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SYNTHESIS OF 3-NITRO-L-[$^2H_3$]TYROSINE FOR USE AS AN INTERNAL STANDARD FOR QUANTIFICATION OF 3-NITRO-L-TYROSINE BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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Summary

3-Nitro-L-tyrosine is produced in vitro and in vivo by the nitration of tyrosine and tyrosine residues in proteins. Plasma 3-nitro-L-tyrosine has been suggested as an index of oxidative damage in living organisms that depend on nitrating species. For use in the quantitative determination of 3-nitro-L-tyrosine in biological fluids by gas chromatography-mass spectrometry (GC-MS) we describe the chemical synthesis of 3-nitro-L-[$^2H_3$]tyrosine by nitration of commercially available L-[$^2H_4$]tyrosine by nitrate and sulphuric acid. GC-MS analysis revealed a ^2H isotopic purity of about 98%. The isolated reaction product did not contain any detectable amounts of L-[$^2H_4$]tyrosine. The utility of 3-nitro-L-[$^2H_3$]tyrosine as an internal standard for the quantitative analysis of 3-nitro-L-tyrosine by GC-MS is shown.

Keywords

3-Nitro-L-tyrosine; 3-nitro-L-[$^2H_3$]tyrosine; nitration; gas chromatography-mass spectrometry

Introduction

Tyrosine residues in proteins and free tyrosine can be nitrated by various reactive nitrogen species in vitro and in vivo to give 3-nitro-tyrosine and other nitr(osyl)ated products [1-7]. In this area special scientific interest has focussed on peroxynitrite ($ONOO^-$) which is one of the most

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powerful in vivo oxidants and nitrating agents formed by reaction of nitric oxide (NO) with superoxide anions [reviewed in 8]. Peroxynitrite has been shown to be not the only species that converts tyrosine to 3-nitro-tyrosine [9,10]. Nevertheless, 3-nitro-tyrosine is considered as a general biomarker for nitrating species [10]. 3-Nitro-L-tyrosine was detected in serum and synovial fluid from rheumatoid patients [11]. It has also been assessed as an indicator of oxidative stress in human brain [12]. Various analytical techniques have been used to detect free and protein-associated 3-nitro-tyrosine. Immunohistochemical techniques were used to localise 3-nitro-L-tyrosine within tissues [13]. A high-performance liquid chromatographic (HPLC) assay with electrochemical detection for 3-nitro-L-tyrosine was developed by Shigenaga et al. [14]. Other quantitative approaches based on HPLC [11] or gas chromatography-mass spectrometry (GC-MS) [5] have not offered the sensitivity or convenience needed. Recently, Leeuwenburgh et al. described a method for the quantification of 3-nitro-L-tyrosine by GC-MS using 3-nitro-[$^{13}C_6$]tyrosine as internal standard [3], the latter being sunthesized using the extremely toxic and explosive tetranitromethane [15] and the expensive [$^{13}C_6$]tyrosine. The increasing interest in 3-nitro-L-tyrosine and the lack of suitable internal standards for its quantitative determination by GC-MS prompted us to synthesise the standard by a substantially less hazardous and costly route.

Experimental

Chemicals and reagents

L-[$^2H_4$]Tyrosine was obtained from Isotec (Miamisburg, Ohio, USA) at a ^2H isotopic purity of 98%. Unlabelled 3-nitro-L-tyrosine was purchased from Aldrich (Steinheim, Germany). Pentafluoropropionic anhydride (PFPA) and *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were obtained from Pierce (Rockford, IL, USA). All other chemicals were purchased from Merck (Darmstadt, Germany).

Preparation of 3-nitro-L-[$^2H_3$]tyrosine

L-[$^2H_4$]Tyrosine (30 μmoles) and two-molar excess of $NaNO_3$ were suspended in 600 μl of ice-cold water. Upon slow addition of 300 μl of sulphuric acid (97%) under stirring the colour of the cool solution changed to yellow. After one hour of incubation in the ice-bath HPLC analysis showed the reaction to be complete. 5 M Aqueous sodium hydroxide was added and the barium sulphate precipitated upon addition of barium chloride, was removed by centrifugation. The pH of the remaining yellow-coloured 3-nitro-L-[$^2H_3$]tyrosine stock solution was adjusted to 5 and

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diluted with distilled water to achieve a final concentration of about 3 mM. HPLC analysis showed this solution to be stable for at least 6 months when stored at 4 °C.

Derivatization procedures

Derivatization of 3-nitro-L-tyrosine and its deuterium-labelled analog was performed by minor modifications of the method described by Leeuwenburgh et al. [3] for amino acids. Instead of the n-propyl ester the methyl (Me) ester was prepared. After acylation with pentafluoropropionic (PFP) anhydride (PFPA) the residue was treated with 50 µl of BSTFA and heated at 60 °C for 60 min to obtain the trimethylsilyl (TMS) ether derivatives.

Mass spectrometric analysis

GC-MS and GC-MS/MS analysis in the negative-ion chemical ionisation (NICI) mode was performed on a triple-stage quadrupole mass spectrometer Finnigan MAT TSQ 45 directly interfaced with a Finnigan MAT gas chromatograph 9611 (San Jose, CA, USA). The gas chromatograph was equipped with a fused-silica capillary column DB-5 MS (30 m x 0.25 mm I.D., 0.25 µm film thickness) from J & W Scientific (Rancho Cordova, CA, USA). The following oven temperature program was used with helium (70 kPa) as the carrier gas: 2 min at 80°C, then increased to 320°C at a rate of 25°C/min and finally kept at 320°C for 5 min. The interface, injector and ion source were kept at 280°C, 280°C and 140°C, respectively. Electron energy and electron current were set to 90 eV and 220 µA, respectively. Methane (65 Pa) and argon (0.2 Pa collision pressure) were used as reagent and collision gases, respectively. The collision energy was set at 14 eV and the electron multiplier voltage was 1 – 2 kV. Aliquots of 1 µl were injected in the splitless mode.

Results and discussion

HPLC analysis of dilutied stock solutions of the reaction product, with detection at 278 nm, showed a single peak with the retention time of synthetic unlabeled 3-nitro-L-tyrosine and no detectable amounts of L-[$^2H_4$]tyrosine (Fig. 1). The retention times were as follows: 4.42 ± 0.01 min for L-[$^2H_4$]tyrosine, 7.04 ± 0.09 min for 3-nitro-L-tyrosine and 6.94 ± 0.03 min for 3-nitro-L-[$^2H_3$]tyrosine. The UV-vis spectrum of 3-nitro-L-[$^2H_3$]tyrosine in 0.015 M aqueous sodium hydroxide solution in 0.01 M NaCl showed a maximum at 428 nm. The absorbance at 428 nm divided by the absorbance at 340 nm gave a factor of 4.9 compared to a literature value of 5.1 for 3-nitro-L-tyrosine [2].

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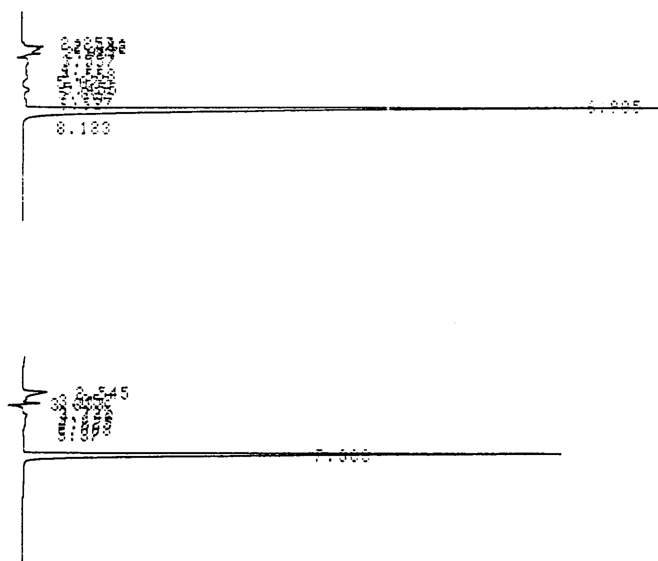

Figure 1. HPLC chromatograms of unlabelled (left) and ^{2}H-labelled 3-nitro-L-tyrosine (right).

The structure of the synthesised reaction product and its isotopic purity was determined by mass spectrometry. GC-MS analysis of the Me-PFP-TMS derivatives of unlabeled and ^{2}H-labelled L-tyrosine in the NICI mode each showed elution of a single peak with virtually identical retention times (Fig. 2). The GC-MS mass spectrum derived from the Me-PFP-TMS derivative of unlabelled 3-nitro-L-tyrosine includes prominent ions at m/z 438 $[M - HF]^-$, m/z 368 $[M - TMSOH]^-$ and m/z 351 $[M - TMSOH - OH]^-$. Prominent ions of labelled 3-nitro-L-tyrosine were found at m/z 441 $[M - HF]^-$, m/z 371 $[M - TMSOH]^-$ and m/z 354 $[M - TMSOH - OH]^-$. These results clearly indicate that the isolated reaction product is identical with 3-nitro-L-[$^2H_3$]tyrosine. The loss of one deuterium atom in the ring of L-[$^2H_4$]tyrosine results from its replacement by the nitro group at C-3.

The parent ions $[M - HF]^-$ of the unlabelled and ^{2}H-labelled 3-nitro-L-tyrosine were subjected to collisionally activated dissociation (CAD) with argon as the collision gas. The most intense daughter ions were observed at 351 m/z for unlabelled and 354 m/z for 3-nitro-L-[$^2H_3$]tyrosine.

The concentration of 3-nitro-L-[$^2H_3$]tyrosine in the stock solution was determined as follows: unlabelled 3-nitro-L-tyrosine was accurately weighed and a 2 mM stock solution was prepared. The absorbance at 428 nm of dilutions of this stock solution in 0.015 M aqueous sodium

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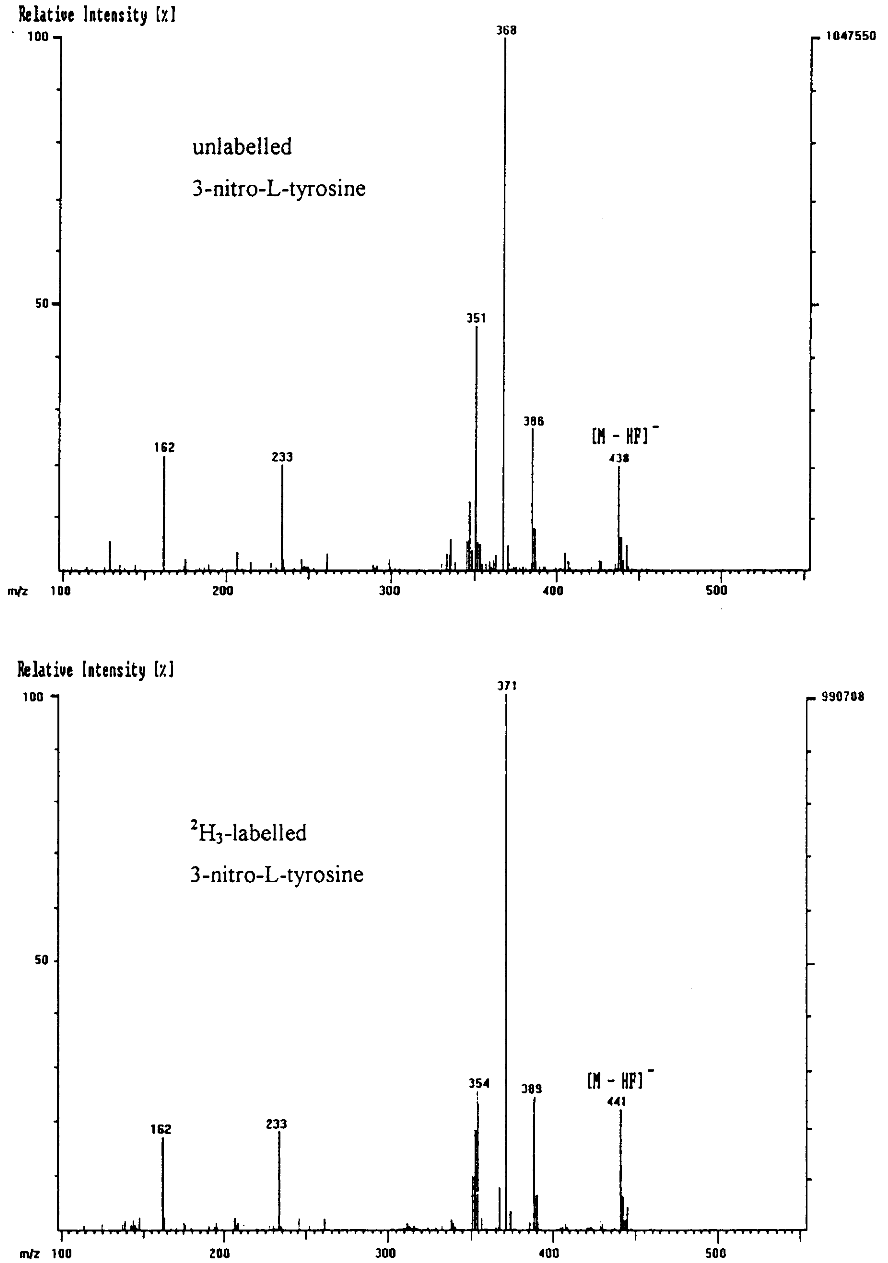

Figure 2. GC-MS mass spectra of the Me-PFP-TMS derivatives of unlabelled (upper panel) and ^{2}H-labelled 3-nitro-L-tyrosine (lower panel).

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hydroxide/0.01 M NaCl was plotted vs. the concentration of 3-nitro-L-tyrosine (Fig. 3). An appropriate dilution of the 3-nitro-L-[$^2H_3$]tyrosine stock solution was quantified by means of this curve. By this procedure the concentration of 3-nitro-L-[$^2H_3$]tyrosine in the stock solution was determined as 3.0 mM. This is identical with the expected value obtained by HPLC. The final yield of the isolated 3-nitro-L-[$^2H_3$]tyrosine was determined to be 80 %. This result was verified by GC-MS using the following standardisation procedure: Each 100 pmoles of accurately weighed unlabeled 3-nitro-L-tyrosine were mixed with different amounts of 3-nitro-L-[$^2H_3$]tyrosine (0 – 500 pmoles), then converted to their Me-PFP-TMS derivatives and analysed by selected reaction monitoring of m/z 351 for 3-nitro-L-tyrosine and m/z 354 for 3-nitro-L-[$^2H_3$]tyrosine.

Linear regression analysis between the ratio of the peak areas at m/z 354 and 351 (y) and the ratio of the expected amounts of 3-nitro-L-[$^2H_3$]tyrosine to that of 3-nitro-L-tyrosine (x) resulted in a straight line with the regression equation $y = 0.010 + 1.026\,x$ (Fig. 4, $r^2 = 0.999$). From the slope of the straight line a mean concentration of 3-nitro-L-[$^2H_3$]tyrosine in the stock solution of 3.08 mM was calculated (1.026 x 3.0 mM = 3.078 mM).

The isotopic purity of 3-nitro-L-[$^2H_3$]tyrosine was determined as follows: 500 pmoles of 3-nitro-L-[$^2H_3$]tyrosine were converted to its Me-PFP-TMS derivative without any addition of

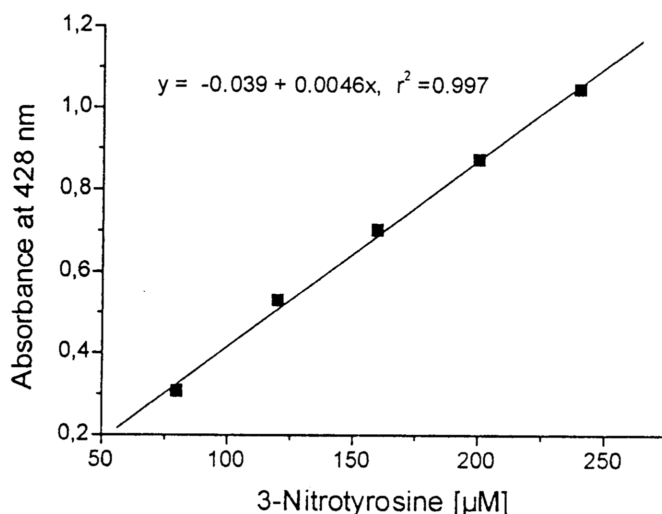

Figure 3. UV-vis calibration curve for 3-nitro-L-tyrosine used for standardisation of 3-nitro-L-[$^2H_3$]tyrosine.

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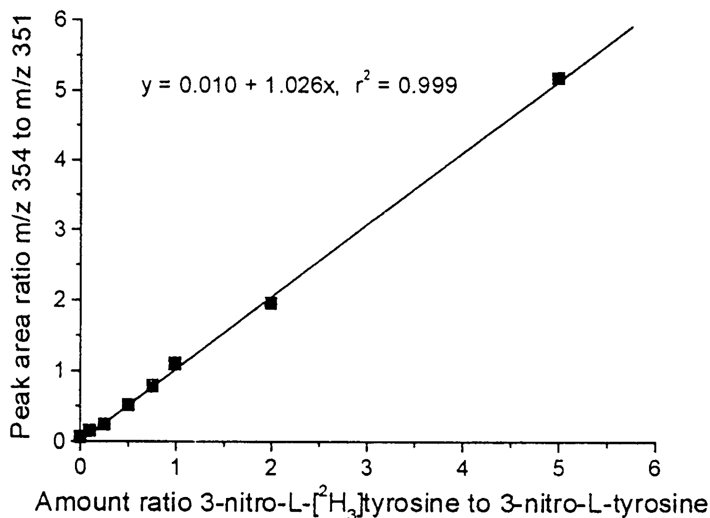

Figure 4. Standardisation of 3-nitro-L-[$^2H_3$]tyrosine by GC-MS.

3-nitro-L-tyrosine and analysed by GC-MS/MS. The amount of unlabelled 3-nitro-L-tyrosine in 3-nitro-L-[$^2H_3$]tyrosine was determined as 2 % which corresponds to the isotopic impurity of the starting material L-[$^2H_4$]tyrosine.

Conclusions

We describe here the sulphuric acid-catalysed nitration of L-[$^2H_4$]tyrosine by nitrate to yield 3-nitro-L-[$^2H_3$]tyrosine in high isotopic purity and yield. This synthetic route is simple, safe and reveals a single reaction product. It is considerably less costly than a previously reported method which uses tetranitromethane and [$^{13}C_6$]tyrosine. 3-Nitro-L-[$^2H_3$]tyrosine should be useful as an internal standard for quantification of 3-nitro-L-tyrosine in biological samples by GC-MS.

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