (injected) of 3-NT by SIM of the ions at *m/z* 404 for 3-NT and *m/z* 410 for 3-nitro-[$^{13}C_6$]-tyrosine by GC–MS in the NIEC mode. Morton et al. [17] prepared an oxazolinone derivative of 3-NT after its reduction to 3-aminotyrosine.

3. Applications to biological samples

3.1. Quantification of basal free 3-NT in human plasma

The concentrations of free 3-NT have been proposed to indicate "nitro-oxidative" stress. Despite considerable efforts, our knowledge of the role of 3-NT in proteins and the significance of circulating free 3-NT concentrations in humans is still very fragmentary. Thus, at this stage we have no consensus on circulating

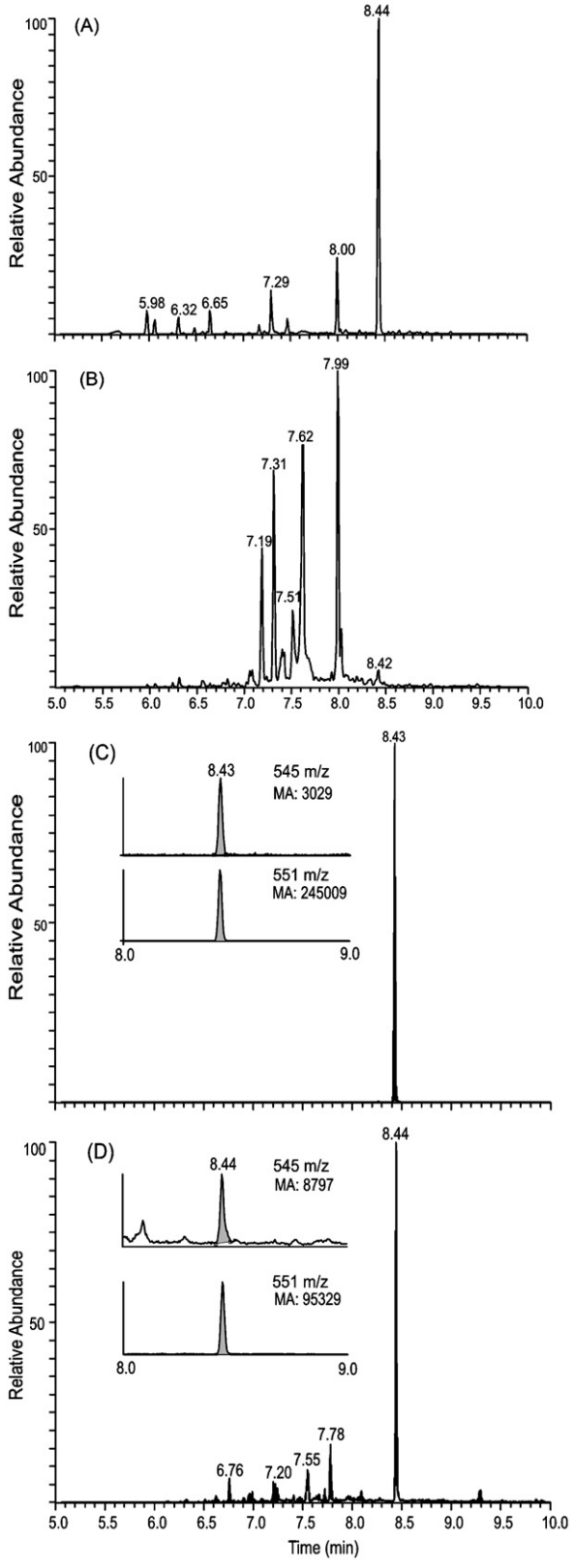

Fig. 3. Comparison of GC–MS and GC–MS/MS analysis of 3-NT in human plasma sample or in a water sample (from Söderling et al. [73]: A derivatization assay using gas chromatography/negative chemical ionization tandem mass spectrometry to quantify 3-nitrotyrosine in human plasma. Copyright John Wiley & Sons Limited 2003; reproduced with permission). (A) GC–MS SIM mode and (C) GC–MS/MS SRM mode total ion current (TIC) chromatograms of 3-nitro-[$^{13}C_6$]-tyrosine in non-ultrafiltered aqueous solution (12.5 nM). (B) GC–MS SIM mode and (D) GC–MS/MS SRM mode TIC chromatograms of 3-NT of in ultrafiltered human plasma sample spiked with 3-nitro-[$^{13}C_6$]-tyrosine (12.5 nM). Sample aliquots of 100 μl were derivatized and dissolved in 100-μl aliquots of acetonitrile, and 1-μl aliquots were loaded on the column. The ions (A, B) at *m/z* 582, 576, 551, and 545 and the product ions (C, D) of *m/z* 551, 545 were recorded consecutively for all runs.

free 3-NT concentrations, nor on their biological significance. The main reason for this is the difficulty to establish reliable and validated methods for quantitative measurements of free 3-NT in complex biological matrices such as plasma or serum samples from humans. The reliability of the measurements can be compromised by a number of factors such as falsely high concentrations due to artifactual nitration of tyrosine during sample processing and lack of selectivity and specificity. Comprehensive reviews and discussion of analytical problems associated with the quantitative determination of 3-NT have been published recently [30,87].

The determination of 3-NT concentrations in human plasma is of special interest as this biological matrix is one of the best investigated and one of the most frequently used in the clinic. Further, human plasma is widely available and can serve as a suitable matrix for method development and validation. To date, there are approximately 19 studies reporting on free 3-NT concentrations in human plasma (Table 1). A closer examination of the reported data provides some interesting information. The concentrations reported vary considerably between different methods, with the difference between the highest and lowest reported concentration being several orders of magnitude. There is a clear tendency that methodologies with higher inherent selectivity such as LC–MS/MS and GC–MS/MS have provided considerably lower basal concentrations of 3-NT compared to less selective methodologies such as HPLC-UV and GC–MS. Thus, selectivity is a decisive factor for method reliability. This issue has also been addressed previously [73,77,87]. One of the most sensitive methods, i.e. GC–MS, has generated one of the highest reported 3-NT concentrations [81]. Consequently, sensitivity per se is not a guarantee for the generation of reliable results for 3-NT.

Another interesting notion is that the same analytical method generated basal 3-NT concentrations that differ by a factor higher then 10, when applied to different studies or used by different researchers, despite low inter-batch variation. Thus, one group reported that basal concentrations of 3-NT in human plasma amount to 64 nM ($n = 8$) [81]. Using the same GC–MS method, another study from the same research group reported 3-NT basal concentrations of 5.44 nM ($n = 29$) [83]. It is not clear whether this variation is due to an inherent problem of the method such as lack of selectivity or due to other factors such as difference in sample handling. By using a LC–MS/MS method, another group reported 3-NT basal concentrations at 6.5 nM [64] and 9.4 nM [88], while later it was reported that 3-NT concentrations were below 0.4 nM [89], i.e. the LOQ of the method. The latter is especially surprising since the LC–MS/MS methodology is considered to be selective with respect to 3-NT.

Table 1
Summary of reported basal concentrations of free 3-NT in human plasma using different chromatographic and mass spectrometric methods and their sensitivity values

Method	LOQ (nM)	3-NT concentration (nM)	Number of subjects	Reference
Hitachi amino acid analyzer	100	<100	10	[109]
HPLC-Fluorescence	100 fmol	31 ± 6	9	[44]
HPLC-UV 355 nm	Not stated	14 ± 6	9	[23]
HPLC-UV 274 nm	200 nM (LOD)	5300 ± 400	19	[110]
HPLC UV 274 nm	600 nM (LOD)	<600 (LOD)	10	[40]
HPLC-ECD	0.1 pmol (LOD)	<5 (LOD)	5	[52]
LC–MS/MS	4.4	<4.4	5	[31]
LC–MS/MS	3.2 fmol (LOD)	<1.6 (LOD)	3	[62]
LC–MS/MS	Not stated	0.6 ± 0.04	10	[90]
LC–MS/MS, Hypercarb column	0.022 pmol (LOD)	6.5 ± 2.5	5	[64]
LC–MS/MS, Hypercarb column	0.022 pmol (LOD)	9.4 ± 0.4	21	[88]
LC–MS/MS, Hypercarb column	0.022 pmol (LOD)	<0.4 (LOQ)	12	[89]
LC–MS/MS	100 fmol (LOD)	1	1	[111]
GC–MS, *Frost* derivative	0.004 fmol (LOD)	64 ± 3	8	[81]
GC–MS, *Frost* derivative	0.07 fmol (LOD)	11 ± 2	3	[62]
GC–MS, *Frost* derivative	0.004 fmol (LOD)	5.44 ± 1.19	29	[83]
GC–MS/MS, *Schwedhelm* derivative	0.125	2.8 ± 0.84	11	[76]
GC–MS/MS, *Schwedhelm* derivative	0.125	0.73 ± 0.53	18	[77]
GC–MS/MS, *Schwedhelm* derivative	0.125	0.64 ± 0.15	20	[78]
GC–MS/MS, *Söderling* derivative	0.3	0.74 ± 0.6	12	[73]

Two different methods using GC–MS/MS have been reported free 3-NT concentrations in human plasma in the range 0.64–2.8 nM in four different studies [73,76–78]. One method uses HPLC fractioning prior to derivatization and the other uses the reduction of the nitro-group, followed by a derivatization step that also targets the aromatic amino group. These steps minimize the risk of falsely high concentrations due to the nitration of tyrosines during sample processing, but the steps also contribute with extra selectivity to the methods. The concentrations measured by GC–MS/MS for free 3-NT in human plasma below 3 nM are supported by a LC–MS/MS method which reported basal 3-NT concentrations of about 0.7 nM [90], and indirectly by HPLC-ECD and LC–MS/MS methods which were not able to detect 3-NT concentrations below the respective LOQ values (or LOD if LOQ was not reported), corresponding to concentrations in the lower nM-range. Thereby, it has been stated that the concentrations should be lower than 5 nM [52], 4.4 nM [31], and even 1.6 nM [62].

3.2. Quantification of free 3-NT in human urine

Urine provides a source of samples in which biomarkers can be measured non-invasively. Therefore, free 3-NT in urine could serve as a practical marker of "nitro-oxidative" stress in different conditions. However, only very scant and highly diverging data have been reported for free 3-NT in human urine, so that the significance of urinary 3-NT is largely uncertain.

The first reported method used to measure basal concentrations of free 3-NT in human urine was GC with flame ionization detection (FID). 3-NT was extracted from the urine with SPE and derivatized using *N*-(*t*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide. Apart from linearity and LOD (8.8 nM), no validation data have been provided for this method. The mean excretion rate of 3-NT was measured at 248 ± 15 nmol/day in 10 healthy subjects [91]. Another method utilized HPLC-UV (274 nM) for the measurement of urinary 3-NT. The urine samples were first filtered with a 30-kDa cut-off filter, followed by a 3-kDa cut-off filter prior to analysis. The LOD of this method was determined as 0.12 μM. Within- and between-assay precision for the method have also been reported. With this method the basal concentration of free 3-NT in urine from 20 healthy individuals was measured to be 5.5 μM corresponding to a creatinine-corrected excretion rate of 11.3 μmol/mmol creatinine [92]. These concentrations should be compared with the concentrations provided by mass spectrometric methods. By using a triple-stage quadrupole LC–MS/MS method with ESI and Hypercarb columns the concentration of free 3-NT in human urine was determined to be 38 ± 9 nM in 6 healthy subjects [64]. Using the same LC–MS/MS method the 3-NT excretion rate was determined as 3.84 nmol/mmol creatinine in 12 healthy subjects [89]. Even considerably lower urinary concentrations of 8.4 ± 10.4 nM and excretion rates of 0.46 ± 0.49 nmol/mmol creatinine for 3-NT were determined using a GC–MS/MS method, which had been previously applied to plasma 3-NT [79] (Fig. 4). The highest (5.5 μM) and the lowest (8.4 nM) concentrations of 3-NT determined in human urine by HPLC-UV and GC–MS/MS, respectively, therefore differ by a factor of 650 in healthy subjects.

3.3. Quantification of free 3-NT in human cerebrospinal fluid

The cerebrospinal fluid (CSF) is a clear fluid that fills the ventricles and surrounds the brain. The fluid can be sampled by

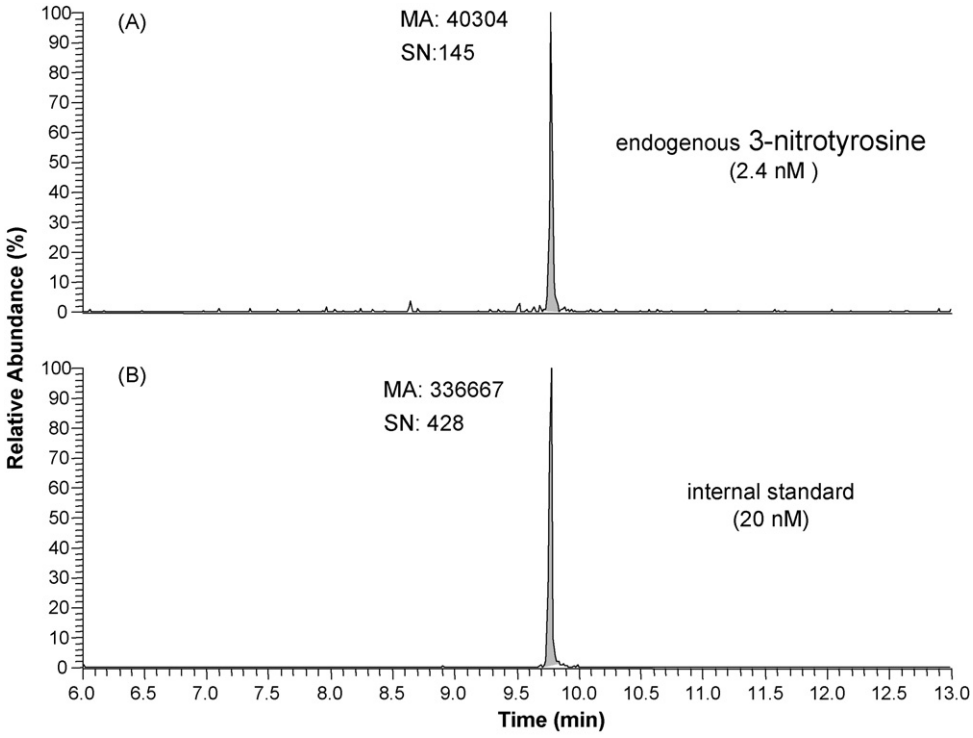

Fig. 4. GC–MS/MS analysis of 3-NT in human urine as described in ref. [79] (by courtesy of D. Tsikas). The human urine samples were spiked with 20 nM of 3-nitro-L-[$^2H_3$]-tyrosine (internal standard) and fractionated using HPLC prior to derivatization. The 3-NT was derivatized into an *n*-propyl-pentafluoropropionyltrimethylsilyl ether derivative and the analyses were performed in the electron capture negative-ion chemical ionization mode on a triple-stage quadrupole mass spectrometer (ThermoQuest TSQ 7000). Quantification by GC–MS/MS was performed by selected-reaction monitoring (SRM) of the characteristic product ions at *m/z* 379 and 382, which were obtained by collision-activated dissociation of the parent ions at *m/z* 396 and 399 of the derivatives. Chromatogram A is the endogenous 3-NT in the urine sample and was recorded using the *m/z* 379 product ion (from *m/z* 396 parent ion). Chromatogram B is the 3-nitro-L-[$^2H_3$]-tyrosine internal standard and was recorded using the *m/z* 382 product ion (from *m/z* 399 parent ion).

a harmless procedure called lumbar puncture and the withdrawn sample can be used for biochemical analyses. Analyses of this kind have proven very useful in the diagnosis of a large number of diseases or for obtaining other important information regarding the chemical or biological state of the central nervous system [93]. A few studies have investigated the usefulness of free 3-NT as a biomarker in human CSF (Table 2). One group used HPLC-ECD for measurements of 3-NT in the CSF of healthy controls and patients with the neurodegenerative disorders amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD) [94,95]. Apart from the LOD of the method for 3-NT, which was 0.2 nM, no other validation data were presented in these articles. It was reported that the basal concentrations of free 3-NT in human CSF were 1.4 ± 0.7 nM ($n = 19$) [95], and 1.6 ± 0.4 ($n = 24$) nM in another study [94]. 3-NT concentrations were determined to be 9.0 ± 4.2 nM in ALS patients ($n = 19$) [95], and 11.4 ± 5.4 nM in AD patients ($n = 25$) [94]. We applied a validated GC–MS/MS method, which has been previously applied to plasma 3-NT [73], to measure free 3-NT in human CSF from healthy controls and found 3-NT at concentrations of 0.35 ± 0.019 nM ($n = 19$) [74]. Application of the method to CSF of patients provided 3-NT concentrations of 0.38 ± 0.034 nM in ALS patients ($n = 14$) and 0.44 ± 0.031 nM in AD patients ($n = 17$). Another research group used LC–MS/MS to quantify the concentrations of free 3-NT in the CSF. The 3-NT concentrations determined were 0.4 ± 0.28 nM ($n = 18$) for healthy controls and 1.03 ± 0.46 nM ($n = 32$) for patients with AD [96]. Thus, the basal concentrations of free 3-NT in CSF measured by GC–MS/MS and LC–MS/MS were 4 times lower in healthy humans and 11 times [96] to 25

Table 2
Summary of reported concentrations of free 3-NT in human cerebrospinal fluid (CSF) samples using different chromatographic and mass spectrometric methods

Method	Number of subjects	3-NT concentration (nM)	Reference
Controls			
HPLC-ECD	19	1.4 ± 0.7	[95]
HPLC-ECD	24	1.6 ± 0.4	[94]
GC–MS/MS	19	0.35 ± 0.02	[74]
LC–MS/MS	18	0.4 ± 0.28	[96]
ALS			
HPLC-ECD	19	9.0 ± 4.2	[95]
GC–MS/MS	14	0.38 ± 0.03	[74]
AD			
HPLC-ECD	25	11.4 ± 5.4	[94]
GC–MS/MS	17	0.44 ± 0.03	[74]
LC–MS/MS	35	1.03 ± 0.4	[96]

Controls are healthy normal subjects, ALS group is CSF from patients with amyotrophic lateral sclerosis and AD group is CSF from patients with Alzheimer's disease.

times [74] lower in patients as compared to those provided by the HPLC-ECD method.

3.4. Quantification of free 3-NT in human breath condensate

Efforts have been made to follow treatment effects in respiratory diseases by monitoring general or airway inflammation. Exhaled $NO^{\bullet}$ has attained much interest, but studies so far have failed to prove reduced exacerbation rate when steering prevention by this measure [97]. Other biomarkers of disease activity are therefore sought [98], and oxidative stress markers in breath condensate constitute candidates, among these 8-isoprostane [99–101], H_2O_2 [99,102,103] and 3-NT [65,75,104–107]. Early studies detected 3-NT, by using an enzyme immunoassay considered to be specific for free 3-NT, in breath condensate of normal subjects (6.3 ± 0.8 ng/ml or about 28 nM), 2.4 times higher concentrations with mild (but not with more advanced) asthma [107], and 4 times higher concentrations with cystic fibrosis [104]. However, LC–MS/MS with an LOQ of 3.9 pg/ml (17 pM) has shown concentrations between LOQ and 184 pg/ml (0.81 nM) in normals with no difference between smokers and nonsmokers [65]. Applying GC–MS/MS, with LOD of 3 amol (per μl sample injected) and LOQ 1.7 pM, we found concentrations of 31 pM with no difference between controls and asthmatic patients [75]. Thus, LC–MS/MS and GC–MS/MS yield distinctly lower concentrations of 3-NT in breath condensate than those detected by immunoassays, which indicates a potential overestimation by immunoassays. Recent data also confirm that GC–MS in the NIEC mode is not sufficient to detect breath condensate differences between normal children and those with asthma or cystic fibrosis [106]. LC–MS/MS was capable, however, when calculating the 3-NT/tyrosine ratio, to discriminate between asthmatic children (five times higher concentrations) and normal children [105]. A relation between 3-NT and exhaled $NO^{\bullet}$ was detected by immunoassay [107], but not with GC–MS/MS or LC–MS/MS techniques [75,105]. One hypothesis might be that the immunoassay is less specific and detects not only 3-NT but also inflammatory components with a closer relation to $NO^{\bullet}$ production. Evidently, further studies are needed to define the role of 3-NT as biomarker in respiratory disease.

3.5. Quantification of free 3-NT in protein hydrolysates from human samples

Chromatographic and mass spectrometric methods have been applied to analyze 3-NT in hydrolysates generated from proteins by acid-, base- or enzyme-catalyzed hydrolysis. For this purpose e.g. HCl acid, NaOH solutions or a cocktail of unspecific proteases have been used. 3-NT concentrations in plasma proteins from healthy subjects have been reported by various groups (Table 3). Two of these studies measured 3-NT concentrations in human serum albumin after affinity extraction and enzymatic hydrolysis. There are apparent discrepancies between the 3-NT values provided by the MS/MS methodology and those obtained from the application of the MS methodology or HPLC with fluorescence detection. Two different LC–MS/MS methods and two different GC–MS/MS methods have reported consistent 3-NT concentrations in human plasma proteins in the range of 0.17–0.8 pmol/mg protein [73,77,78,89,108]. Four studies using the GC–MS technique have reported 3-NT concentrations in the range of 2.2–11.9 pmol/mg protein [44,81–84], i.e. 3 to 34 times higher compared with the MS/MS technique (see Table 3). The study using HPLC with fluorescence detection reported concentrations above 28 pmol/mg protein, which is more than 35 times higher than those obtained by MS/MS methods.

Some studies have also reported on the content of 3-NT in human lipoproteins in atherosclerosis. Using a GC–MS method, the content of 3-NT in circulating low density lipoprotein in atherosclerosis patients was determined as 9 ± 7 μmol/mol tyrosine ($n = 6$) or 2.5 ± 1.9 pmol/mg protein [66]. In the atherosclerotic tissue, the concentrations of 3-NT in the same proteins were 840 ± 140 μmol 3-NT/mol tyrosine ($n = 10$, 232 ± 38.7 pmol 3-NT/mg protein). A similar study determined the content of 3-NT in high density lipoprotein in atherosclerosis [84]. Using a GC–MS method the circulating concentrations in control subjects was determined to be 57 ± 10 μmol 3-NT/mol tyrosine ($n = 5$, 15.75 ± 2.76 pmol/mg protein). Circulating con-

Table 3
Summary of reported basal content for 3-NT in human plasma proteins using different chromatographic and mass spectrometric methods

Method	Reported content, number of subjects	Re-calculated as pmol/mg protein	Kind of hydrolysis	Reference
HPLC-fluorescence	0.01–0.07% of tyrosine, $n = 9$	28–193	HCl hydrolysis	[44]
GC–MS	8 ± 6 μmol 3-NT/mol tyrosine, $n = 5$	2.2 ± 1.7	Sulfonic acid hydrolysis	[84]
GC–MS	1.78 ± 0.21 ng 3-NT/mg protein, $n = 23$	7.9 ± 0.93	NaOH hydrolysis	[82]
LC–MS/MS	0.6 μmol 3-NT/mol tyrosine, $n = 12$	0.17 ± 0.083	Enzymatic hydrolysis	[89]
LC–MS/MS	1–4 ng 3-NT/mg tyrosine, $n = 22$	0.2–0.8	Enzymatic hydrolysis	[108]
GC–MS	1.31 ± 0.14 ng 3-NT/mg protein, $n = 29$	5.8 ± 0.62	NaOH hydrolysis	[83]
GC–MS/MS	0.60 ± 0.41 pmol/mg protein, $n = 12$	0.60 ± 0.41	Enzymatic hydrolysis	[73]
GC–MS	2.7 ± 0.4 ng 3-NT/mg protein, $n = 8$	11.9 ± 1.77	NaOH hydrolysis	[81]
GC–MS/MS	1.55 ± 0.54 3-NT/10^6 tyrosine, $n = 18$	0.44 ± 0.15	Enzymatic hydrolysis[a]	[77]
GC–MS/MS	1.21 ± 0.57 3-NT/10^6 tyrosine, $n = 20$	0.34 ± 0.16	Enzymatic hydrolysis[a]	[78]

Note: In some calculations it was assumed that plasma proteins contain 0.05 mg tyrosine per mg protein used.
[a] In these studies 3-NT levels were measured in serum albumin extracted from human plasma by affinity column extraction.

centrations in atherosclerosis patients were reported to be 104 ± 39 μmol 3-NT/mol tyrosine ($n = 17$, 28.7 ± 10.8 pmol 3-NT/mg protein), whereas the concentrations in the atherosclerotic tissue were determined to be 619 ± 178 μmol 3-NT/mol tyrosine (171 ± 49.2 pmol 3-NT/mg protein). One study used HPLC-UV for the measurement of 3-NT in total proteins from control and atherosclerotic vessels [37]. The concentrations were reported to be $15{,}800 \pm 2500$ pmol 3-NT/mg protein ($n = 10$) in the control group and $46{,}600 \pm 23{,}300$ pmol 3-NT/mg protein in the atherosclerotic tissue. Thus, HPLC-UV yielded 200 times higher values for 3-NT as compared to GC–MS.

Finally, another study has reported on the content of 3-NT in brain tissue as measured by the HPLC-ECD technique [50]. The concentrations reported were 0.5–4 mmol 3-NT/mol tyrosine (138–552 pmol 3-NT/mg protein) in the control subjects and 0.5–16 mmol 3-NT/mol tyrosine ($n = 11$, 138–2210 pmol 3-NT/mg protein) in Alzheimer's disease patients.

4. Conclusions

Protein nitration might be an important mechanism in cell death in conditions with an increased production of $NO^{\bullet}$ and superoxide. Our understanding of the biological significance of the nitration of proteins is largely dependent on reliable analytical methods for the identification and quantification of 3-NT. Availability of such methods is absolutely required to prove the utility of 3-NT as a marker for "nitro-oxidative" stress. In this article we reviewed and discussed published chromatographic and mass spectrometric methods available to date for the quantitative analysis of 3-NT. The application of these methods to different biological samples from humans including plasma, urine, breath condensate and cerebrospinal fluid samples were also reviewed and discussed.

In agreement with two recent review articles on 3-NT [30,87], we ascertained that reported basal concentrations for 3-NT in a certain biological matrix vary up to a factor of 1000 between different methods. Such large differences demand plausible and convincing explanations. Differences in the quality of the samples or in the composition of the study groups may contribute to varying 3-NT concentrations, but fail to explain divergences of three orders of magnitude. By contrast, artifactual nitration of tyrosine by ubiquitous nitrate and nitrite under low pH conditions has previously been shown to produce falsely high concentrations [31], and may, at least in part, account for the high concentrations of 3-NT concentrations measured by some methods. However, our conclusion, after comparing the basal concentrations of free 3-NT and 3-NT in proteins from different sample types such as human plasma, CSF and urine, is that the selectivity of the analytical method is a very important factor, which decisively determines the reliability of the analytical result. This review clearly reveals that analytical methods involving methodologies of inherent and undisputable selectivity, i.e. LC–MS/MS and GC–MS/MS, have constantly provided lower basal concentrations for 3-NT than analytical methods that are based on methodologies lacking selectivity, i.e. GC, HPLC-UV, and GC–MS.

There is increasing evidence that the plasma concentrations of free 3-NT in healthy humans are of the order 1 nM. This very low concentration indicates that high sensitivity, i.e. very low LOD and LOQ values, is a further important analytical factor which must be provided by the analytical method intended for the quantitative determination of 3-NT in human plasma and other biological samples. Thus, besides avoidance of artifactual formation of 3-NT during sample treatment and method selectivity, satisfactory sensitivity and precision are further requirements for the reliable quantitative determination of 3-NT in biological samples.

In recent years, considerable chromatographic and instrumental advances have been achieved, especially in the LC–MS/MS technology. Thus, the LOD of the LC–MS/MS methodology for 3-NT has been improved by a factor of about 10. This great improvement makes a derivatization step of 3-NT superfluous, thus eliminating the potential for artifactual formation of 3-NT. For methods using liquid chromatography, column materials with improved chromatographic properties have been developed. Thus, porous carbon and stable bond phenyl have been reported to allow higher resolution. Also derivatization techniques have improved. By utilizing the specific chemical and chromatographic properties of 3-NT, at least two different strategies have been developed and used to avoid and minimize artifactual formation of 3-NT during derivatization, thus minimizing the risk for falsely high concentrations.

Accurate quantitative determination of 3-NT in plasma and other biological samples is an analytical challenge. It requires the application of sophisticated analytical procedures and demanding instrumental techniques allowing specific and sensitive detection. At present, LC–MS/MS and GC–MS/MS performed on triple-stage quadrupole instruments or instruments with similar features represent the most useful methodologies for the reliable analysis of 3-NT in biological samples. The highly diverging and contradictory data available to date for 3-NT do not yet provide conclusive evidence for the suitability of 3-NT as a biomarker of oxidative stress. However, recent determinations suggest that 3-NT is normally present and detectable in human samples, but at low concentrations indicating that only few of available tyrosine residues are nitrated. The importance of specific and unspecific nitration of tyrosines, under normal circumstances as well as in different pathological situations, is still unsettled. To what extent nitration/denitration constitutes a regulatory mechanism remains to be proven by protein site specific techniques not discussed in this review. Further investigations applying the most sensitive and specific techniques in various conditions are necessary to establish 3-NT as an indicator of the extent of oxidative stress. At present, LC–MS/MS and GC–MS/MS seem to be best suited for such studies.

Hopefully, the present review provides satisfactory information on the analytical methods currently available for 3-NT, sensitizes the reader for particular problems in the analysis of 3-NT, and helps the reader both in interpreting published analytical and clinical data and in choosing the most appropriate analytical approach for use in future studies on 3-NT.

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