Analysis of free and protein-bound nitrotyrosine in human plasma by a gas chromatography/mass spectrometry method that avoids nitration artifacts

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Measurement of nitrotyrosine in biological fluids and tissues is increasingly being used to monitor the production of reactive nitrogen species *in vivo*. The detection of nitrotyrosine *in vivo* has been reported with the use of a variety of methods including immunoassay, HPLC and GLC/MS. The validity of HPLC and immunoassays have been questioned with regard to their selectivity and sensitivity limits. In principle, the measurement of nitrotyrosine by GLC/MS permits a highly specific, highly sensitive and fully quantitative assay. The nitration of tyrosine under acidic conditions in the presence of nitrite is well documented. Derivatization for the full quantification of nitrotyrosine by using GLC/MS can lead to the artifactual nitration of tyrosine if performed under acidic conditions in the presence of nitrite. We describe a novel alkaline method for the hydrolysis and derivatization of nitrotyrosine and tyrosine, and demonstrate its applicability to the measurement of plasma concentrations of both free and protein-bound nitrotyrosine and tyrosine. A detection limit of 1 pg for nitrotyrosine and 100 pg for tyrosine has been achieved. Our method allows, for the first time, the analysis of free and protein-bound nitrotyrosine and tyrosine in biological samples. The plasma concentrations (means ± S.E.M.) of free tyrosine and nitrotyrosine in eight normal subjects were 12 ± 0.6 μg/ml and 14 ± 0.7 ng/ml respectively. Plasma proteins contained tyrosine and nitrotyrosine at 60.7 ± 1.7 μg/mg and 2.7 ± 0.4 ng/mg respectively.

Key words: alkaline hydrolysis, nitric oxide, oxidative stress, reactive-nitrogen species, tyrosine.

INTRODUCTION

Tyrosine nitration might have important consequences for cell function [1], and the measurement of nitrotyrosine in biological fluids and tissues is increasingly being used to monitor the production of reactive nitrogen species *in vivo* [2–4]. Initially it was believed that nitrotyrosine was formed almost exclusively by the reaction of tyrosine with peroxynitrite, a potent oxidant formed by the reaction of nitric oxide and the superoxide anion [5]. However, it has increasingly become recognized that other reactions, for example those involving hypochlorous acid and peroxidase enzymes, might give rise to nitration reactions *in vivo* [6].

The tissue localization and semiquantitative estimation of nitrotyrosine has been performed extensively by immunohistochemistry. For example, increased staining of nitrotyrosine has been observed in renal allograft rejection, chronic hepatitis C, atherosclerotic plaques and inflammatory bowel disease [7]. However, whereas immunological methods provide data on the localization of nitrotyrosine, analytical methods are necessary for an accurate quantification of the levels found. The quantitative estimation of nitrotyrosine (either free or bound to proteins) by HPLC and UV detection is too insensitive to measure the low concentrations found in many fluids or tissues and has been found to be influenced by artifacts [8]. Significant amounts of free nitrotyrosine have been reported in body fluids from patients with rheumatoid arthritis [9] and in tissues from lesions of amyotrophic lateral sclerotics [10]. To monitor the formation of nitrotyrosine requires an accurate and reproducible method for its determination that can be applied to plasma and tissue (if available). There has been one report of free nitrotyrosine concentration in plasma [11] and only a few reports of the plasma concentrations of protein-bound nitrotyrosine measured by immunoassay [12,13].

The determination of plasma nitrotyrosine concentrations by GLC/MS should be the ideal method. There are several potential problems associated with this approach. Analysis of nitrotyrosine as the *N*-(t-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (TBDMS) derivative, in which all three reactive groups (NH_2, CO_2H and OH) are silylated by TBDMS and the fragment ions are measured by positive electron-impact MS, lacks the sensitivity required to quantify the low levels found in plasma (M. T. Frost, K. P. Moore and B. Halliwell, unpublished work). The most sensitive method, recently described for measurement in tissue [14], in which the negative-ion chemical-ionization (NICI) method is used, has the disadvantage that most derivatization reactions are performed under extremely acidic conditions in which nitrite, always present in biological samples, can cause the nitration of tyrosine [15]. This problem can be exacerbated during the analysis of samples obtained from patients with increased synthesis of nitric oxide.

To circumvent these problems we have developed a method for the hydrolysis of protein and the subsequent derivatization of nitrotyrosine and tyrosine under alkaline or neutral conditions. By using this method, which incorporates an internal control to detect the nitration of tyrosine as an artifact, we have measured the concentration of free and protein-bound nitrotyrosine in plasma and plasma proteins in normal volunteers.

MATERIALS AND METHODS

Chemicals

All chemicals, including [2,3,5,6-^{2}H]tyrosine, were purchased from Sigma Aldrich (Poole, Dorset, U.K.) unless stated other-

Abbreviations used: NICI, negative-ion chemical-ionization; TBDMS, *N*-(t-butyldimethylsilyl)-*N*-methyltrifluoroacetamide; TFA, trifluoroacetic acid.

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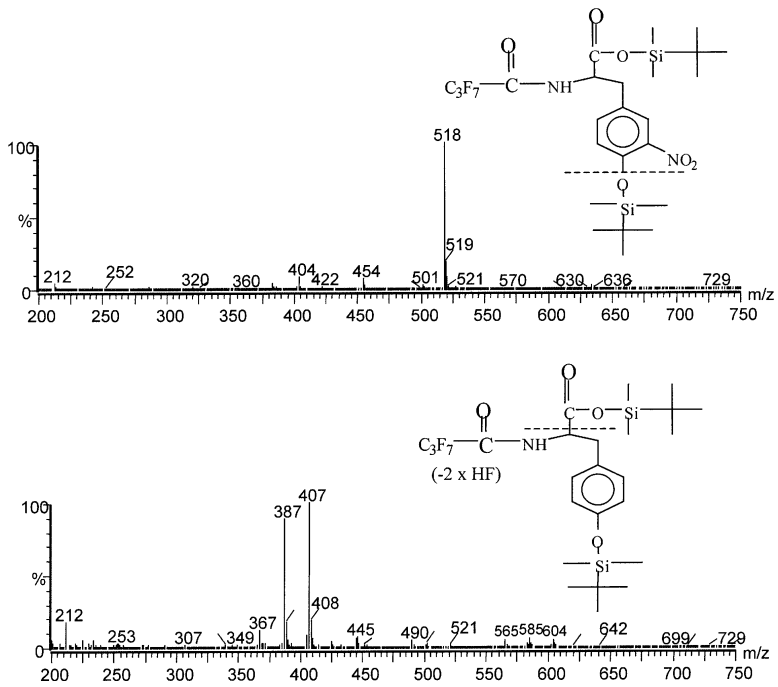

Figure 1 Structures and spectra from NICI scans of the heptafluoro derivative of nitrotyrosine (upper panel) and the heptafluoro derivative of tyrosine (lower panel)

The NICI scans produced predominantly single ions. The molecular mass of the final deprotonated derivative was 649 mass units ($M-H^-$) and 604 mass units ($M-H^-$) for nitrotyrosine and tyrosine respectively. In NICI mode, nitrotyrosine gave a predominant ion at 518 mass units, formed by the loss of 131 mass units (comprising TBDMS-OH) from the parent molecule as shown above. The tyrosine derivative gave dominant ions at 407 and 387 mass units, formed by the loss of 197 and 217 mass units from the parent molecule. The fragmentation of the tyrosine derivative was more complex. It involved the initial loss of two HF groups from the heptafluoro derivative to give rise to ions at *m/z* 585 and 565. The ion formed (*m/z* 565) then lost 158 mass units comprising the TBDMS group together with the carboxy group as shown above, to give the major ion at 407 mass units. The ion at 387 mass units represents the further loss of HF from the fluorinated derivative. Different fragmentation patterns of nitrotyrosine and tyrosine occur because of the presence of the nitro group and its influence on the silylated hydroxy goup on the benzene ring. The formation of predominantly single ions for both nitrotyrosine and tyrosine derivatives increases the sensitivity of the assay.

wise. [$^{13}C_9$]Tyrosine was purchased from Cambridge Isotope Laboratories (Andover, MA, U.S.A.). Ethyl heptafluorobutyrate was obtained from Fluorochem (Old Glossop, Derbyshire, U.K.), trifluoroacetic acid (TFA) from Lancaster (Morecambe, Lancashire, U.K.) and methanol from Merck Ltd. (Lutterworth, Leicestershire, U.K.). Anhydrous solvents were prepared by storage over a layer of CaH_2.

Subjects

Blood (10 ml) was taken from from normal laboratory male personnel (mean age 31 years; range 24–41 years) into tubes containing EDTA as an anticoagulant and centrifuged at 2300 ***g*** and 4 °C for 30 min; plasma was analysed immediately.

Standards

For internal standards both [2,3,5,6-^{2}H]tyrosine and [$^{13}C_9$]nitrotyrosine were employed. [$^{13}C_9$]Nitrotyrosine was synthesized by reacting 1 mg of [$^{13}C_9$]tyrosine with 25 μl of a 1:4 dilution of tetranitromethane in ethanol in 1 ml of 0.5 M sodium carbonate buffer, pH 9.0, for 30 min at room temperature. The yellow solution was aspirated, avoiding any unreacted tetranitromethane (visible as a small oily droplet), and extracted on a LC_{18} SupelcoClean cartridge (Sigma) as described for the extraction of nitrotyrosine (see below). To remove any unreacted tyrosine and by-products, the freeze-dried extract was purified by HPLC on a Techsphere C_{18} column (25 mm × 4.6 mm; HPLC Technology, Macclesfield, Cheshire, U.K.). This employed a gradient of water (Waters system, 610 pumps) containing 0.1 % (v/v) TFA (solution A) and 0.1 % (v/v) TFA/acetonitrile (3:1) (solution B). Initial conditions were 100 % A, changing to 90 % A/10 % B over 15 min, then to 50 %A/50 %B from 15 to 30 min. The fractions containing nitrotyrosine were identified by their retention times (approx. 28 min for nitrotyrosine, approx. 23 min for tyrosine) and characteristic UV spectra (λ_{max} at 216, 276 and 354 nm) with a Waters photodiode array system (Millenium; Waters, Watford, Hertfordshire, U.K.). Fractions were pooled; the concentration of nitrotyrosine was confirmed initially by measurement of its absorbance at 276 nm and subsequently by analysis together with authentic preweighed nitrotyrosine by GLC/MS (see below) after derivatization.

Extraction of nitrotyrosine and tyrosine from plasma

The internal standards (10 ng of [$^{13}C_9$]nitrotyrosine and 1 μg of [2,3,5,6-^{2}H]tyrosine) were added to 1 ml of plasma, which was immediately filtered by centrifugation at 9000 ***g*** in a Microfuge through a 30 kDa molecular mass cut-off centrifugal membrane (Ultrafree; Millipore, Bedford, MA, U.S.A.) to remove high-molecular-mass proteins. The filtrate was applied to a reverse-phase column (3 ml, LC_{18} SPE Tube; Supelco, Bellafonte, PA, U.S.A.) that had been prewashed with 2 ml of methanol, 2 ml of water, and 6 ml 0.1 % (v/v) TFA (adjusted to pH 5.0 with ammonia solution). The column was washed with 2 ml of water; nitrotyrosine and tyrosine were eluted with 2 ml of 25 % (v/v) methanol in water and dried under vacuum before derivatization.

Extraction, hydrolysis and purification of nitrotyrosine and tyrosine from plasma proteins

The plasma proteins were extracted from 1 ml of plasma after the addition of 2 ml of water and 12 ml of a 2:1 (v/v) mixture of chloroform/methanol, vortex-mixed and then centrifuged at 9000 ***g***. The protein pellet was obtained at the interface by aspiration of the aqueous and organic layers, then dried under vacuum. Freeze-dried plasma proteins (1–2 mg) were weighed into polypropylene tubes (Sarstedt Ltd., Beaumont Leys, Leicester, U.K.) and 1 ml of 4 M NaOH was added. Internal standards (20 ng of [$^{13}C_9$]nitrotyrosine and 10 μg of [2,3,5,6-^{2}H]tyrosine) were added to the tubes, which were then purged with argon to create an inert atmosphere and sealed with poly(tetrafluoroethylene) tape and screw caps. Samples were heated to 120 °C for 16 h. After hydrolysis, the pH was adjusted to 5.0 with HCl; samples were then handled and processed as outlined above for the extraction of tyrosine and nitrotyrosine from filtered plasma on the LC_{18} column. For the measurement of tyrosine concentrations, 50 μl of the 2 ml eluate was derivatized after being dried under nitrogen directly, as described below, and analysed. However, for the analysis of nitrotyrosine we observed multiple interfering peaks in protein hydrolysates; further purification was required. Therefore the remaining 1.95 ml of eluate was freeze-dried under vacuum, resuspended in 200 μl of water [containing 1 % (v/v) TFA and corrected to pH 4.0 with ammonia solution], loaded on an ENV$^+$ column (50 mg; Jones Chromatography, Hengoed, Mid-Glamorgan, Wales, U.K.). The ENV$^+$ cartridge is a non-polar column with a larger absorbant capacity than the LC_{18} column; retention of polar compounds that were not retained from an aqueous matrix by C_{18} silica absorbants is achieved. The greater retention of the nitrotyrosine enabled the sample to be washed with 1 % (v/v) methanol, which obtained the required purity of the analyte. The ENV$^+$ column was preconditioned with 1 ml of methanol followed by 2 ml of water (pH 4.0) containing 1.0 % (v/v) TFA. The fraction containing nitrotyrosine was washed with 1 ml of 1 % (v/v) methanol, eluted with 1 ml of 50 % (v/v) methanol in water, dried under vacuum and derivatized as described below.

Derivatization of amino acids

After extraction and freeze-drying, nitrotyrosine and tyrosine were converted into the respective amides by the addition of 200 μl of anhydrous dimethylformamide and 20 μl of di-isopropyl ethylamine. Samples were left for at least 5 min on ice, after which 40 μl of ethyl heptafluorobutyrate was added. After being left for a further 30 min, samples were sonicated for 1 h. Unreacted and excess reagents were evaporated under nitrogen. The amino acid amides were converted into the t-butyl-(dimethyl)silyl ester or ether by the addition of 30 μl of acetonitrile and 30 μl of TBDMS containing 1 % (v/v) *N*-(t-butyldimethylsilyl)-*N*-methyltrifluoroacetamide chlorosilane at room temperature for 30 min. Derivatized samples were dried under nitrogen and redissolved in 16 μl of undecane containing 25 % (v/v) TBDMS. Samples were analysed on a CarloErba GC 8000 equipped with a 15 m RH-5ms$^+$ capillary column (0.25 mm internal diam., 0.2 μm film thickness; Capital Analytical) interfaced with a VG Trio 1000 mass spectrometer. The ion source and interface were set at 200 and 300 °C respectively. Samples were analysed in NICI mode with ammonia as the reagent gas. Considerable problems were encountered with peak shape and recovery when using conventional injection of the analyte into a glass-lined injection port during the analysis of nitrotyrosine. All samples were therefore subsequently injected with the on-column injection technique. On-column injection is the method by which a sample is directly injected manually into the analytical column from a fine-bore microlitre syringe. This technique avoids problems that can arise from the interactions between the analyte and the glass injection liner. The initial column temperature was maintained at 180 °C for 1 min and then increased to 300 °C at 20 °C/min for nitrotyrosine analysis. For tyrosine analysis the initial column temperature was maintained at 180 °C for 1 min and then increased to 250 °C at 10 °C/min.

Quantification of nitrotyrosine and tyrosine

The amino acid derivatives were quantified by stable-isotope-dilution GLC/MS and calculated from the known $^{13}C_9$- or 2,3,5,6-^{2}H-labelled internal standards. For nitrotyrosine and for tyrosine, these are respectively 9 and 4 mass units heavier. Ions were monitored at 518 and 527 mass units for nitrotyrosine and its $^{13}C_9$-labelled standard respectively, and at 407 and 411 mass units for tyrosine and [2,3,5,6-^{2}H]tyrosine respectively.

RESULTS

Characterization of the derivatives of nitrotyrosine and tyrosine

The structures of the heptafluorobutyl amide, TBDMS-derivatized nitrotyrosine and tyrosine are shown in Figure 1. The molecular mass of derivatized nitrotyrosine is 650 Da, which is 45 mass units heavier than derivatized tyrosine (605 Da). Analysis of the heptafluoro derivatives of nitrotyrosine and tyrosine by full scan mode (monitoring ions at m/z 200–750) (Figure 1) showed that nitrotyrosine has a dominant ion at m/z 518 (a loss of 131 mass units) and tyrosine has a dominant ion at m/z 407 (a loss of 197 mass units).

The deprotonated molecular ion for the tyrosine derivative is evident at m/z 604 ($M-H^-$) and for nitrotyrosine at 649 ($M-H^-$) (not visible on the spectra shown). To confirm initially that the nitrotyrosine derivative formed gave rise to the peak at m/z 518, we also performed a similar derivatization with ethyl trifluoroacetate and ethyl pentafluoropropionate in place of ethyl heptafluorobutyrate. This resulted in the observation of the major ion for nitrotyrosine at 418 and 468 mass units, an expected 100 and 50 mass units shift in the derivative formed (results not shown). However, formation of the trifluoroacetyl amide with tyrosine was inefficient and peak resolution was poor with greater fragmentation; however, the deprotonated molecular ion was clearly evident at m/z 504 ($M-H^-$). For the pentafluoropropionyl derivative the major ions were at m/z 554 ($M-H^-$), 434 and 357 (50 mass units different from the heptafluoro derivative). Excessive fragmentation of the pentafluoro derivative was still evident. On the basis of these results, all further work focused on developing the analytical method for nitrotyrosine and tyrosine as the heptafluorobutyric derivative with selective ion monitoring at 518 and 407 mass units for nitrotyrosine and tyrosine respectively.

After gas chromatography, a single peak was eluted over 3–6 s for both the tyrosine and nitrotyrosine derivatives along with their respective internal standards (Figure 2).

Standard curves of nitrotyrosine and tyrosine

Standard curves generated from nitrotyrosine and tyrosine showed good reproducibility and linearity. The most critical method of evaluating an analytical assay is that the method can accurately measure known amounts of added analyte. Known amounts (0–2.5 μg of tyrosine or 0–100 ng of nitrotyrosine) were therefore added to plasma and the samples were analysed as unknowns. There was excellent correlation between the added and measured amounts of nitrotyrosine ($r = 0.99$) and tyrosine

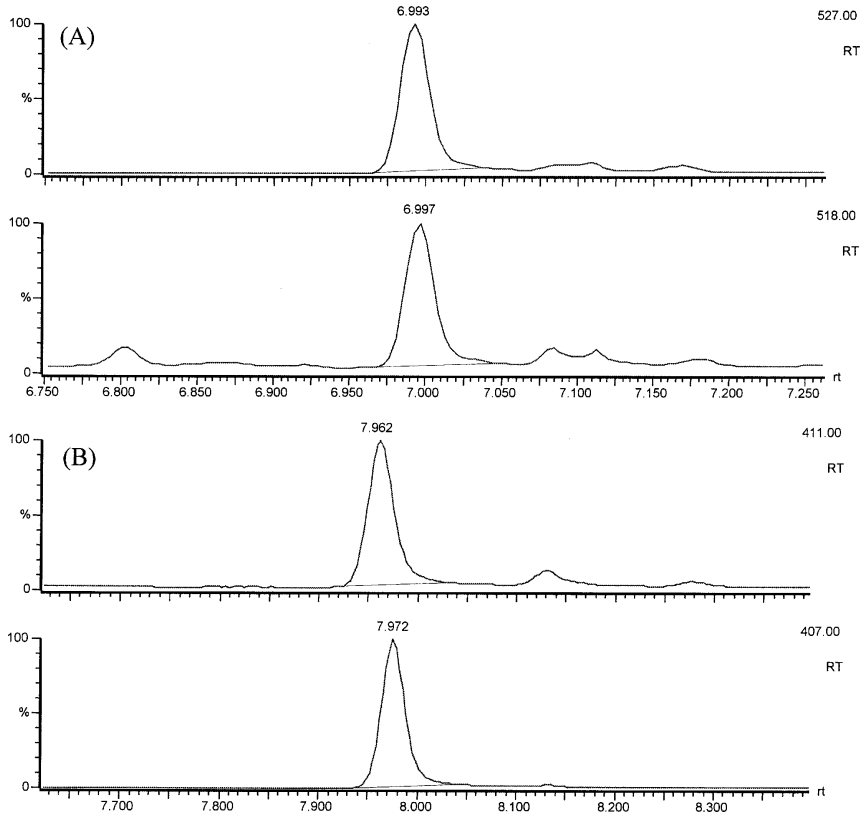

Figure 2 Typical chromatogram showing peak shape and resolution for both nitrotyrosine (A) and tyrosine (B) in a hydrolysed plasma protein sample

Excellent chromatography and resolution can be seen. The $^{13}C_9$-labelled nitrotyrosine and [2,3,5,6-2H]tyrosine resulted in derivatives that were respectively 9 and 4 mass units heavier than the parent compound. Nitrotyrosine (upper panel) was quantitated by monitoring ions of *m/z* 518 and 527, and tyrosine (lower panel) by monitoring ions of *m/z* 407 and 411. The *x*-axis represents time.

($r = 0.98$), with a linear standard curve superimposed on the basal concentrations of these analytes (Figure 3). Known amounts of nitrotyrosine were also added to different weights of plasma proteins. The correlation again was excellent ($r = 0.99$) (Figure 4). The recoveries of added nitrotyrosine and tyrosine were $94 \pm 1\,\%$ and $84 \pm 1\,\%$ (means $\pm$ S.E.M.) respectively.

Levels of free and bound nitrotyrosine and tyrosine in plasma

The normal range of plasma free nitrotyrosine and free tyrosine in eight individuals is shown in Figure 5. The concentrations (means $\pm$ S.E.M.) of free nitrotyrosine and tyrosine in normal individuals were 14.4 ± 0.7 ng/ml and 12.2 ± 0.6 μg/ml respectively, equivalent to 64 ± 3 nM and 67 ± 3 μM respectively. The mean ratio of nitrotyrosine to tyrosine was 1:1073 (930 μmol/mol of tyrosine). The coefficients of variation between assays were determined on a single plasma sample as 3.1 % and 3.8 % for nitrotyrosine and tyrosine respectively ($n = 10$ determinations).

Nitration of tyrosine as an artifact

During the work-up and preparation of samples, which invariably contained low levels of nitrite, conventional acid hydrolysis or the use of derivatization techniques with strongly acidic conditions could theoretically lead to the nitration of endogenous tyrosine (free or protein-bound) and lead to the generation of nitrotyrosine. To investigate this, tyrosine (10 μg) was incubated for 2 h at room temperature with or without sodium nitrite (10 μM), with either 6 M HCl (as used in the acid hydrolysis of proteins) or 1 % (w/v) TFA buffered to pH 3.0, 4.0 or 5.0 with ammonia solution. These concentrations of TFA were used because they reflected the most extreme conditions used in the current assay. To monitor the formation of nitrotyrosine, [$^{13}C_9$]nitrotyrosine (20 ng) was added as an internal standard. Each experiment was performed in duplicate. In the presence of HCl and nitrite, approx. 1 % of the tyrosine was converted into nitrotyrosine, which increased from a basal level of undetectable to 137 ± 53 ng. However, no formation of nitrotyrosine as an artifact was observed when tyrosine was incubated with TFA at pH 3.0–5.0. To confirm that the method described did not cause the nitration of tyrosine as an artifact during the preparation of plasma samples, we monitored the formation of deuterated nitrotyrosine from the [2,3,5,6-^{2}H]tyrosine used as an internal standard. The nitration of [2,3,5,6-^{2}H]tyrosine results in the formation of [3,5,6-^{2}H]nitrotyrosine, with the loss of the 2′ deuterium atom. No [3,5,6-^{2}H]nitrotyrosine was detected after either alkaline hydrolysis or after the extraction and derivatization of free nitrotyrosine (results not shown).

The normal range of bound nitrotyrosine in plasma proteins was determined in samples from the same subjects ($n = 8$; Figure 6), and expressed both in absolute terms and as a ratio to tyrosine. The level of nitrotyrosine and tyrosine present in plasma proteins was determined as 2.7 ± 0.4 ng/mg and 60.7 ± 1.7 μg/mg of protein, with a ratio of 1:28000 (35.4 μmol/

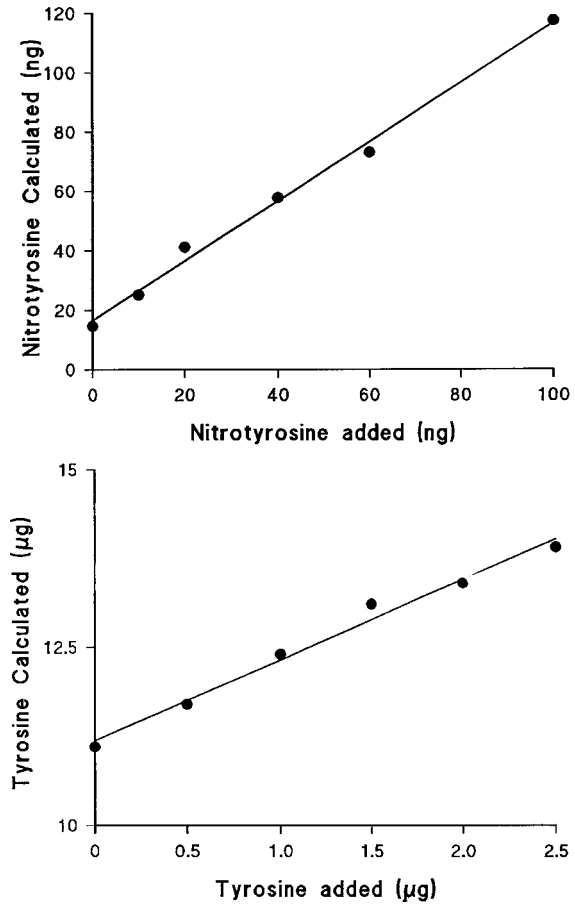

Figure 3 Internal standard curves for free nitrotyrosine (upper panel) and free tyrosine (lower panel) in plasma

The addition of known amounts of free nitrotyrosine and free tyrosine to 1 ml of plasma showed the expected elevated linear response when analysed by GLC/MS.

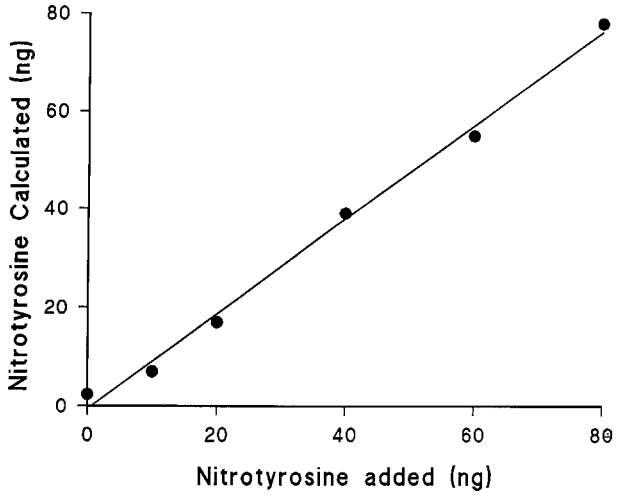

Figure 4 Internal standard curve for protein-bound nitrotyrosine

The addition of known amounts of free nitrotyrosine to 1–2 mg of plasma protein showed excellent correlation and yielded the expected elevated linear response when analysed by GLC/MS after hydrolysis, sample preparation and derivatization.

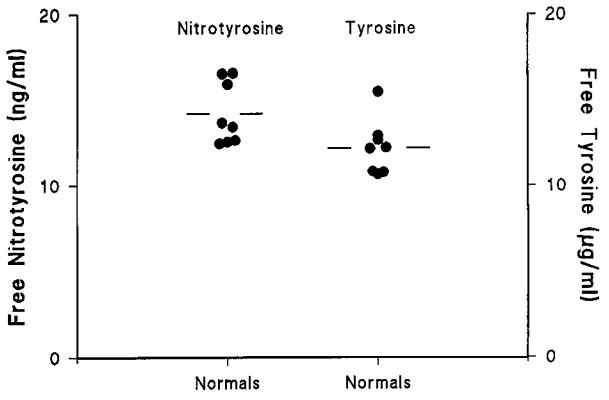

Figure 5 Levels of free nitrotyrosine and tyrosine in 1 ml of plasma from normal volunteers ($n = 8$)

Lines indicate mean values.

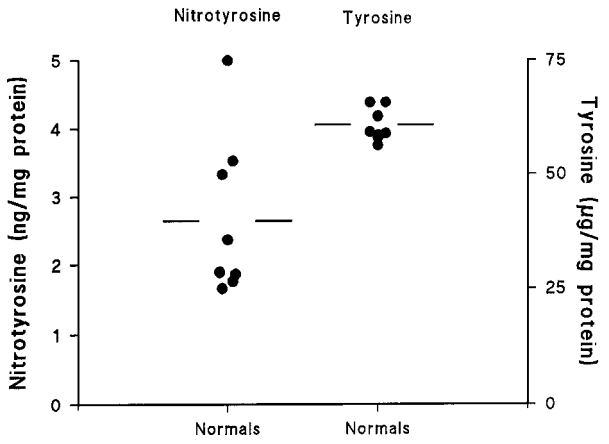

Figure 6 Levels of nitrotyrosine and tyrosine in 1–2 mg of plasma proteins from normal volunteers ($n = 8$)

Lines indicate mean values.

mol of tyrosine). The coefficient of variation between assays was determined on a single plasma protein sample as 5.1% for tyrosine and 19.4% (1.64 ± 0.5 ng/mg, range 1.3–2.1 ng/mg) for nitrotyrosine ($n = 10$ determinations). The coefficient of variation of the assay was higher for the protein samples than for free nitrotyrosine or tyrosine, presumably reflecting the greater number of steps in this procedure.

DISCUSSION

The development of analytical methods that are accurate, reproducible and applicable to biological samples is crucial to our understanding of nitration reactions in pathological processes. Our early attempts at measuring the concentration of free nitrotyrosine in plasma after acidic derivatization yielded results in which the concentrations were high and variable. It was the realization that our high but variable results were due to the nitration of tyrosine by nitrite in the sample under the acidic conditions employed that led us to develop the analytical method described here. This derivatization method employs slightly acidic or alkaline conditions, is simple to perform and is highly efficient.

These conditions prevent the generation of nitrotyrosine as an artifact from the reaction of tyrosine with nitrite present in biological fluids, as shown by the absence of nitration of the [2,3,5,6-^{2}H]tyrosine internal standard. Moreover, the volatility of the derivatization reagents and by-products permits their removal by evaporation under nitrogen.

Conventionally, acid hydrolysis has been used for the digestion of tissues and proteins [16] but this also causes the nitration of tyrosine as an artifact. The addition of 1 % (w/v) phenol to act as a surrogate species for nitration decreases but does not eliminate this problem. The enzymic digestion of proteins, coupled with the measurement of free nitrotyrosine, has been used to determine the nitration ratio (ratio of nitrotyrosine to tyrosine) in tissues [17,18]. A potential disadvantage with this method is that the autohydrolysis of the protease itself might contribute to the levels of tyrosine and nitrotyrosine released into the medium. We have therefore employed alkaline hydrolysis, a procedure that is rarely used in protein studies and has primarily been used for the determination of tryptophan [19]. The main disadvantage of this method is that total or partial decomposition of amino acids such as cysteine, serine, arginine and threonine occurs. However, both tyrosine and nitrotyrosine are stable under these conditions, and the decomposition of unrelated amino acids improves the clean-up and removal of related compounds from the hydrolysate.

In the present study we determined plasma free nitrotyrosine as 14 ± 0.7 ng/ml. There are very few reports of the concentrations of free nitrotyrosine in the plasma of normal individuals. Kaur and Halliwell [9] reported the level of free nitrotyrosine as less than 100 nM (23 ng/ml). Most reports concern the measurement of nitrotyrosine in particular disease states [2,20], usually by immunoassay. However, it is difficult to compare the values obtained with immunoassays (e.g. ELISA) because the results are generally reported as nitrated BSA equivalents [13]. The concentration of free nitrotyrosine in plasma has been reported by Kamisaki et al. [11] as approx. 31 nM as determined by HPLC coupled with electrochemical detection. This is equivalent to approx. 7 ng/ml, which is comparable to the value observed in the present study (14 ng/ml). However, the peak shape and baseline reported in the above study could make it difficult to obtain reproducible quantitative data. In the present study we determined plasma free tyrosine as 12.2 ± 0.6 μg/ml, which is consistent with the published results for this amino acid.

Plasma proteins precipitated by our procedure include albumin, globulins and other high-molecular-mass proteins. Albumin represents approx. 60 % of human plasma proteins. On the basis of the known structure of human albumin, which contains 19 tyrosine molecules per molecule of albumin, one can estimate that albumin would contain tyrosine at approx. 50 μg/mg. In the present study we observed tyrosine values of 60.7 ± 1.7 μg/mg of plasma protein, which confirms the efficiency of the hydrolysis procedure and reflects the presumed content of tyrosine in the other plasma proteins. Moreover, this technique can be and is being applied to the measurement of the nitrotyrosine content in different tissues such as the heart, liver and kidney (M. T. Frost, K. P. Moore and B. Halliwell, unpublished work).

The level of protein-bound nitrotyrosine in plasma from normal controls was below the detection limit of one immunoassay [12]. HPLC is the most widely used chromatographic technique for the analysis of nitrotyrosine. HPLC coupled with electrochemical detection is reported to be the most sensitive method [21] but has the disadvantage that nitrotyrosine is reduced to 3-aminotyrosine to ensure satisfactory sensitivity [22]. Moreover, 3-aminotyrosine is reported to be produced *in vivo* (0.1–1.5 mol of 3-aminotyrosine/10 kmol of tyrosine) [23] via tyrosine amination, so endogenous 3-aminotyrosine might cause interference with the sample of interest. HPLC with electrochemical detection has been used to measure 3-nitrotyrosine in a range of disease states [17–24]. In one study the concentration of nitrotyrosine in human plasma proteins was reported as 2.3 μmol/mol of tyrosine [18] (0.2 ng/mg) and in another as being below the limit of detection to 4.2 ng/mg [2]. In comparison, the present study has estimated the level of nitrotyrosine to be 35 μmol/mol of tyrosine (2.7 ng/mg). The reason for these differences is not clear but is presumably related to the sensitivity and recoveries of the different assays. The development of assays that are sensitive, accurate and applicable to a wide variety of biological samples will provide an important step in developing our understanding of the importance of nitration reactions and reactive nitrogen species in disease processes.

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