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Determination of 3-nitrotyrosine in human urine at the basal state by gas chromatography–tandem mass spectrometry and evaluation of the excretion after oral intake

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Abstract

3-Nitrotyrosine (NO_2Tyr) is a potential biomarker of reactive-nitrogen species (RNS) including peroxynitrite. 3-Nitrotyrosine occurs in human plasma in its free and protein-associated forms and is excreted in the urine. Measurement of 3-nitrotyrosine in human plasma is invasive and associated with numerous methodological problems. Recently, we have described an accurate method based on gas chromatography (GC)–tandem mass spectrometry (MS) for circulating 3-nitrotyrosine. The present article describes the extension of this method to urinary 3-nitrotyrosine. The method involves separation of urinary 3-nitrotyrosine from nitrite, nitrate and L-tyrosine by HPLC, preparation of the *n*-propyl-pentafluoropropionyltrimethylsilyl ether derivatives of endogenous 3-nitrotyrosine and the internal standard 3-nitro-L-[$^2H_3$]tyrosine, and GC–tandem MS quantification in the selected-reaction monitoring mode under negative-ion chemical ionization conditions. In urine of ten apparently healthy volunteers (years of age, 36.5 ± 7.2) 3-nitrotyrosine levels were determined to be 8.4 ± 10.4 nM (range, 1.6–33.2 nM) or 0.46 ± 0.49 nmol/mmol creatinine (range, 0.05–1.30 nmol/mmol creatinine). The present GC–tandem MS method provides accurate values of 3-nitrotyrosine in human urine at the basal state. After oral intake of 3-nitro-L-tyrosine by a healthy volunteer (27.6 μg/kg body weight) 3-nitro-L-tyrosine appeared rapidly in the urine and was excreted following a biphasic pharmacokinetic profile. Approximately one third of administered 3-nitro-L-tyrosine was excreted within the first 8 h. The suitability of the non-invasive measurement of urinary 3-nitrotyrosine as a method of assessment of oxidative stress in humans remains to be established.

Keywords: Reactive-nitrogen species; 3-Nitrotyrosine; Human; Urine; Biomarker

1. Introduction

Reactive-nitrogen species (RNS) originating from nitrogen monoxide (NO) such as nitrogen dioxide (NO_2), peroxynitrite ($ONOO^-$), and nitryl chloride (NO_2Cl) react readily with tyrosine and protein-associated tyrosine to form free 3-nitrotyrosine (NO_2Tyr) and protein-associated 3-nitrotyrosine, respectively. Therefore, detection of 3-nitrotyrosine provides evidence for generation of RNS [1,2]. However, the measurement of 3-nitrotyrosine in biological fluids, notably in human plasma, is associated with numerous methodological problems. Artifactual formation of 3-nitrotyrosine during sample setup, insufficient detection sensitivity and lack of specificity are the most serious and widely recognized analytical shortcomings (discussed in Refs. [3–6]).

Artifactual formation of 3-nitrotyrosine from tyrosine in the presence of nitrate and/or nitrite occurs from acidification of biological samples [3–6]. In addition, performance of derivatization reactions under acidic conditions, such as the acid-catalyzed esterification of carboxylic groups of amino acids in particular in methods based on mass spectrometry (MS), may lead to artifactual formation of 3-nitrotyrosine [7–12]. Therefore, separation of 3-nitrotyrosine from tyrosine for instance by HPLC [7] or reduction of

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3-nitrotyrosine to 3-aminotyrosine by dithionite [12] are absolutely required. Several reported methods based on HPLC with UV absorbance or fluorescence detection and even some LC–tandem MS methods lack of sufficient sensitivity to detect basal plasma levels of 3-nitrotyrosine which are of the order of 1 nM [6,7,12]. At present only the gas chromatography (GC)–tandem MS methodology seems to provide the required sensitivity and specificity for the accurate quantification of circulating NO_2Tyr [6].

Besides circulating 3-nitrotyrosine considerable attention has been paid to urinary 3-nitrotyrosine. Various groups reported that 3-nitrotyrosine is excreted in the urine of healthy humans [13–16]. Reported excretion rates and urinary concentrations of 3-nitrotyrosine vary between 60 nmol/day [15] and 248 nmol/day [13], i.e. 4-fold, and between 36 nM [14] and 5 μM [16], i.e. 139-fold, respectively, strongly suggesting serious methodological problems in the quantification of urinary 3-nitrotyrosine, too. As far as we know, the GC–tandem MS methodology has not been applied for urinary 3-nitrotyrosine. In the present article we report the development, validation and application of a GC–tandem MS method for specific, interference-free and accurate quantitative determination of 3-nitrotyrosine in human urine. This method represents an expansion and modification of the GC–tandem MS method originally reported for circulating free and protein-associated plasma 3-nitrotyrosine [7,17]. The 3-nitrotyrosine levels measured in urine of healthy humans by the present GC–tandem MS method are below 33 nM and the lowest reported for urinary 3-nitrotyrosine thus far. The method was also applied to evaluate urinary excretion of 3-nitrotyrosine after oral intake by a healthy volunteer.

2. Experimental

2.1. Materials and chemicals

3-Nitro-L-tyrosine, *p*-nitro-L-phenylalanine, L-tyrosine and creatinine were purchased from Sigma (Deisenhofen, Germany). 3-Nitro-L-[$^2H_3$]tyrosine (d_3-NO_2Tyr) had been synthesized previously by nitration of L-[$^2H_4$]tyrosine (98 at.% at ^{2}H; Isotec, Miamisburg, OH) [7]. Pentafluoropropionic anhydride (PFPA) and *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) were obtained from Pierce (Rockford, IL, USA). Sodium nitrate and sodium nitrite were purchased from Riedel-de Haën (Seelze, Germany). Ammonium sulfate and *n*-propanol were obtained from Merck (Darmstadt, Germany). Methanol of HPLC gradient grade was from Baker (Deventer, The Netherlands).

2.2. Sample preparation and derivatization procedures

Urine samples from spontaneous micturition were collected into polypropylene tubes and stored immediately in an ice-bath. For the HPLC analysis of creatinine (see below), urine aliquots (10 μl) were diluted with aliquots (990 μl) of the HPLC mobile phase. From these dilutions aliquots (200 μl) were injected into the HPLC apparatus. For the analysis of 3-nitrotyrosine, aliquots of urine samples (1 ml) were spiked with aliquots (10 μl) of a 2-μM stock solution of the internal standard d_3-NO_2Tyr in the mobile phase to achieve a final concentration of 20 nM. These solutions (100 μl) were diluted with the mobile phase (900 μl), and aliquots (200 μl) of the dilutions were injected into the HPLC apparatus. The HPLC fraction (approximately 2 min) eluting with synthetic 3-nitro-L-tyrosine (see below) was collected into a polypropylene tube and solvents were evaporated to dryness by means of a nitrogen stream in a water-bath at 40 °C. The pH value of the HPLC fraction was found to decrease from 5.5 (at the beginning) up to approximately 3 (immediately prior to complete solidification). The residue was treated with absolute ethanol (500 μl) and mixed by vortexing. Ammonium sulfate was removed by centrifugation (1600 × *g*, 5 min, 4 °C). The supernatant was transferred into an autosampler glass vial and ethanol was evaporated under nitrogen. Derivatization of 3-nitrotyrosine to its *n*-propyl ester-pentafluoropropionyl amide-trimethylsilyl ether (*n*-propyl-PFP-TMS) derivative was performed as described previously [7]. Briefly, amino acids were converted to their *n*-propyl ester derivatives by heating with 3 M HCl in *n*-propanol (100 μl) for 1 h at 80 °C. Subsequently, the sample was evaporated to dryness, the residue was treated with PFPA in ethyl acetate (1:4, v/v; 100 μl), and the sample was heated for 30 min at 65 °C. After cooling to room temperature the sample was evaporated to dryness, the residue was treated with borate buffer (0.4 M, pH 8.5, 200 μl) and toluene (500 μl), and derivatives were extracted immediately by vigorous vortex-mixing for 1 min. After centrifugation (1600 × *g*, 5 min) the toluene phase was decanted, the solvent evaporated to dryness by means of a nitrogen stream, the residue treated with BSTFA (50 μl) and the sample heated for 1 h at 60 °C.

2.3. Prepurification of 3-nitrotyrosine by HPLC

HPLC analyses were performed by using a Pharmacia LKB pump model 2248 and an analytical column (250 mm × 4 mm I.D.) packed with Nucleosil 100-5C_{18} AB (5-μm particle size) from Macherey-Nagel (Düren, Germany). The mobile phase consisted of 50 mM ammonium sulfate in water-methanol (95:5, v/v), at a pH value of 5.5 (not adjusted), and was pumped at a flow rate of 1 ml/min. The variable ultraviolet-visible detector model Spectroflow 783 from Kratos Analytical (Ramsey, NJ) was set to 205 nm for nitrite and nitrate, 236 nm for creatinine, and 276 nm for 3-nitrotyrosine, *p*-nitro-L-phenylalanine, and L-tyrosine. Analyses were performed at ambient temperature (22–26 °C).

In this HPLC system nitrite and nitrate eluted at approximately 2 min, whereas creatinine and L-tyrosine eluted at 2.7 and 3.4 min, respectively. Injection of 200-μl aliquots of a 100-nM solution of 3-nitro-L-tyrosine in the mobile phase gave a 3-nitrotyrosine peak with a signal-to-noise