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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

Dimitrios Tsikas *, Anja Mitschke, Maria-Theresia Suchy, Frank-Mathias Gutzki, Dirk O. Stichtenoth

Institute of Clinical Pharmacology, Hannover Medical School, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany

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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

* Corresponding author. Tel.: +49 511 532 3959; fax: +49 511 532 2750.
E-mail address: tsikas.dimitros@mh-hannover.de (D. Tsikas).

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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

(S/N) ratio of 8:1. *p*-Nitro-L-phenylalanine (100 nM in the mobile phase) was used to determine the retention time of 3-nitrotyrosine prior to analyze urine samples. HPLC analysis of 200-μl aliquots of a mixture consisting of *p*-nitro-L-phenylalanine and 3-nitrotyrosine (each 100 nM in the mobile phase) on different days resulted in retention times of 8.363 ± 0.200 min for 3-nitrotyrosine and 9.586 ± 0.227 min for *p*-nitro-L-phenylalanine (mean ± S.D., $n = 8$). From these analyses the ratio of the retention times of 3-nitrotyrosine and *p*-nitro-L-phenylalanine was determined to be 0.872 (R.S.D., 0.4%). The retention time of 3-nitrotyrosine was calculated by multiplying the currently measured retention time of *p*-nitro-L-phenylalanine by the factor of 0.872.

2.4. Gas chromatography–tandem mass spectrometry

GC–tandem MS analyses were performed in the electron capture negative-ion chemical ionization mode on a triple-stage quadrupole mass spectrometer model ThermoQuest TSQ 7000 (Finnigan MAT, San Jose, CA) directly interfaced with a Trace 2000 series gas chromatograph equipped with an autosampler AS 2000 (CE Instruments, Austin, TX). The gas chromatograph was equipped with a fused-silica capillary column Optima 5-MS (30 m × 0.25 mm I.D., 0.25-μm film thickness) from Macherey-Nagel (Düren, Germany). The following oven temperature program was used with helium (at a constant flow rate of 1 ml/min) as the carrier gas: 1 min at 90 °C, then increased to 340 °C at a rate of 25 °C/min, and kept at 340 °C for 1 min. Interface, injector and ion source were kept at 280, 280 and 180 °C, respectively. Electron energy and electron current was set to 200 eV and 300 μA, respectively. Methane (530 Pa) and argon (0.27-Pa collision pressure) were used as reagent and collision gases, respectively. Collision energy was set to 6 eV. Electron multiplier voltage was set to 2.8 kV. Aliquots (1 μl) were injected in the splitless mode by the autosampler.

Quantification by GC–tandem MS was performed by selected-reaction monitoring (SRM) of the characteristic product ions [7] at *m*/*z* 379 and 382 which were obtained by collision-activated dissociation (CAD) of the parent ions at *m*/*z* 396 and 399 of the *n*-propyl-PFP-TMS derivatives of NO_2Tyr and d_3-NO_2Tyr, respectively. Quantification by GC–MS was performed by selected-ion monitoring (SIM) of the parent ions at *m*/*z* 396 and 399. The dwell-time was 0.4 s for each ion in both techniques. GC–tandem MS analysis of three aliquots of a 20-nM solution of d_3-NO_2Tyr by SRM of *m*/*z* 379 and 382 revealed a mean peak area ratio of 0.0069 (R.S.D., 1.4%). The contribution of 0.138 nM by d_3-NO_2Tyr to endogenous NO_2Tyr was subtracted from calculated concentrations.

2.5. Validation of the method

Three experiments were performed to determine intra- and inter-day accuracy (recovery, %) and precision (R.S.D., %) of the method for 3-nitrotyrosine in urine from healthy volunteers. Recovery was calculated for each added concentration by means of the equation: Recovery (%) = [(measured value − basal value): added value] × 100. In the first experiment intra-day recovery and precision were determined. Quantification was performed by GC–tandem MS, the unspiked urine sample contained 3-nitrotyrosine at a mean concentration of 4.5 nM, and synthetic 3-nitro-L-tyrosine was added at concentrations of 10 and 20 nM. In the second experiment, 3-nitrotyrosine was quantified both by GC–tandem MS and GC–MS. The unspiked urine sample contained 3-nitrotyrosine at a mean concentration of 2.4 nM (by GC–tandem MS) and 1.6 nM (by GC–MS), and synthetic 3-nitro-L-tyrosine was added at concentrations of 3.6, 7.2, 14.4 and 43.2 nM. Inter-day recovery and precision were determined in the third experiment on 4 days. Quantification was performed by GC–tandem MS, the unspiked urine sample contained 3-nitrotyrosine at a mean concentration of 3.2 nM, and synthetic 3-nitro-L-tyrosine was added at concentrations of 10 and 20 nM. In all validation experiments triplicate analyses were performed, and the concentration of the internal standard was 20 nM throughout.

3. Results

3.1. Separation and isolation of urinary 3-nitrotyrosine by HPLC

The method described in the present article for urinary 3-nitrotyrosine is based on a method originally developed, validated and used for the determination 3-nitrotyrosine in human plasma at the basal state [7,17]. The essential part of the previous method was the HPLC separation of 3-nitrotyrosine from tyrosine, nitrite and nitrate from plasma ultrafiltrate. Indeed, HPLC separation of circulating 3-nitrotyrosine was found to effectively avoid artifactual formation of 3-nitrotyrosine from nitrite, nitrate and tyrosine. We assumed that accurate quantification of urinary 3-nitrotyrosine would also require HPLC separation of 3-nitrotyrosine from urinary tyrosine and nitrate. It should be noted that both tyrosine and nitrate are present in human urine, with nitrate occurring at much higher concentrations (100–4000 μM) than in plasma (20–60 μM) [18].

The HPLC system and the solid-phase extraction (SPE) procedure used to isolate plasma 3-nitrotyrosine from HPLC fractions [7,17] could not be simply expanded to the quantification of urinary 3-nitrotyrosine, but required considerable modification. The major modifications concern the mobile phase and the renunciation of the SPE step. This was achieved by using as HPLC mobile phase aqueous methanol (5 vol.%) which was buffered (pH 5.5) by $(NH_4)_2SO_4$ (50 mM). 3-Nitrotyrosine could be derivatized after complete removal of solvents and constituents from the respective HPLC fraction without preceding SPE.

The feasibility of this approach was checked as follows. Dilutions in the HPLC mobile phase (1 ml) containing

varying concentrations of synthetic 3-nitro-L-tyrosine (0, 25, 50, 75, 100, 200 nM) and a fixed concentration of the internal standard d_3-NO_2Tyr (60 nM) were evaporated to dryness, analytes were converted to their *n*-propyl-PFP-TMS derivatives and analyzed by GC–tandem MS in the SRM mode. Plotting of the peak area ratio of *m*/*z* 379 to *m*/*z* 382 (*y*) versus the molar ratio of NO_2Tyr (i.e. d_0-NO_2Tyr) to d_3-NO_2Tyr (*x*) resulted in a straight line ($R = 0.999$) with the regression equation $y = 0.01 + 1.00x$. This finding underlines the method's practicability. During evaporation of the HPLC mobile phase (1 ml), a drop of the pH value from initially 5.5 to approximately 3 was noted, most likely due to removal of ammonia. However, this pH drop was found not to contribute to artifactual formation of 3-nitrotyrosine. Thus, a solution (200 μl) of L-tyrosine (100 μM) and nitrate (800 μM) in the mobile phase was completely evaporated. The residue was reconstituted in an aliquot (200 μl) of the mobile phase and the solution was analyzed by HPLC and GC–tandem MS for 3-nitrotyrosine. Both analyses did not reveal formation of 3-nitrotyrosine above the limit of detection (LOD) of 6 pmol by HPLC and 4 amol by GC–tandem MS [7].

Injection of native urine spiked with d_3-NO_2Tyr and subsequent GC–tandem MS analysis revealed abundant peaks for urinary 3-nitrotyrosine and d_3-NO_2Tyr and 3-nitrotyrosine basal levels far above those prevailing in human plasma. However, accuracy, precision and chromatography were unacceptable (data not shown). Thus, we decided to dilute urine samples by 1:10 (v/v) with the mobile phase of the HPLC system prior to injection. Sample dilution was possible because of the very high sensitivity of the method – low LOD (4 amol) and limit of quantitation (LOQ) of 125 pM of the original GC–tandem MS method for plasma 3-nitrotyrosine [7] – and because of the relatively high urinary levels of 3-nitrotyrosine in comparison with its plasma levels. Dilution of urine samples with the mobile phase also resulted in good chromatography and relatively stable retention times in HPLC.

Table 1
Intra-day accuracy (recovery) and imprecision (R.S.D.) of the GC–tandem MS method for 3-nitrotyrosine in urine of a healthy volunteer

Added	Measured (mean ± S.D., $n = 3$)	Recovery (%)	Imprecision (%)
0.0	4.5 ± 0.43	N.A.	9.5
10.0	15.0 ± 1.23	105	8.2
20.0	25.2 ± 1.39	104	5.5

N.A., not applicable.

Unlike in the previous HPLC system [7], in the present method synthetic *p*-nitro-L-phenylalanine eluted in HPLC immediately after synthetic 3-nitro-L-tyrosine. However, the ratio of the retention times of these compounds was very stable, so that the actual retention time of 3-nitrotyrosine could be calculated from that of *p*-nitro-L-phenylalanine. Synthetic 3-nitro-L-tyrosine (at 100 nM) was not used for this purpose due to considerable *memory* effects (data not shown).

3.2. Validity of the GC–tandem MS method

Tables 1–3 summarize the results from the validation experiments on the quantification of 3-nitrotyrosine in urine from healthy volunteers. The results show that quantification of 3-nitrotyrosine is accurate by GC–tandem MS

Table 2
Intra-day accuracy (recovery) and imprecision (R.S.D.) of the method for 3-nitrotyrosine in urine of a healthy volunteer quantified by GC–tandem MS (i.e. MS–MS) and GC–MS (i.e. MS)

Added (nM)	Measured (nM)		Recovery (%)		Imprecision (%)	
	MS–MS (mean ± S.D., $n = 3$)	MS (mean ± S.D., $n = 3$)	MS–MS	MS	MS–MS	MS
0.0	2.38 ± 0.25	1.6 ± 0.20	N.A.	N.A.	10.5	12.8
3.6	5.72 ± 0.59	4.5 ± 0.34	92.8	80.6	10.4	7.6
7.2	8.6 ± 0.61	6.6 ± 0.41	86.4	69.4	7.1	6.2
14.4	16.3 ± 1.35	13.2 ± 0.83	96.7	80.6	8.3	6.3
43.2	43.5 ± 5.88	33.9 ± 3.90	95.2	74.8	13.5	11.5

All analyses were performed within a day. N.A., not applicable.

Table 3
Intra- and inter-day accuracy (recovery) and imprecision (R.S.D.) of the GC–tandem MS method for 3-nitrotyrosine in urine of a healthy volunteer

Day	3-Nitrotyrosine added (nM)								
	0			10			20		
	Mean ± S.D.[a] (nM)	Recovery (%)	Imprecision (%)	Mean ± S.D[a] (nM)	Recovery (%)	Imprecision (%)	Mean ± S.D.[a] (nM)	Recovery (%)	Imprecision (%)
A	3.6 ± 0.5	N.A.	13.9	13.3 ± 1.05	96.5	7.9	23.9 ± 0.93	102	3.9
B	3.1 ± 0.53	N.A.	17.3	13.3 ± 1.78	102	13.4	24.1 ± 1.65	105	6.8
C	3.4 ± 0.37	N.A.	10.9	13.7 ± 0.85	103	6.2	21.2 ± 0.52	89	2.5
D	2.7 ± 0.36	N.A.	13.2	11.5 ± 0.30	88	2.6	20.9 ± 0.06	91	0.3
Mean ± S.D.	3.2 ± 0.39	N.A.	14 ± 3	13 ± 1	97.4 ± 7	7.5 ± 4	22.5 ± 1.7	96.8 ± 8	3.4 ± 3

[a] Measured concentration of 3-nitrotyrosine. All analyses (each 9 samples per day) were performed in triplicate. N.A., not applicable.

and considerably less accurate by GC–MS (Table 2) in the tested relevant concentration range; imprecision was comparable in both techniques. 3-Nitrotyrosine values obtained by GC–MS were constantly lower compared to those measured by GC–tandem MS. In the relevant concentration range urinary 3-nitrotyrosine was quantified by GC–tandem MS with intra- and inter-day recovery values closed to 100% (range, 86–105%) and imprecision values below 18%. It is noted that the GC–tandem MS quantification of 3-nitrotyrosine in urine at concentrations below 5 nM is accurate but associated with considerable imprecision.

Representative chromatograms from the GC–tandem MS and GC–MS quantification of 3-nitrotyrosine in urine of a healthy volunteer are shown in Figs. 1 and 2, respectively. The chromatograms from the GC–tandem MS analyses do not show other peaks except for those corresponding to the *n*-propyl-PFP-TMS derivatives of endogenous 3-nitrotyrosine (Fig. 1A, upper trace) and endogenous plus externally added synthetic 3-nitro-L-tyrosine (Fig. 1B, upper trace) and the internal standard (Fig. 1, lower traces). By contrast, the chromatograms from the GC–MS analyses show many other peaks of considerably higher intensity. Endogenous 3-nitrotyrosine at 2.4 nM was detected with a S/N ratio of 145:1 by GC–tandem MS (Fig. 1A, upper trace, peak eluting at 9.77 min). This peak was produced from the injection of an approximate amount of 1 fmol of 3-nitrotyrosine, under the assumption that 3-nitrotyrosine was completely recovered from the diluted urine after HPLC separation and derivatization. GC–MS analysis of the same unspiked urine sample revealed a peak with S/N ratio of approximately 1:1 (Fig. 2A, upper trace, peak eluting at 9.93 min). Thus, this peak can not be discriminated from baseline noise, so that endogenous urinary levels of 3-nitrotyrosine of the order of 1.6 nM can not be quantified by GC–MS (see also Table 2).

The identity of urinary 3-nitrotyrosine and the lack of any interferences in the present GC–tandem MS method was demonstrated by generating a product ion mass spectrum from endogenous 3-nitrotyrosine (Fig. 3). Subjection of the parent ion at *m*/*z* 396 (P^-; $[M - TMSOH]^-$) of the *n*-propyl-PFP-TMS derivative of the GC peak eluting with the retention time of synthetic 3-nitrotyrosine generated a product ion mass spectrum virtually identical with that of synthetic 3-nitrotyrosine, with the characteristic product ions at *m*/*z* 379 ($[P–17]^-$) and *m*/*z* 261 ($[n$-propyl-CO_2=CH-N-PFP$]^-$) [7]. Thus, unlike circulating free 3-nitrotyrosine [6], no other compounds were found to co-elute with urinary 3-nitrotyrosine in the present method.

3.3. Basal levels of 3-nitrotyrosine in human urine

The GC–tandem MS method was applied to measure 3-nitrotyrosine in urine of healthy young volunteers without history of cardiovascular, renal or other diseases. 3-Nitrotyrosine was found by GC–tandem MS to be physiologically present in all urine samples analyzed (Table 4). Basal concentration as well as creatinine-corrected excretion rate of 3-nitrotyrosine was found to vary greatly, i.e. between 1.6 and 33.2 nM, and between 0.05 and 1.3 nmol/mmol creatinine, respectively. There was no statistically significant difference ($P = 0.993$, unpaired t test) between females (mean 0.459 nmol/mmol creatinine) and males (mean 0.462 nmol/mmol creatinine). On the basis of a mean excretion rate of 10 mmol creatinine per day, our findings suggest that healthy humans excrete in the urine approximately 4.6 nmol of 3-nitrotyrosine per day at the basal state.

3.4. Urinary excretion of orally taken synthetic 3-nitro-L-tyrosine by a healthy volunteer

The pharmacokinetics of 3-nitrotyrosine in humans is not yet investigated. In a preliminary study we applied the present GC–tandem MS method to evaluate the excretion of 3-nitrotyrosine in humans after oral intake. One of the authors took 2.9 mg (12.8 μmol) of 3-nitro-L-tyrosine diluted in drinking water, and urine samples were collected subsequently within the period of 2 h before until 7.5 h after administration. Urinary 3-nitrotyrosine levels increased from approximately 6 nM (before administration) to levels up to 5300 nM (after administration). Fig. 4 shows that orally administered 3-nitro-L-tyrosine appeared in the urine rapidly at relatively high amounts. A biphasic pharmacokinetic profile was observed. 3-Nitrotyrosine excretion rate in the urine amounted to 0.9 μmol/h in the first 2 h, and 0.35 μmol/h after this time. Approximately 29% of administered 3-nitro-L-tyrosine were excreted in the urine within the observation time of 7.5 h. 3-Nitrotyrosine could not be quantified in any urine sample (diluted with the mobile phase by 1:10, v/v) from this study by HPLC with UV absorbance detection at 276 nm (data not shown).

4. Discussion

4.1. The GC–tandem MS method

Circulating 3-nitrotyrosine in its free and protein-associated forms received special attention due to its potential suitability as a biomarker of reactive-nitrogen species including peroxynitrite in humans. For this purpose different methodologies were developed and applied [3]. However, even mass spectrometry-based analytical methods such as GC–MS revealed basal plasma levels for free 3-nitrotyrosine in the very wide range of 0.7–64 nM [6]. Several factors likely contribute to this high variation. They include artifactual formation of 3-nitrotyrosine from tyrosine and nitrite/nitrate from endogenous and exogenous sources, as well as lack of sensitivity and selectivity. This issue has been thoroughly discussed recently, and recommendations were done to overcome existing methodological problems [5,6]. At present, only the GC–tandem MS methodology allows for the accurate quantification of free 3-nitrotyrosine in human plasma at the basal state (discussed in Ref. [6]).

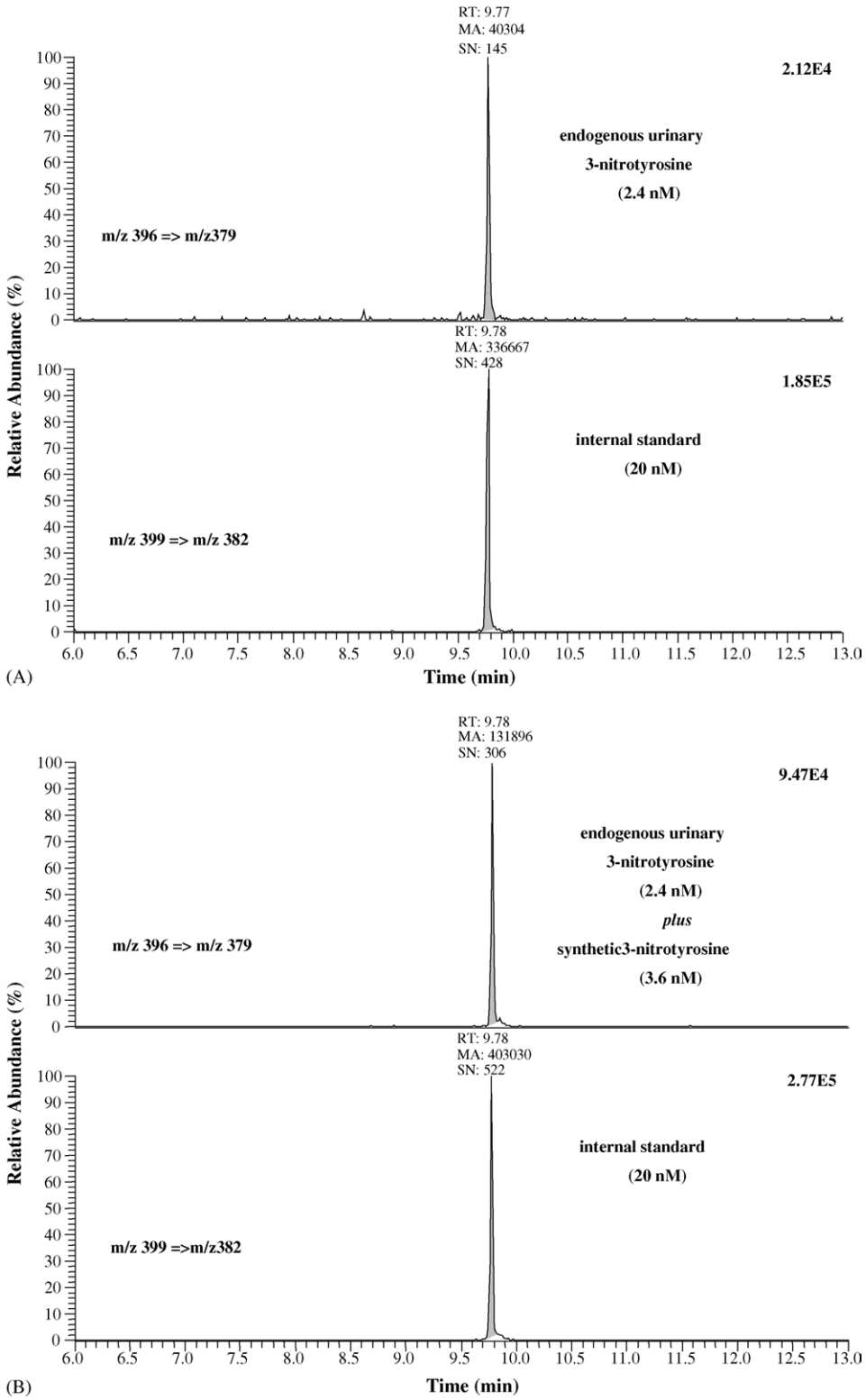

Fig. 1. Partial chromatograms from the GC–tandem MS analysis of 3-nitrotyrosine in an unspiked (A) and in a spiked (B) urine sample from a healthy volunteer. SRM of *m/z* 379 (from *m/z* 396) for endogenous NO_2Tyr and *m/z* 382 (from *m/z* 399) for d_3-NO_2Tyr of the *n*-propyl-PFP-TMS ether derivatives was performed. Endogenous 3-nitrotyrosine mean concentration in this urine amounted to 2.4 nM (A). The urine was spiked with 3.6 nM of synthetic 3-nitrotyrosine (B). See also Fig. 2.

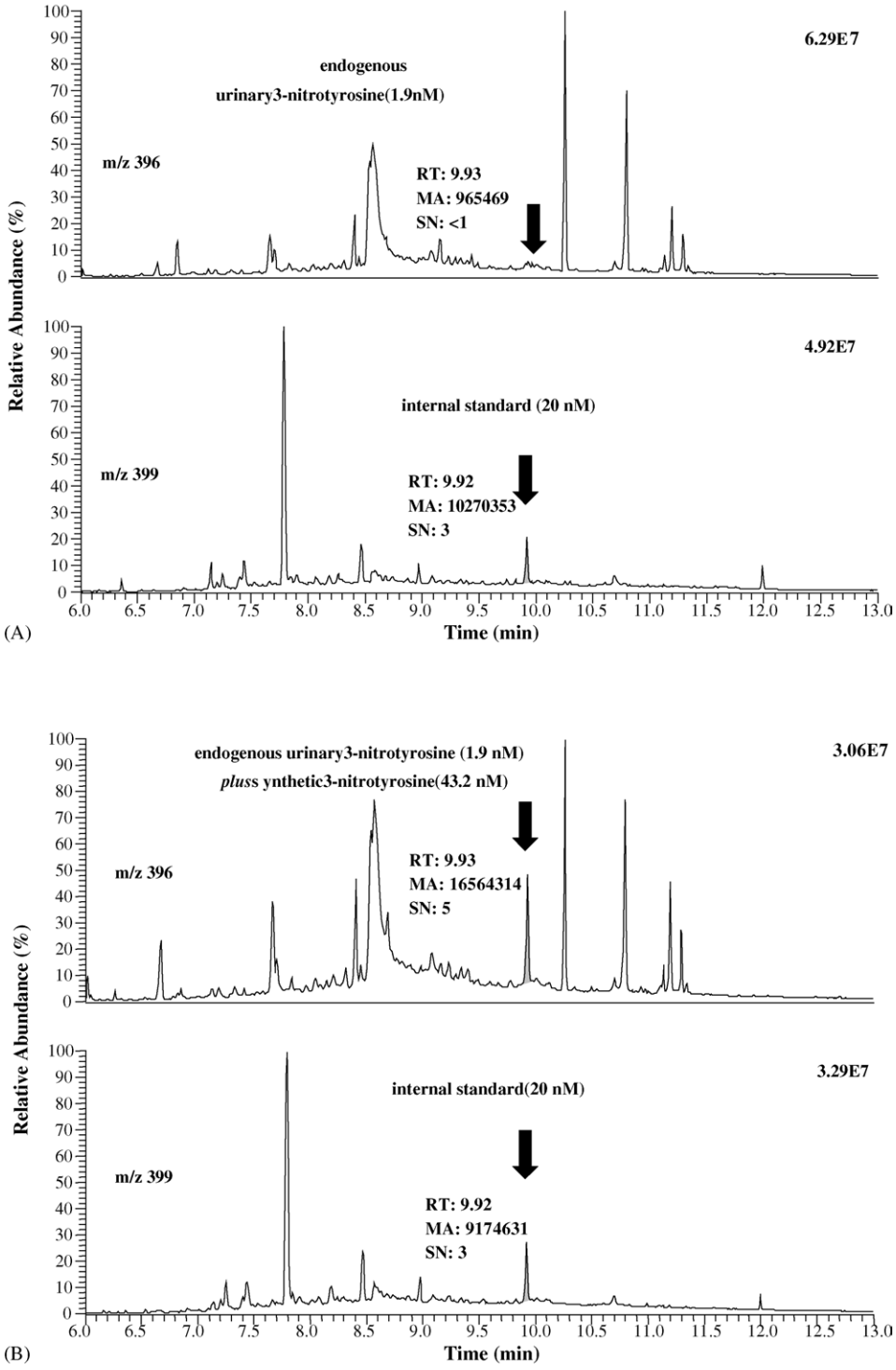

Fig. 2. Partial chromatograms from the GC–MS analysis of 3-nitrotyrosine in an unspiked (A) and in a spiked (B) urine sample from a healthy volunteer. SIM of *m*/*z* 396 for endogenous NO_2Tyr and *m*/*z* 399 for d_3-NO_2Tyr of the *n*-propyl-PFP-TMS ether derivatives was performed. The same sample was injected as in Fig. 1. Endogenous 3-nitrotyrosine concentration amounted to 1.9 nM (A). Note that in this case the urine was spiked with 43.2 nM of synthetic 3-nitro-L-tyrosine (B). Arrows indicate the retention time of the *n*-propyl-PFP-TMS ether derivatives of 3-nitrotyrosine. See also Fig. 1.

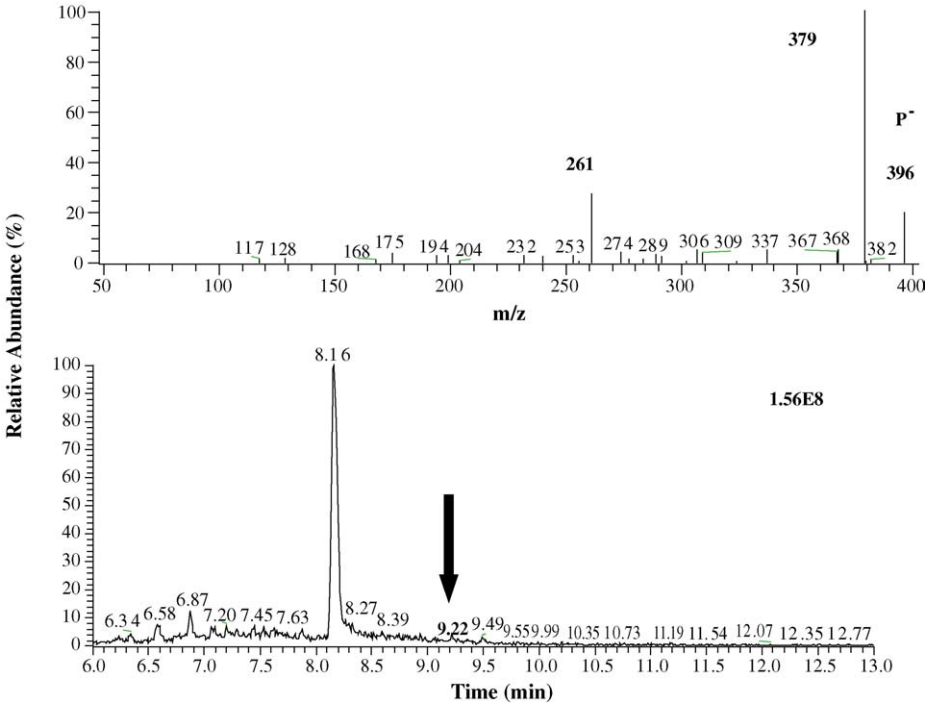

Fig. 3. Product ion mass spectrum (upper trace) of the *n*-propyl-PFP-TMS ether derivative, peak indicated by an arrow in the total ion current chromatogram (lower trace), eluting with the retention time of *n*-propyl-PFP-TMS ether derivative of synthetic 3-nitro-L-tyrosine (peak with the retention time of 9.22 min). Human urine (1 ml) was spiked with d_3-NO_2Tyr (20 nM), diluted with the mobile phase (1:10, v/v), analyzed by HPLC, and the HPLC fraction with the retention time of synthetic 3-nitro-L-tyrosine was collected. After complete derivatization the parent ion P^- ($[M - TMSOH]^-$) at *m/z* 396 was subjected to CAD at a collision energy of 6 eV and the product ions were scanned between *m/z* 50 and *m/z* 400. TMSOH, trimethylsilanol. It should be noted that differences in the retention times of 3-nitrotyrosine in the figures are the result of sample analysis within a relatively long period of time.

In the present article we describe a GC–tandem MS method for the quantification of 3-nitrotyrosine in human urine at the basal state. This method is a modification of a GC–tandem MS method originally developed for and applied to quantify free 3-nitrotyrosine in human plasma at the basal state [7]. The previous method included preparation of plasma ultrafiltrate under mild conditions, HPLC analysis of an aliquot (200 μl) of the ultrafiltrate, and isolation of 3-nitrotyrosine from the respective HPLC fraction by SPE. In the present method urine is diluted with the mobile phase (1:10, v/v), an aliquot (200 μl) of this dilution is analyzed by a HPLC system with a mobile phase comprising of 50 mM ammonium sulfate in water-methanol (95:5, v/v), which allows renunciation of the SPE step to isolate 3-nitrotyrosine from the HPLC fraction. This proceeding enables isolation of urinary 3-nitrotyrosine without artifactual formation from endogenous tyrosine and nitrate which is abundantly present in human urine [18]. Samples injected into the HPLC

Table 4
Basal levels of 3-nitrotyrosine in urine of healthy humans[a]

Subject	Sex	Age (years)	Creatinine (mM)	3-Nitrotyrosine	
				nM	nmol/mmol creatinine
1	Female	33	12.8	13.1	1.023
2	Female	41	9.53	2.46	0.258
3	Female	35	15.7	4.53	0.289
4	Female	43	3.38	3.61	1.068
5	Female	28	39.5	2.04	0.052
6	Female	26	26.2	1.61	0.061
7	Male	43	25.5	33.2	1.302
8	Male	29	43.4	2.09	0.048
9	Male	46	42.9	18.7	0.436
10	Male	41	36.1	2.18	0.060
Mean ± S.D.		36.5 ± 7.2	25.5 ± 14.7	8.4 ± 10.4	0.46 ± 0.49

[a] 3-Nitrotyrosine was quantified by GC–tandem MS. The concentration of the internal standard, i.e. d_3-NO_2Tyr, was 20 nM in all urine samples. Urine from spontaneous micturition was used.

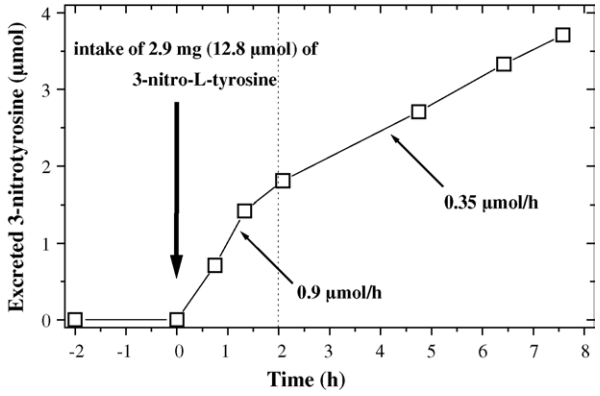

Fig. 4. Excretion of 3-nitrotyrosine in the urine after oral intake of 2.9 mg (12.8 μmol) of synthetic 3-nitro-L-tyrosine by a healthy volunteer (one of the authors). Two hours and immediately before administration urine was collected. At time "zero" 2.9 mg of 3-nitro-L-tyrosine diluted in 200 ml of drinking water were taken orally (indicated by the arrow). In addition, further 200 ml of drinking water were drunk subsequently. Urine samples were collected as indicated. Urine samples were spiked with d_3-NO_2Tyr (20 nM), treated and quantified by GC–tandem MS as described in Experimental except for dilution (1:10, v/v) by BSTFA of derivatized samples from urine samples collected after 3-nitro-L-tyrosine administration. Basal 3-nitrotyrosine levels amounted to 5.9 nM (at "−2 h") and 4.6 nM (at "0 h").

apparatous contain endogenous 3-nitrotyrosine at a concentration similar to that present in plasma ultrafiltrate, i.e. of the order of 0.8 nM (see Table 4) [7], and the internal standard at 2 nM. 3-Nitrotyrosine at these levels can be easily quantified by this GC–tandem MS method which has a LOD of 4 amol [7]. Typically, endogenous 3-nitrotyrosine from plasma is detected by our GC–tandem MS method at an S/N ratio of 200:1 [6]. This sensitivity is comparable to that of the present method for urinary 3-nitrotyrosine and the internal standard (Fig. 1). To test the applicability of the modified GC–tandem MS method to plasma 3-nitrotyrosine, we analyzed a plasma sample from a healthy volunteer for 3-nitrotyrosine. The chromatogram shown in Fig. 5 indicates that the modified method is well suited to quantify plasma free 3-nitrotyrosine at the basal state. The concentration of endogenous 3-nitrotyrosine in the plasma sample analyzed was determined to be 0.4 nM which is close to the 3-nitrotyrosine levels measured in human plasma by the original GC–tandem MS method [6]. Thus, the modified GC–tandem MS methods seems to be equally suited for the quantification of free 3-nitrotyrosine in plasma and urine of humans at the basal state.

Plasma 3-nitrotyrosine cannot be accurately quantified by GC–MS or LC–MS because of lack of selectivity [6]. In the present work GC–MS revealed considerably higher peaks than GC–tandem MS, but the S/N ratio was much higher in

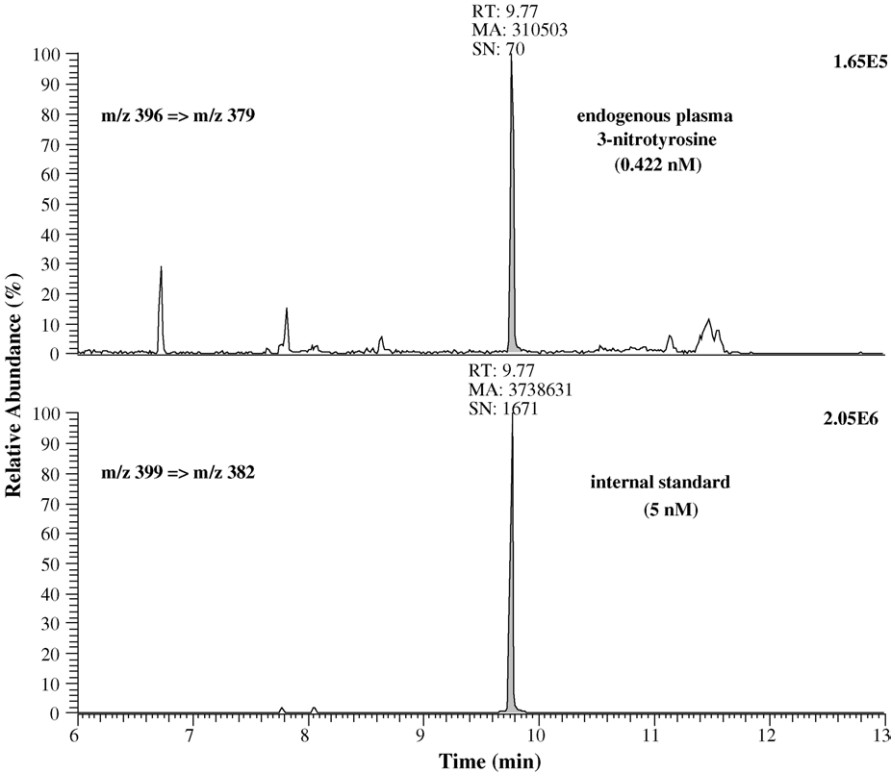

Fig. 5. Partial chromatogram from the GC–tandem MS analysis of an unspiked plasma sample from a healthy volunteer for free 3-nitrotyrosine. SRM of *m/z* 379 (from *m/z* 396) for endogenous NO_2Tyr and of *m/z* 382 (from *m/z* 399) for d_3-NO_2Tyr of the *n*-propyl-PFP-TMS ether derivatives was performed. Endogenous free 3-nitrotyrosine mean concentration amounted to 0.422 nM. The internal standard (d_3-NO_2Tyr) was added to human plasma at a final concentration of 5 nM. Plasma (2 ml) was ultrafiltered by centrifugation as described [7]. An aliquot of 200-μl was analyzed by the HPLC system described in the present work but without dilution. Further sample treatment was the same as described here for urine samples.

GC–tandem MS, emphasizing the higher sensitivity of the GC–tandem MS methodology for biological samples. The recovery values summarized in Table 2 suggest that the LOQ of urinary 3-nitrotyrosine is much lower in GC–tandem MS compared to GC–MS. Thus, we may reasonably expect that quantification of urinary 3-nitrotyrosine at the basal state by GC–MS by the present method can not be performed by the same degree of accuracy as by GC–tandem MS.

4.2. Basal levels of 3-nitrotyrosine in human urine

In urine of apparently healthy volunteers we estimated by GC–tandem MS a mean urinary daily excretion of 4.6 nmol of 3-nitrotyrosine. To the best of our knowledge this value is the first to be measured by a MS-based methodology and the lowest so far reported for urinary 3-nitrotyrosine in healthy humans. By means of a HPLC method with fluorescence detection the mean daily urinary excretion rate of 3-nitrotyrosine by healthy humans was reported to be 60 nmol [15]. A GC assay with flame ionization detection revealed values for urinary 3-nitrotyrosine of 248 nmol per day [13]. This value is approximately 54 times higher than that measured by the present GC–tandem MS method. Moreover, by means of HPLC with UV absorbance detection, similar to that used here (i.e. with a LOD of approximately 100 nM for 3-nitrotyrosine), 3-nitrotyrosine was measured in urine of patients administered with radiocontrast media at approximately 5.5 μM or 11.3 μmol/mmol creatinine [16]. These values are more than 1000 times higher than those measured by GC–tandem MS in the present study in healthy humans. The HPLC–UV method applied in that study has been originally developed to measure 3-nitrotyrosine in coronary sinus effluent in rats [19]. This HPLC–UV method has not been validated for this matrix or urine and plasma from humans [16]. Remarkably, urinary 3-nitrotyrosine concentrations of the order of 5 μM were obtained in our study only after oral intake of 12.8 μmol of 3-nitrotyrosine, an amount that is excreted by humans within an approximate period of 8 years. These concentrations could not be measured by HPLC in our study. Application of GC–tandem MS to clinical studies revealed that changes in plasma 3-nitrotyrosine concentrations are only very moderate [6]. Thus, even in patients with chronic renal failure free 3-nitrotyrosine is elevated by a factor of only 2 in comparison with healthy subjects [6]. We may, therefore, reasonably assume that the extremely high urinary levels of 3-nitrotyrosine measured by HPLC–UV [16] were produced by co-eluting interfering compounds.

The GC–tandem MS methods previously described for circulating free 3-nitrotyrosine and newly reported here for urinary 3-nitrotyrosine are characterized by high specificity, selectivity and sensitivity and allow for accurate quantification of 3-nitrotyrosine in these biological fluids. LC–tandem MS is greatly supporting of the GC–tandem MS data with regard to circulating 3-nitrotyrosine. The suitability of LC–tandem MS to urinary 3-nitrotyrosine has not been shown so far. On the basis of the tandem mass spectrometry methodology some of the high levels and dramatic changes of 3-nitrotyrosine in human plasma observed in earlier work has not proved real [5,6]. It may, therefore, be assumed that urinary levels obtained from the use of non-MS methods, notably those based on GC and HPLC–UV, may also not prove real.

4.3. Evaluation of urinary excretion of 3-nitro-L-tyrosine after oral intake

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 29% of administered 3-nitro-L-tyrosine was excreted in the urine within the observation time of 7.5 h. In the present study we did not investigate the metabolic pathway of 3-nitro-L-tyrosine and the urinary excretion of putative metabolites. 3-Nitro-L-tyrosine orally given to rats (667 μg/kg body weight), i.e. at a much higher dose than in the present study, was found to be metabolized to 3-nitro-4-hydroxyphenylacetic acid (NHPA) and 3-nitro-4-hydroxyphenyllactic acid (NHPL) [20]. Of the administered dose, about 44% and 5% were excreted in the urine after 5 days as NHPA and NHPL, respectively, revealing NHPA as the major urinary metabolite of 3-nitro-L-tyrosine in the rat [20]. It should be, however, pointed out that in that study NHPA was determined after extraction from strongly acidified urine, i.e. from pH 1, so that abundant artifactual formation of NHPA from 4-hydroxyphenylacetic acid and nitrate can not be excluded [20]. In that study urinary excretion of unchanged 3-nitro-L-tyrosine had not been investigated. It has been reported that NHPA is excreted in the urine of smokers and non-smokers at comparable mean excretion rates of 2.7 μg/day (17.8 nmol/day) and 2.9 μg/day (19.1 nmol/day), respectively [20], suggesting a mean urinary NHPA concentration of 15 nM in humans. In young healthy non-smoking volunteers a urinary excretion rate of NHPA of 2.3 ± 2.7 nmol/mmol creatinine was measured by GC–tandem MS [21]. Interestingly, creatinine-corrected NHPA excretion is five times higher [21] than the creatinine-corrected excretion of 3-nitrotyrosine measured in the present study. Further investigations are needed to evaluate the high variation of the urinary excretion of 3-nitrotyrosine and NHPA. Studies on the relationship between 3-nitrotyrosine and its metabolite NHPA in urine may answer the question whether the precursor or the metabolite is the better biomarker of oxidative stress in humans.

5. Conclusions

The present method, which is an extension of a previous method successfully applied to quantify circulating 3-nitrotyrosine, allows for the accurate quantification of 3-nitrotyrosine in human urine. This GC–tandem MS method is complicated by the use of a HPLC step for sample purification

which hampers high-throughput analysis. Following the application of our method we believe that the use of earlier, less sensitive and unspecific methods to quantify 3-nitrotyrosine led to erroneous conclusions. We argue that the choice of analytical quantitative methods by investigators should be directed to reliability rather than to simplicity and rapidity, in particular when these methods are used in clinical studies.

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