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Bollenbach, Alexander; Bakker, Stephan J. L.; Tsikas, Dimitrios

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ORIGINAL ARTICLE

GC–MS measurement of biological N^G-hydroxy-L-arginine, a stepmotherly investigated endogenous nitric oxide synthase substrate and arginase inhibitor

Alexander Bollenbach[1] · Stephan J. L. Bakker[2,3] · Dimitrios Tsikas[1]

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Abstract

L-Arginine is converted by nitric oxide synthase (NOS) to L-citrulline and nitric oxide (NO). N^G-Hydroxy-L-arginine (NOHA) is the isolable intermediate of this reaction. NOHA has been identified in biological samples by gas chromatography–mass spectrometry (GC–MS) and quantified by high-performance liquid chromatography (HPLC). Reportedly, NOHA concentrations in human plasma and serum range over four orders of magnitude (e.g., 2 nM–34 µM). The natural occurrence of NOHA in urine has not been reported thus far. Here, we report a validated stable-isotope dilution GC–MS method for the quantitative determination of NOHA in 10-µL aliquots of human serum and urine samples. The method is based on a two-step derivatization of NOHA to the methyl ester pentafluoropropionyl (PFP) derivatives using newly synthesized trideuteromethyl ester NOHA (d_3Me-NOHA) as the internal standard and GC–MS quantification. NOHA was found to form a methyl ester-N^G,N^δ,N^α-pentafluoropropionyl derivative, i.e., Me-$(PFP)_3$ (M, 642) with the N^G-hydroxy group remaining non-derivatized. Selected-ion monitoring of mass-to-charge (m/z) ratio of 458 for endogenous NOHA and m/z 461 for d_3Me-NOHA in the negative-ion chemical ionization mode revealed NOHA concentrations of the order of 0.2 µM in human serum and 3 µM in urine samples. Accuracy (recovery, %) was 91.6 ± 1.6% in serum and 39.9 ± 4.5% in urine. Inorganic nitrate was found to decrease NOHA recovery from urine presumably through the reaction of the OH group of NOHA with nitric acid. Imprecision (RSD, %) ranged between 1.4 and 14.8% in serum, and between 5.3 and 18.4% in urine in the investigated concentration range (0–15 µM NOHA). Ten healthy kidney donors excreted in the urine (mean ± SEM) 13.9 ± 1.81 µmol NOHA per day before and 10.9 ± 1.4 µmol NOHA per day after kidney donation ($P = 0.24$). Similar results were observed for dimethylamine (DMA), the major urinary metabolite of asymmetric dimethylarginine (ADMA). Changes in NOHA and DMA correlated positively ($r = 0.718$, $P = 0.019$). This is the first report on the occurrence and measurement of NOHA in human urine and on the effect of human unilateral nephrectomy on urinary NOHA and DMA. Healthy kidney donation may be useful as a model for kidney disease.

Keywords Arginase · L-Arginine · Asymmetric dimethylarginine · Dimethylamine · N^G-Hydroxy-L-arginine · Mass spectrometry · Nitrate · Nitric oxide · Nitric oxide synthase

Abbreviations

ADMA	Asymmetric dimethylarginine
DDAH	Dimethylarginine dimethylaminohydrolase
DMA	Dimethylamine
EI	Electron ionization
GC–MS	Gas chromatography–mass spectrometry

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✉ Dimitrios Tsikas
tsikas.dimitros@mh-hannover.de

[1] Institute of Toxicology, Core Unit Proteomics, Hannover Medical School, Carl-Neuberg-Strasse 1, 30623 Hannover, Germany

[2] Division of Nephrology, Department of Internal Medicine, University Medical Center, Groningen, The Netherlands

[3] Groningen Kidney Center, Groningen, The Netherlands

IS	Internal standard
LC–MS	Liquid chromatography–mass spectrometry
LC–MS/MS	Liquid chromatography–tandem mass spectrometry
LLOQ	Lower limit of quantitation
LPS	Lipopolysaccharide
MeOH	Methanol
m/z	Mass-to-charge
N^G	Guanidine nitrogen
NO	Nitric oxide
NOHA	N^G-Hydroxy-L-arginine
NOS	Nitric oxide synthase
iNOS	Inducible nitric oxide synthase
OPA	*o*-Phthaldialdehyde
PAR	Peak area ratio
PFP	Pentafluoropropionyl
PFPA	Pentafluoropropionic anhydride
PRMT	Protein arginine methyltransferase
RSD	Relative standard deviation
SDMA	Symmetric dimethylarginine
TNF-α	Tumor necrosis factor alpha

Introduction and historical retrospect

Nitric oxide synthase (NOS; EC 1.14.13.39) oxidizes one of the two guanidine nitrogen (N^G) atoms of L-arginine (Arg) to nitric oxide (NO) and L-citrulline (Förstermann and Sessa 2012). In humans, less than 0.1% of Arg is converted by NOS to NO (Tsikas 2008). N^G-Hydroxy-L-arginine (NOHA) is an isolable intermediate of this reaction (Fig. 1). This is supported by the observation that NOHA itself serves as a substrate for all NOS isoforms (Zembowicz et al. 1991; Moali et al. 1998). The inducible NOS (iNOS) isoform is Ca^{2+}-independent and possesses the highest activity among the NOS isozymes. This is presumably the reason why NOHA could be isolated in vitro from macrophages and be detected in vivo in rat and possibly in human serum at concentrations in the lower µM-range (Hecker et al. 1995a; Meyer et al. 1997; Wigand et al. 1997). NOHA was reported to be an inhibitor (K_i, 42 µM) of rat liver arginase (EC 3.5.3.1) (Daghigh et al. 1994) and of bovine liver arginase (K_i, 150 µM; Boucher et al. 1994). NOHA was moreover found to inhibit arginase activity ($IC_{50} \geq 15$ µM) and Arg transport ($IC_{50} \geq 500$ µM) in rabbit and rat alveolar macrophages (Hecker et al. 1995b). NOHA was detected by high-performance liquid chromatography (HPLC) and radiochemical detection in human endothelial cells, with phorbol dibutyrate having a stronger effect on NOHA formation than lipopolysaccharide (LPS), tumor necrosis factor alpha (TNF-α) or rapamycin in human endothelial cells (Barilli et al. 2012).

In serum of rats treated with bacterial LPS, the NOHA concentration was reported to increase from about 4 µM to 16 µM as measured by HPLC with fluorescence detection; this increase was accompanied by a more than 14-fold increase in the serum concentration of nitrite and nitrate (Hecker et al. 1995a), the major NO metabolites (Tsikas 2008). Decreased plasma concentrations of NOHA have been measured in patients with combined cardiovascular risk factors (6 µM) compared to healthy subjects (11 µM); therefore, NOHA has been proposed as a marker of diminished NO synthesis (Garlichs et al. 2000). NOHA was found in the aqueous humor of nondiabetic (0.4 µM) and diabetic (0.8 µM) patients independent of retinopathy (Hattenbach et al. 2000). Median plasma NOHA concentrations in healthy subjects and in patients with sickle cell disease were reported to be of the order of 2 µM, while the median plasma NOHA concentration was 34 µM in patients with renal insufficiency (creatinine > 220 µM) (Kato et al. 2009), as measured by a previously reported liquid chromatography–tandem mass spectrometry (LC–MS/MS) method (Nicholls et al. 2007). Yet, this LC–MS/MS method has been originally reported for Arg and some of its metabolites, but not for NOHA (Nicholls et al. 2007). In addition, the authors did not report any validation data of the method for NOHA (Nicholls et al. 2007; Kato et al. 2009).

Ishizaka and colleagues measured by HPLC with fluorescence detection NOHA concentrations in rabbit endothelial cells from thoracic aorta of 5.1 µM in normoglycemia and 2.3 µM in hyperglycemia (Ishizaka et al. 2007). Asymmetric dimethylarginine (ADMA) is an endogenous NOS inhibitor produced by protein arginine methyltransferase (PRMT; EC 2.1.1.125), which catalyzes the asymmetric dimethylation of Arg residues in proteins (Tsikas 2008) (Fig. 1). The intracellular concentration of ADMA was 1.5 µM in case of normoglycemia and 3.0 µM in case of hyperglycemia. This suggests hyperglycemia-induced elevation of the synthesis of ADMA and/or decrease of the activity of dimethylarginine dimethylaminohydrolase (DDAH; EC 3.5.3.18), which hydrolyzes ADMA to dimethylamine (DMA) and L-citrulline (Tsikas 2008) (Fig. 1). DMA is the major metabolite of ADMA (Achan et al. 2003). Urinary DMA may, therefore, serve as a measure of whole-body asymmetric dimethylation of Arg residues in proteins. The comparably very small and statistically insignificantly lower (by 12%) intracellular Arg concentrations of 313 µM in normoglycemic and of 275 µM in hyperglycemic rabbits suggests that NOHA does not only act as an arginase inhibitor, but may also be associated with the activity of DDAH. Yet, this is not known at present. It is worthy to mention that many N^G-methoxyalkyl analogs of Arg are inhibitors of DDAH activity (Leiper et al. 2007; Murphy et al. 2016). In addition, small synthetic NOHA-containing peptides including histone H4 have been recently

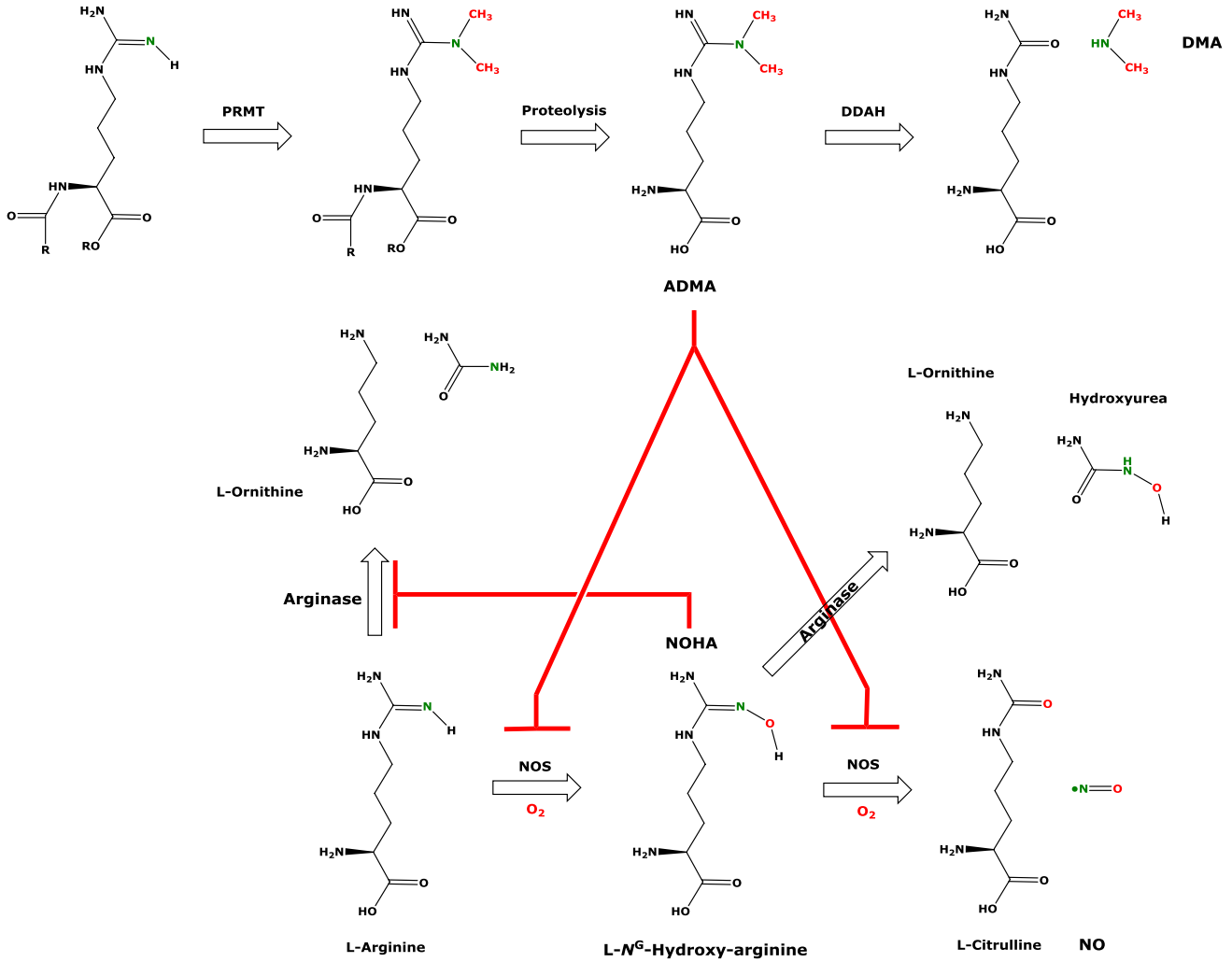

Fig. 1 Simplified schematic of few pathways involving L-arginine. Nitric oxide synthase (NOS) catalyzes the conversion of L-arginine to L-citrulline and nitric oxide (NO) via intermediate formation of L-N^G-hydroxy-arginine (NOHA). Arginine residues in proteins are asymmetrically methylated by protein arginine methyltransferase (PRMT). Proteolysis of these proteins releases asymmetric dimethylarginine (ADMA) which inhibits NOS activity. ADMA is hydrolyzed by dimethylarginine dimethylaminohydrolase (DDAH) to L-citrulline and dimethylamine (DMA). Non-metabolized ADMA and DMA are excreted in the urine. NOHA inhibits arginase activity. It is hypothesized that arginase hydrolyzes NOHA to L-ornithine and hydroxyurea which circulates in blood and is excreted in the urine

reported to inhibit PRMT1 activity in vitro (Brown et al. 2018).

NOHA has been proposed as an ethnic specific indicator of estrogen-receptor negative (ER^-) breast cancer (Mohan et al. 2012, 2016, 2018a, b). By means of an LC–MS/MS method, NOHA was found to be present in ER^+ breast cancer tumor cells at concentrations of about 54 nM, at concentrations in the range 16–25 nM in ER^- breast cancer tumor cells, and at concentrations in the order of 62 nM in control mammary cells, while in plasma of healthy and breast cancer subjects the NOHA concentration was reported to range between 2 and 10 nM (Mohan et al. 2016). The lower limit of quantification (LLOQ) of the LC–MS/MS method was reported to range between 1.5 and 5.0 pM for Arg, NOHA, L-ornithine and L-citrulline (Mohan et al. 2016). The validation of the LC–MS/MS method used in that study has been reported later by this group (Mohan et al. 2018a). In that study, the previously reported NOHA concentrations (Mohan et al. 2016) were confirmed (Mohan et al. 2018a). In Fig. 1 of the article, the authors reported that the concentrations of Arg and NOHA are of the nM-order, whereas in the systemic circulation the Arg concentration was stated to be ≥ 1 mM and that of NOHA in the nM-range (Mohan et al. 2018a). This group reported tissue contents of 10–400 pg/µg protein for NOHA and 1000–3000 µg/µg protein for Arg in cell lysates (Mohan et al. 2018b), suggesting that the concentration of NOHA in the cells is about 10^7–10^8 times lower than the Arg concentration. The reported LLOQ

ranges of the LC–MS/MS method of 1.5–5.0 pM for Arg, NOHA, L-ornithine (Orn) and L-citrulline (Cit) (Mohan et al. 2016) are unique and the lowest ever reported NOHA concentrations. To the best of our knowledge, these extremely low LLOQ values cannot be reached by LC–MS/MS, especially not for endogenous substances such as the amino acids Arg, Orn and Cit, which are present in µM-concentrations in plasma, serum and other biological samples (Davids et al. 2012; Martens-Lobenhoffer and Bode-Böger 2014). We speculate that at least the actual plasma concentrations of endogenous NOHA measured by Mohan and colleagues are in the lower µM-range (µM-to-nM interchange).

Thus far, GC–MS was used to identify NOHA in biological samples (Meyer et al. 1997; Garlichs et al. 2000). To the best of our knowledge, GC–MS has not been used thus far for the quantitative determination of NOHA in biological fluids, although GC–MS is widespread and used in the measurement of amino acids for many decades (Hušek and Macek 1975) including Arg and its metabolites (Kayacelebi et al. 2015). The introduction and the retrospect outlined above point out to the remarkable uncertainty with respect to the concentration of NOHA in human biological samples in health and disease. The aim of the present study was to test the utility of GC–MS for the measurement of NOHA in human serum and urine samples on the basis of procedures previously found to be useful for Arg and Arg metabolites, including symmetric dimethylarginine (SDMA), a surprisingly highly challenging analyte for GC–MS (Bollenbach et al. 2018).

Materials and methods

Chemicals and materials

Tetradeuterated methanol (CD_3OD, 99% at ^2H) was supplied by Aldrich (Steinheim, Germany). Methanol of UHPLC grade, ethyl acetate and 37% hydrochloric acid were purchased from Mallinckrodt Baker (Griesheim, Germany). L-N^G-Hydroxy-arginine acetate, the sodium salt of [^{15}N] nitrate (99 at% at ^{15}N) and toluene were purchased from Sigma (Deisenhofen, Germany). L-N^G-Hydroxy-arginine was used immediately after receipt. The solid material (1 mg) in the original brown glass flask was dissolved in 1 mL deionized water to reach a nominal concentration of 4 mM and the sample was stored in a refrigerator at 4 °C. Aliquots (15 µL, 60 nmol) of this solution were used to generate mass spectra. Pentafluoropropionic anhydride was obtained from Thermo Scientific (Dreieich, Germany). All other chemicals including ethyl acetate and boric acid were obtained from Merck (Darmstadt, Germany). Glass ware (1.5-mL autosampler vials and 0.2-mL microvials) and the fused-silica capillary column Optima 17 (15 m × 0.25 mm I.D., 0.25-µm film thickness) were purchased from Macherey–Nagel (Düren, Germany).

Urinary NOHA in kidney donors before and after donation

NOHA was measured in 10-µL aliquots of 24 h urine samples collected by 10 healthy subjects before and after donation (median [interquartile range, 1.6 [1.6–1.9] months) of one of their kidneys. The study is part of a larger prospective cohort study of renal transplant recipients and healthy donors in the northern regions of the Netherlands (Transplantlines Food and Nutrition cohort, Clinicaltrials.gov No NCT02811835) and has been reported elsewhere in detail (van den Berg et al. 2012; Frenay et al. 2015). The study protocol has been conducted in accordance with the declaration of Helsinki and was approved by the institutional ethical review board (METc 2008/186).

Sample preparation of serum and urine samples

NOHA was measured in 10-µL aliquots of native serum and urine samples. Serum proteins were precipitated by adding 50-µL aliquots of an ice-cold 2 M HCl/methanol (MeOH) solution and mixing by vortexing. Immediately thereafter the samples were centrifuged (4000×*g*, 5 min, 4 °C). Each 40-µL aliquots of the supernatants and each 10-µL aliquots of native urine samples were transferred into autosampler glass vials and the solvents were evaporated to dryness under a stream of nitrogen.

Derivatization procedures for NOHA

Methyl esters of NOHA were prepared as follows. The residues of the serum and urine samples treated as described above were reconstituted in 100-µL aliquots of a 2 M HCl/MeOH solution and the vials were tightly sealed. Esterification was performed by heating the samples for 60 min at 80 °C. After cooling to room temperature, the serum and urine residues were spiked with aliquots of the newly synthesized internal standard trideutero-methyl ester of NOHA (d_3Me-NOHA) to reach relevant concentrations with respect to human serum and urine (see next section). After complete evaporation of the solvents under a stream of nitrogen, aliquots (100 µL) of a freshly prepared pentafluoropropionic anhydride (PFPA) solution in ethyl acetate (1:4, v/v) were added, the glass vials were tightly sealed and heated for 30 min at 65 °C to prepare pentafluoropropionic (PFP) derivatives of the methyl esters. After cooling to room temperature, solvents and reagents were evaporated to dryness under a stream of nitrogen. Subsequently, residues were treated first with 200-µL aliquots of 400 mM borate buffer, pH 8.5, and the derivatives were immediately extracted by

vortex-mixing for 60 s with 200-µL aliquots of toluene. After centrifugation (4000×*g*, 5 min, 18 °C), aliquots (150 µL) of the upper organic phase were transferred into autosampler glass vials equipped with microinserts. The samples were sealed and subjected to GC–MS analyses. Aliquots (1 µL) of the toluene phase were injected in the splitless mode.

Method validation in human serum and urine

Method validation was performed on a single day, within a single run in parallel in urine and pooled serum from blood donated by a healthy volunteer. The concentration range of NOHA in serum and urine used in the present work in method validation was chosen considering reported concentration ranges for NOHA in human plasma and serum of healthy and diseased humans as outlined in the introductory section.

Method validation in serum was performed as follows. Five 10-µL aliquots of serum were treated each with 50 µL of 2 M HCl/MeOH to precipitate proteins. After centrifugation, 40-µL aliquots of the supernatants were decanted and spiked with 0, 2.5, 5, 7.5 and 10 µL of a freshly prepared aqueous NOHA solution (15 µM) to reach NOHA added concentrations of 0, 3.75, 7.50, 11.25 and 15 µM with respect to the serum volume. After evaporation under a stream of nitrogen esterification was performed. Then, the freshly prepared internal standard d_3Me-NOHA was added to reach a concentration of 3 µM with the respect to the serum volume.

For the method validation in urine, five 10-µL aliquots of a spot urine sample collected by a healthy volunteer were transferred into autosampler glass vials. Aliquots of 0, 2.5, 5, 7.5 and 10 µL of the same freshly prepared aqueous NOHA solutions (15 µM) used for serum were added to the urine samples to reach NOHA added concentrations of 0, 3.75, 7.50, 11.25 and 15 µM with respect to the urine volume. After evaporation under a stream of nitrogen esterification was performed. Then, the freshly prepared internal standard d_3Me-NOHA was added to reach a concentration of 3 µM with respect to the urine volume.

The serum and urine samples were then subjected to PFPA derivatization, followed by solvent extraction with borate buffer and toluene as described above. From the toluene extracts of the serum and urine samples, 1-µL aliquots were analyzed thrice on two different days by GC–MS as described below.

Precision was determined from multiple analyses in serum and urine and is expressed as relative standard deviation (RSD, %). Accuracy (recovery, %) of the method for added NOHA concentrations in serum and urine was determined by dividing the difference of measured and basal NOHA concentration by the added NOHA concentration and by multiplying the immediate result with 100.

Effect of inorganic nitrate on the NOHA measurement

Seven samples containing a fixed amount of NOHA (0.6 nmol) and increasing amounts of ^{15}N-nitrate (0, 0.6, 1.2, 2.4, 12, 120 and 360 nmol), another seven samples containing a tenfold higher fixed amount of NOHA (6 nmol) and increasing amounts of ^{15}N-nitrate (0, 6, 12, 24, 120, 1200 and 3600 nmol) were subjected to esterification with 2 M HCl/MeOH as described above. The ^{15}N-nitrate-to-NOHA molar ratio in both sets was 0, 1, 2, 4, 20, 200 and 600. Subsequently, all samples were spiked with 0.3 nmol of d_3Me-NOHA and PFPA derivatization was performed as described above. The final toluene extracts were analyzed by SIM of *m/z* 458 for NOHA *m/z* 461 for the internal standard.

GC–MS conditions

GC–MS analyses were performed on a GC–MS apparatus consisting of a single-stage quadrupole mass spectrometer model ISQ, a Trace 1210 series gas chromatograph and an AS1310 autosampler from Thermo Fisher (Dreieich, Germany). Aliquots of 1 µL toluene extracts were injected in the splitless mode. The injector temperature was kept at 280 °C. Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. The oven temperature was held at 40 °C for 0.5 min and ramped up to 210 °C at a rate of 15 °C/min and then to 320 °C at a rate of 35 °C/min; finally the column was kept at 320 °C for 1 min. Interface and ion-source temperatures were set to 300 and 250 °C, respectively. Electron energy was 70 eV and electron current 50 µA. Methane (2.4 mL/min) was used as the reagent gas for both negative-ion chemical ionization (NICI) and positive-ion chemical ionization (PICI). Mass spectra of NOHA derivatives were generated by scanning in the mass-to-charge (*m/z*) range of 50–1000. In quantitative analyses, the dwell time was 100 ms for each ion in the selected-ion monitoring (SIM) mode. The electron multiplier voltage was set to 1900 V.

Results and discussion

GC–MS characterization of NOHA derivatives

GC–MS analysis of the Me-PFP derivatives of unlabelled (d_0Me-PFP) and deuterium-labelled (d_3Me-PFP) NOHA revealed several GC peaks. The mass spectra of three peaks were found to contain corresponding mass fragments, differing by 3 Da, most likely due to the presence of three ^{2}H (D) atoms (i.e., CD_3) in both ionization modes. The retention times of these peaks were 7.43 min (Peak I), 8.66 min (Peak II), and 9.75 min (Peak III) (Fig. 1S). In the NICI and PICI mass spectra of the NOHA derivatives no mass fragments

with a difference of 6 Da were observed. This suggests that the OH group carrying N^G atom of NOHA is not methoxymated and the guanidine group of NOHA is not hydrolyzed to form a dicarboxylic ester during the first esterification step. Previously, we found that SDMA reacts with PFPA to form relatively stable mixed anhydrides with PFPA (Bollenbach et al. 2018). In the present study, NOHA was found not to form mixed anhydrides under the same derivatization conditions (data not shown).

The most likely structure of Peak I is N^{α},N^G,N^G,*O*-pentafluoropropionyl methyl ester; its spectrum contained the most intense ion at *m/z* 276 for d_0Me-$(PFP)_4$ and the ion at *m/z* 279 for d_3Me-$(PFP)_4$ (Fig. 2S). The proposed structures of the derivatives and the ions *m/z* 276 and *m/z* 279 are shown in Fig. 2S. The structure of Peak II for derivatized unlabeled and deuterium-labelled NOHA could not be elucidated (Fig. 3S). In the samples of the validation experiment, no linearity was observed between measured and added NOHA in serum and urine for the Peak I (SIM of *m/z* 276 and *m/z* 279, or SIM of *m/z* 625 and *m/z* 628 in the PICI mode) and for the Peak II (SIM of *m/z* 665 and *m/z* 668 in the NICI mode) (see below for more details). Thus, Peaks I and II seem to be subjects of serious interferences and were not further considered. Below, the GC–MS spectra of Peak III are presented and discussed.

The GC–NICI–MS mass spectra of the Me-PFP derivatives of NOHA (Peak III) with the retention time of 9.79 min are shown in Fig. 2. The most intense ions were *m/z* 187, 233, and 458 for the d_0Me-PFP derivative (Fig. 2a) and *m/z* 187, 236, and 461 for the d_3Me-PFP derivative (Fig. 2b). The proposed structures of these ions and the potential underlying fragmentation mechanisms are illustrated in the Supplement to this article (Fig. 4S). The ions *m/z* 458 and *m/z* 461 are obviously methyl esters which are likely to result from loss of a PFP moiety (147 Da), an OH group (17 Da) from the non-derivatized OH group of the NOHA derivative followed by loss of a HF molecule (20 Da). The anions *m/z* 233 for d_0Me-PFP and *m/z* 236 for d_3Me-PFP differ by 3 Da and are therefore methyl esters produced most likely by a cleavage of the NOHA skeleton at C2 and C3. This type of fragmentation has also been observed for Me-PFP derivatives of other amino acids including ADMA (Tsikas et al. 2003). The most intense anion at *m/z* 187 is common to the d_0Me-PFP and d_3Me-PFP derivatives, and is therefore, not a methyl ester moiety. This ion is most likely a fragment of the guanidine group formed upon loss of the non-derivatized OH group of NOHA as OH (17 Da) followed by a cleavage of the amino acid skeleton between N^{δ} and the C atom of the guanidine group (Fig. 4S). The anion *m/z* 187 has not been observed in the NICI mass spectra of Me-PFP derivatives of other amino acids including ADMA (Tsikas et al. 2003) and SDMA (Bollenbach et al. 2018) and could, therefore, be assumed as specific to NOHA. These NICI mass spectra suggest that the NOHA derivatives with the retention times of 9.79 and 9.77 min are d_0Me-$(PFP)_3$ and d_3Me-$(PFP)_3$, respectively. This would mean that the OH group of NOHA is not derivatized, is stable against hydrochloric acid and heat, and is lost first during the NICI process (Fig. 4S).

The GC–PICI–MS mass spectra of the Me-PFP derivatives of NOHA are shown in Fig. 3. The most intense ions were *m/z* 55, 57, 164 and 479 for the d_0Me-PFP derivative (Fig. 3a), and *m/z* 55, 57, 164 and 482 for the d_3Me-PFP derivative (Fig. 3b). The proposed structures of and possible fragmentation mechanisms leading to these cations are illustrated in the Supplement to this article (Fig. 5S). The corresponding ions *m/z* 479 and *m/z* 482 are obviously methyl esters which are likely to result from loosing each of a PFP moiety (147 Da) and the OH group (17 Da) of NOHA. These PICI mass spectra suggest that the NOHA derivatives with the retention times of 9.79 and 9.77 min (Peak III) are d_0Me-$(PFP)_3$ (*M*, 642) for the unlabeled NOHA and d_3Me-$(PFP)_3$ (*M*, 645) for the deuterium-labelled NOHA derivatives. The OH group of the Me-PFP derivatives NOHA is obviously not derivatized and is lost first during the PICI upon protonation by protons derived from the reactand gas methane (Fig. 5S). Under the same GC–MS conditions including the oven program, the retention times of the Me-PFP derivatives of Orn and Arg were 8.3 min and 9.37 min, respectively. Orn (*m/z* 418 and *m/z* 421) and Arg (*m/z* 586 and *m/z* 589), two potential decomposition products of NOHA, were found in the toluene extracts of the unlabeled and deuterium-labelled NOHA (data not shown), yet they were not quantified.

To our knowledge, the first GC–MS spectrum of NOHA as Me-PFP derivative was reported by Hecker and colleagues (Meyer et al. 1997). The electron ionization (EI) mass spectrum of the Me-PFP derivative contained the following reported mass fragments and intensities with the proposed structures: *m/z* 547 ($[M-(CF_2CF_3)_2]^+$ (8%), *m/z* 402 ($[M-(CF_2CF_3)_3-OCH_3]^+$ (6%), *m/z* 374 ($[M-(CF_2CF_3)_3-CO-CH_3]^+$ (12%), *m/z* 276 $[(CH_2)_3-CH(CO-OCH_3)-NH-COCF_2CF_3]^+$ (5%), and *m/z* 216 ($[(CH_2)_3-CH(NHCOCF_2CF_3)]^+$ (100%). This ion assignment suggests that the NOHA derivative is Me-*N,N,N,O*-$(PFP)_4$ (M, 785), i.e., the OH group of NOHA is esterified by PFPA under conditions similar to those used in our study. Thus, we performed PFPA derivatization for 30 min at 65 °C, while the derivatization procedure described by Meyer et al. was performed for 60 min at 110 °C. The majority of the ions in the reported GC–EI–MS spectrum of the Me-*N,N,N,O*-$(PFP)_4$ derivative have very low intensity, and the base peak at *m/z* 216 is not specific to NOHA. Rather, this ion is found in the GC–EI–MS spectra of the Me-PFP derivatives of many amino acids including Arg and ADMA (Tsikas et al. 2003).

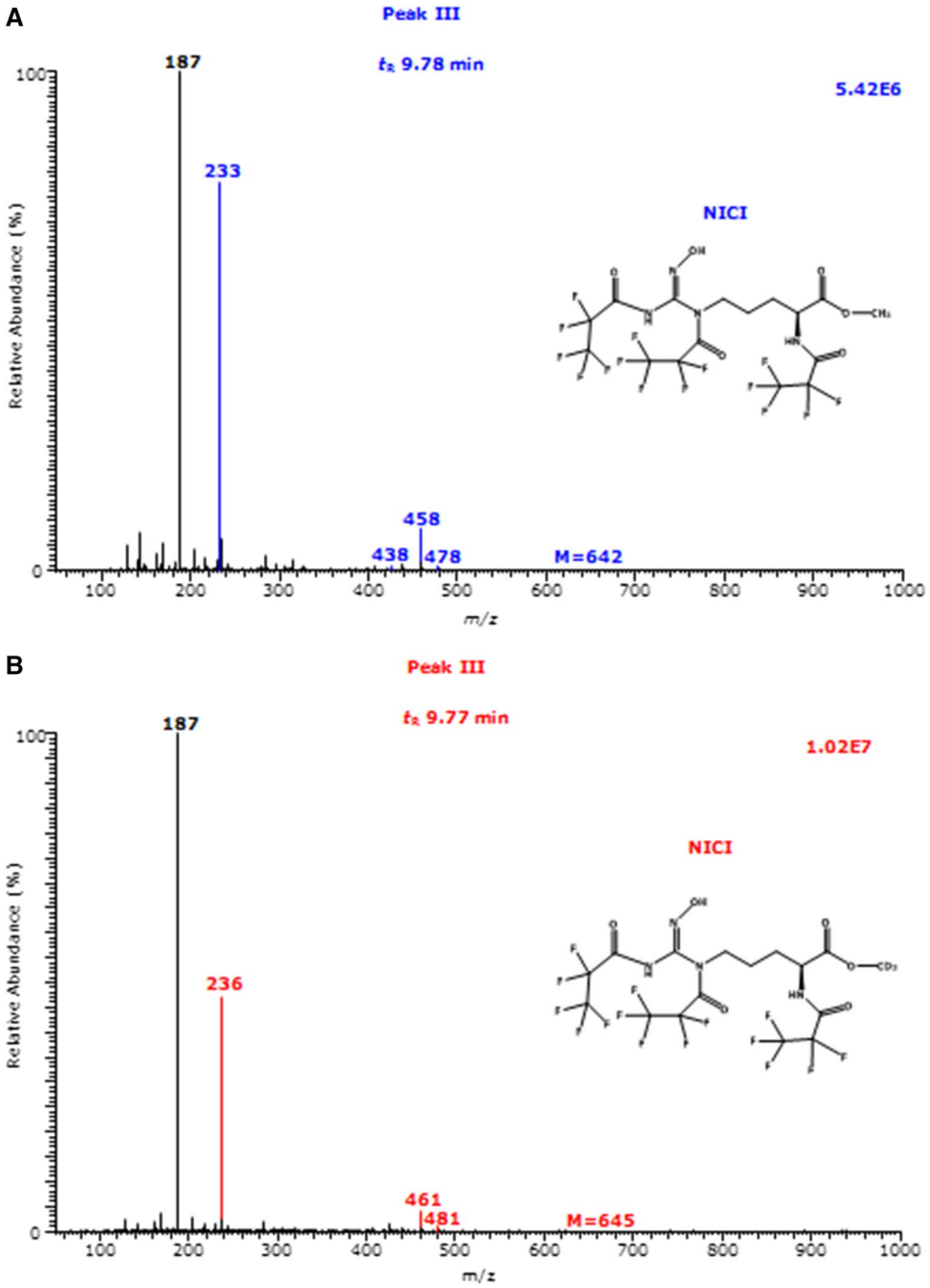

Fig. 2 GC–NICI–MS spectra of **a** synthetic unlabeled NOHA and **b** synthetic deuterium-labelled NOHA after esterification with 2 M HCl in CH_3OH (**a**) or 2 M HCl in CD_3OD (**b**) followed by *N*-acylation with pentafluoropropionic anhydride in ethyl acetate (Peak III; see Fig. 1S). Inserts show the proposed structures of the derivatives

NOHA has been analyzed by GC–PICI–MS as isopropyl ester (iPr) PFP derivative (Garlichs et al. 2000). The GC–PICI–MS spectrum of the iPr-PFP derivative of NOHA was assigned to iPr-$(PFP)_3$ (*M*, 670), with the OH group of NOHA being non-derivatized. The protonated molecular cation has not been observed in the spectrum. The most intense mass fragment in the spectrum was *m/z* 507 which was interpreted as $[(M-NH_2-CO-CF_2CF_3)]^+$ (i.e., $[(M-163)]^+$ (Garlichs et al. 2000). Although loss of NH_2–CO–CF_2CF_3 may indeed produce the cation *m/z* 507, the more likely mechanism leading to *m/z* 507 is loss of the O atom (16 Da) of the OH group of NOHA and loss of a PFP moiety ($COCF_2CF_3$, 147 Da). The cation *m/z* 507 of iPr-$(PFP)_3$ corresponds most likely to the cation *m/z* 479 of Me-$(PFP)_3$ we observed in GC–PICI–MS spectrum of NOHA. The PFPA derivatization step described by Garlichs et al. was performed for 15 min at 110 °C. With respect to the derivative identity obtained from NOHA, our results agree with those reported by Garlichs et al. for the iPr-$(PFP)_3$ derivative (Garlichs et al. 2000), but disagree with those reported by Meyer et al. for Me-$(PFP)_4$ (Meyer et al. 1997).

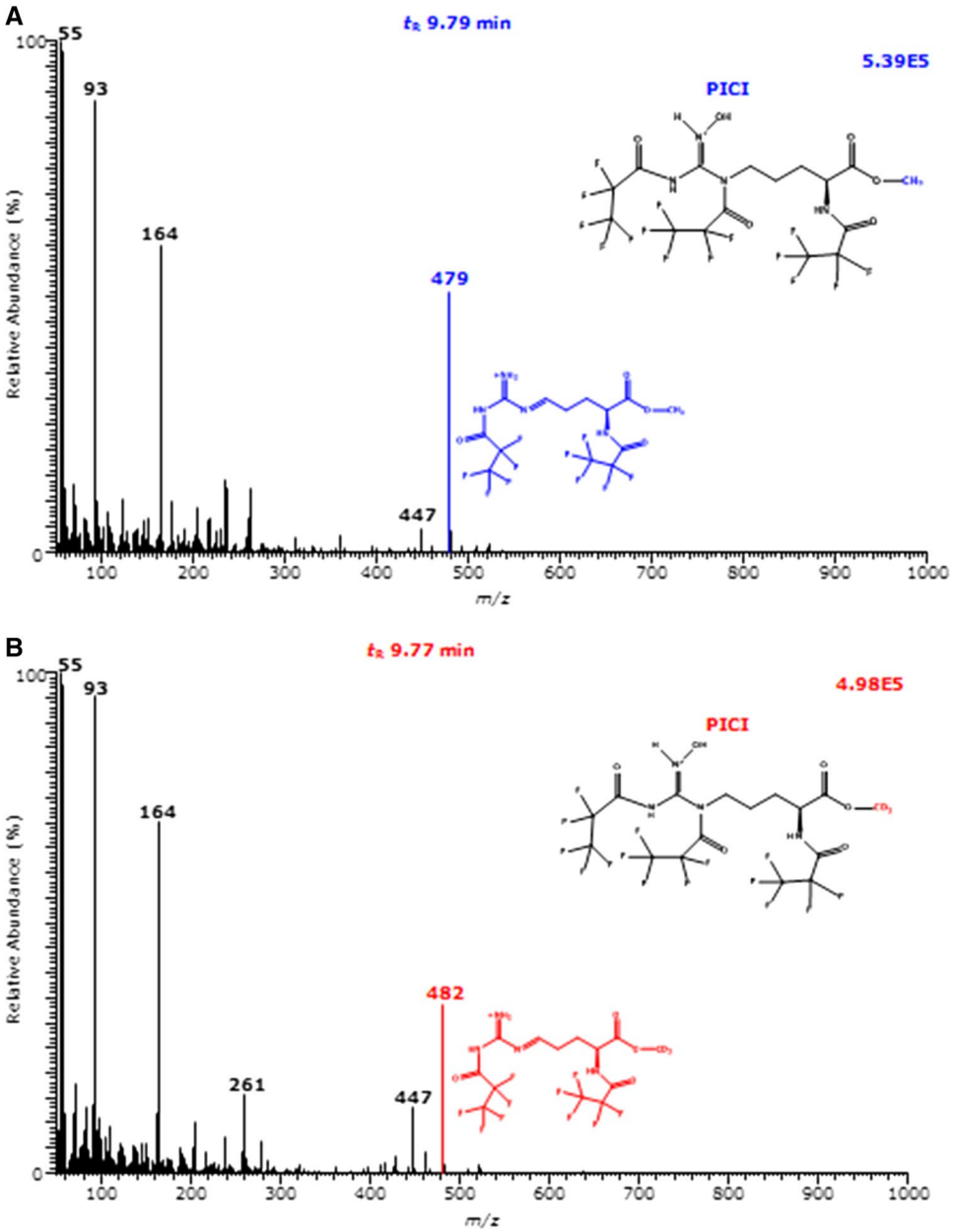

Fig. 3 GC–PICI–MS spectra of **a** synthetic unlabeled NOHA and **b** synthetic deuterium-labelled NOHA after esterification with 2 M HCl in CH_3OH (**a**) or 2 M HCl in CD_3OD (**b**) followed by *N*-acylation with pentafluoropropionic anhydride in ethyl acetate (Peak III; see Fig. 1S). Inserts show the proposed structures of the derivatives and the cations with *m/z* 479 (**a**) and 482 (**b**)

Oximes (R–C=N–OH) and methoximes (R–C=N–OCH_3) may elute in HPLC and GC–MS systems as two partially or entirely separated peaks due to their *syn*- and *anti*-isomers (Tsikas et al. 1998; Cipollina et al. 2009). In the present study we have no indication of the occurrence of *syn* (*Z*) and *anti* (*E*) isomers of synthetic NOHA. NOHA produced enzymatically from Arg is expected to occur rather in a single not yet identified form. About the synthetic NOHA no information is available regarding *syn*-/*anti*-isomerism.

GC–MS measurement of circulating and urinary NOHA—method validation

Given the considerably higher extent of ionization in the NICI mode compared to the PICI mode (by a factor of about 50; see Figs. 2, 3), NOHA can be quantitated much more sensitively in the NICI mode. GC–MS quantification of NOHA in human serum and urine can be performed by selected-ion monitoring (SIM) of *m/z* 233 for unlabeled and

m/z 236 for trideuteromethyl-NOHA which served as internal standard. Yet, higher specificity is achieved by SIM of *m/z* 458 for unlabeled and *m/z* 461 for deuterium-labelled NOHA. The GC–NICI–MS method for NOHA reported in this article was partially validated in 10-µL aliquots of serum and urine samples of a healthy volunteer using trideutero-NOHA as the internal standard. GC–MS chromatograms from the analysis of NOHA in the validation experiments are shown in Fig. 4. Because the NOHA peaks are very small and overshadowed by large peaks eluting in front of the NOHA peak in the chromatograms, only a very narrow retention time window is illustrated as magnification.

The results of the validation experiment in human serum and urine samples are summarized in Table 1. The basal NOHA concentrations were 0.22 µM in the serum and 0.93 µM in the urine samples. In contrast to the basal urinary NOHA concentration of 0.93 µM (Fig. 4b), the serum concentration of NOHA of 0.22 µM was difficult to be quantitated in the serum sample (Fig. 4a) and is likely to be of the order of the LLOQ value of our method. Accuracy (recovery, %) was 91.6 ± 1.6% in the serum and 39.9 ± 4.5% in the urine sample. Imprecision (RSD, %) ranged between 1.4 and 14.8% in serum, and between 5.3 and 18.4% in urine in the investigated concentration range (0–15 µM NOHA). An

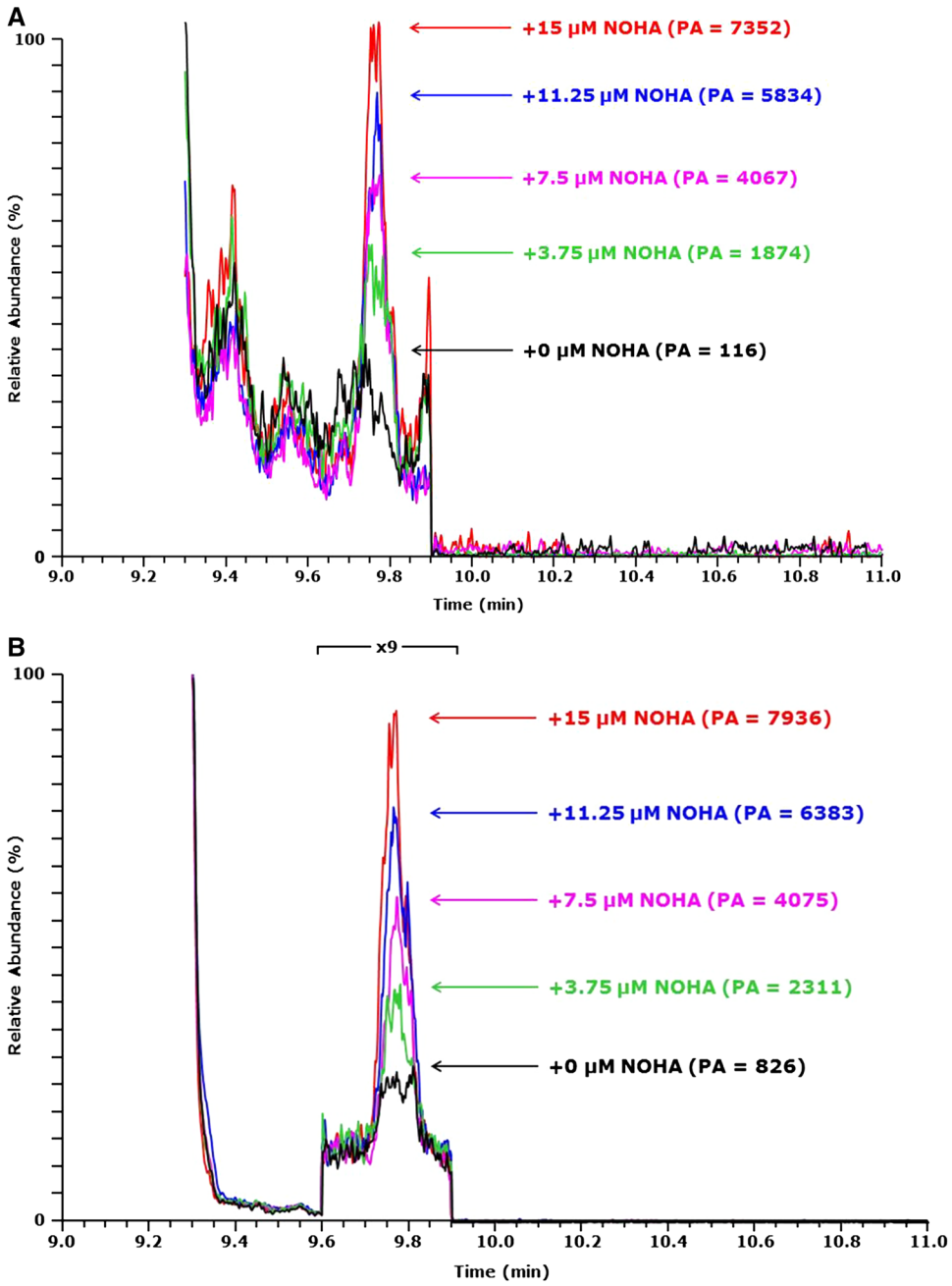

Fig. 4 Chromatograms from the GC–NICI–MS quantification (SIM of *m/z* 458 for endogenous NOHA and *m/z* 461 for the internal standard) of NOHA in human serum (**a**) and urine (**b**) samples before and after addition of synthetic NOHA at the indicated concentrations. The peaks indicate the total ion current of both ions. Note the magnification by a factor of nine in the chromatogram of the urine sample in the window time 9.6–9.9 min

Table 1 Intra-assay accuracy and imprecision of the GC–MS method for NOHA in human serum and urine samples

NOHA added (μM)	NOHA measured (μM)				Accuracy (Recovery, %)	Imprecision (RSD, %)
	1st	2nd	3rd	Mean ± SD		
Serum						
0.00	0.22	0.24	0.19	0.22 ± 0.02	Not applicable	11.6
3.75	3.63	3.70	3.73	3.69 ± 0.05	92.5	1.4
7.5	6.78	8.02	6.65	7.15 ± 0.76	92.4	10.6
11.25	11.1	11.9	8.84	10.6 ± 1.57	92.3	14.8
15.00	14.0	14.5	12.2	13.6 ± 1.19	89.2	8.8
Urine						
0.00	1.00	0.83	0.96	0.93 ± 0.09	Not applicable	9.6
3.75	2.05	2.27	2.22	2.18 ± 0.12	33.3	5.3
7.50	3.92	4.26	3.78	3.99 ± 0.25	40.8	6.2
11.25	5.67	6.49	4.94	5.70 ± 0.77	42.4	13.6
15.00	8.24	8.10	5.82	7.39 ± 1.36	43.1	18.4

explanation for the low recovery rate of NOHA from urine could be reaction of NOHA with presumably abundant urinary constituents. Possible candidates could be nitrite and nitrate, which may react with the OH group of NOHA.

By the present GC–MS method, endogenous NOHA was found to be present at about 220 nM in the pooled serum sample used in method validation. This concentration of NOHA is much lower than the NOHA concentration reported by the majority of the groups which reported on NOHA concentrations in healthy and diseased subjects. Mean serum NOHA concentration was reported to be 9.1 μM in healthy subjects (Meyer et al. 1997). Median NOHA plasma concentrations were reported to be 2.5 μM in healthy subjects, and to be 1.8 μM (range 1.4–2.4 μM) and 34 μM (range 16–63 μM) in patients with sickle cell disease with serum creatinine concentrations < 200 μM and > 200 μM, respectively (Kato et al. 2009). In plasma of healthy subjects, NOHA was measured at 10.4–12.1 μM, while in plasma of patients with metabolic syndrome NOHA was found at 6.4 μM as measured by HPLC with fluorescence detection after pre-column derivatization with *o*-phthaldialdehyde (OPA; Garlichs et al. 2000). On the other hand, another group has measured much lower NOHA plasma concentrations of about 2 nM in healthy subjects and up to 9 nM in patients with breast cancer as measured by LC–MS (Mohan et al. 2016). However, as mentioned above, we estimate that these concentrations are μM but not nM as originally reported (Mohan et al. 2016). Hattenbach and colleagues measured NOHA concentrations in aqueous humor of 0.24–1.88 μM in diabetic patients and 0.04–0.64 μM in non-diabetic patients as measured by pre-column OPA derivatization and HPLC with fluorescence detection (Hattenbach et al. 2000). At present no pharmacokinetic data are available about NOHA. Based on the pharmacokinetic data of nor-NOHA, revealing very short elimination half-life of the order of few minutes in the rat (Havlínová et al. 2014), it could be speculated that NOHA is also a short-lived species in humans, thus resulting in low circulating concentrations presumably in the middle-nM range.

To the best of our knowledge this is the very first report on the occurrence of NOHA in human urine. In the urine sample used in method validation, the basal concentration of NOHA was measured to be about 0.93 μM. Considering a recovery rate of about 33% for NOHA added at 3.75 μM to the urine sample (Table 1), the concentration of endogenous NOHA in the urine sample is estimated to be about 3 μM.

Effect of inorganic nitrate on the NOHA measurement

The concentration of nitrate in human urine may reach low mM-concentrations (Tsikas 2008). We hypothesize that under the esterification conditions used in the present study nitrate could affect the quantitative measurement of NOHA through nitration of the OH of NOHA. To exclude potential effects by urinary compounds, we investigated the effects of nitrate on the measurement of NOHA by the present method in aqueous solutions. The results of this experiment are illustrated in Fig. 5. The peak area of NOHA and the peak area of the internal standard (IS) (Fig. 5a), as well as the peak area ratio of *m/z* 458 (NOHA) to *m/z* 461 (IS) (Fig. 5b) were relatively constant up to nitrate-to-NOHA molar ratios of 20:1 in the higher concentration range for nitrate and NOHA and up to 200:1 in the lower concentration range. A similar pattern was also observed for remaining NOHA (Fig. 5c). The results presented in Fig. 5 suggest that inorganic nitrate in the form of nitric acid indeed affects the measurement of NOHA in a concentration-dependent manner, leading to loss of NOHA. Yet, even in pathophysiological conditions, the concentration of nitrate only very rarely reaches concentrations in the very low mM-range in human urine, while

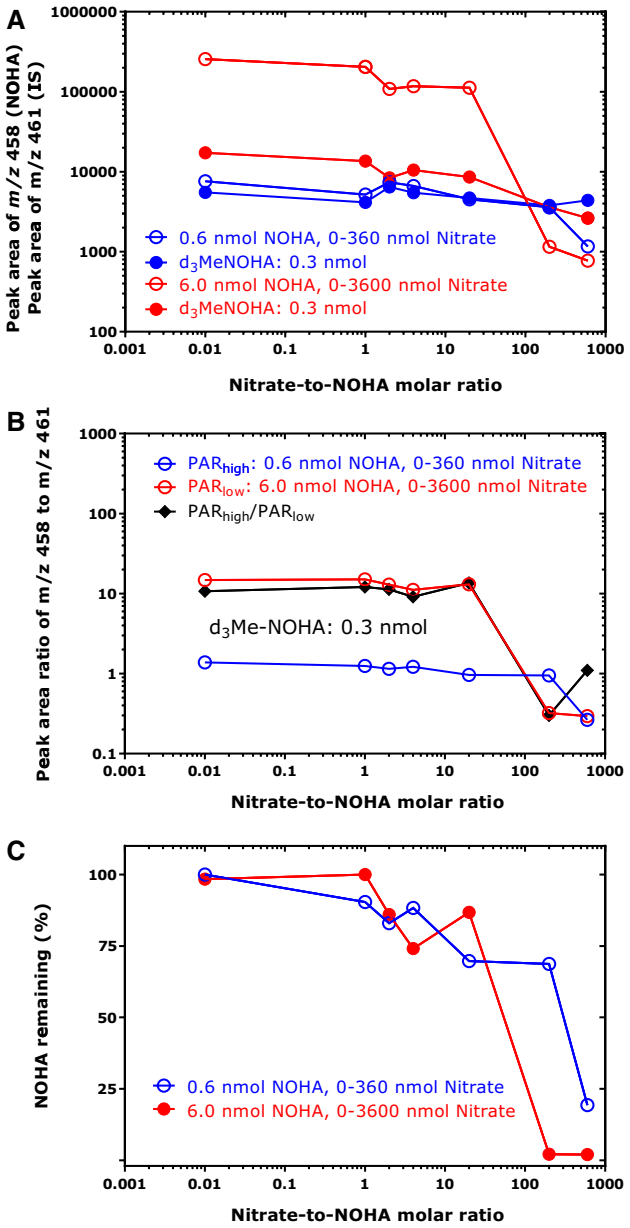

Fig. 5 Effects of ^{15}N-nitrate in two amount ranges (0–360 nmol; 0–3600 nmol) on the analysis of NOHA at two amounts (0.6 nmol; 6.0 nmol) by the present GC–NICI–MS. The amount of the internal standard was 0.3 nmol in both cases. Quantification was performed by SIM of *m/z* 458 for unlabeled NOHA and *m/z* 461 for the internal standard (IS). **a** Peak area of *m/z* 458 and *m/z* 461. **b** Peak area ratio of *m/z* 458 to *m/z* 461. **c** Percentage remaining of NOHA. The NOHA concentration in the sample not spiked with ^{15}N-nitrate was set to 100

in human serum/plasma the nitrate concentration is of the order of 50 µM (Tsikas 2008). For these reasons, the negative effect of nitrate on NOHA measurement is considered minor in serum/plasma as expressed by almost quantitative recovery (Table 1), but may be significant in urine samples that contain high concentrations of nitrate. The results of the validation experiment are supportive of this conclusion.

We did not investigate the underlying mechanism. Yet, under the HCl-acidic conditions (pK_a, − 5.9) of the esterification procedure, the OH group of NOHA is likely to be protonated, eventually leaving the molecule. Loss of NOHA during derivatization is partly compensated by the use of the IS (d_3Me-NOHA). In the presence of nitrate, additional reactions between NOHA and nitrate/nitric acid (pK_a, − 1.4) may occur and include nitration of the OH group of NOHA to form the nitric ester and perhaps subsequent decomposition.

Contribution of the kidney to urinary NOHA in healthy subjects

We used the present GC–MS method to investigate the effect of kidney donation on urinary NOHA in 10 healthy subjects who underwent unilateral nephrectomy. Kidney donation resulted in an average decrease of 22% of urinary NOHA excretion from (mean ± SEM) 13.9 ± 1.8 to 10.9 ± 1.4 µmol per day; yet, this did not reach statistical significance ($P = 0.236$, paired *t* test) (Fig. 6a). In the same subjects, unilateral nephrectomy resulted in an average decrease of 17% of urinary dimethylamine (DMA) excretion from 423 [359–520] to 353 [262–572] µmol per day ($P = 0.311$, Wilcoxon matched-pairs signed rank test) (Fig. 6b). NOHA and DMA excretion correlated with each other (Fig. 6c). There was also a correlation among the ratios of post-to-pre donation excretion rates of NOHA and DMA (Fig. 6d). These observations suggest that the kidney is a considerable contributor to NOHA and DMA, of which the latter is the major urinary metabolite of ADMA (Tsikas 2008).

Conclusions

Our study indicates that NOHA can be quantitated by GC–MS after esterification with 2 M HCl/MeOH and subsequent *N*-acylation with PFPA to form the Me-$(PFP)_3$ derivative which carries the original N^G-OH group of NOHA non-derivatized. In situ prepared trideuteromethyl ester of NOHA (d_3Me-NOHA) turned out to be a useful internal standard for the measurement of NOHA in human serum leading to almost quantitative recovery in reported concentration ranges for circulating NOHA. Our study indicates that the concentration of circulating NOHA is of the order of 0.2 µM, i.e., at least 10 times lower than circulating NOHA concentrations reported by the majority of other groups. From human urine, NOHA is recovered by about 33–43%. Our study indicates that the concentration of urinary NOHA is of the order of 3 µM. At least half of urinary NOHA seems to be lost during the esterification procedure by the reaction of its OH group with endogenous constituents, presumably

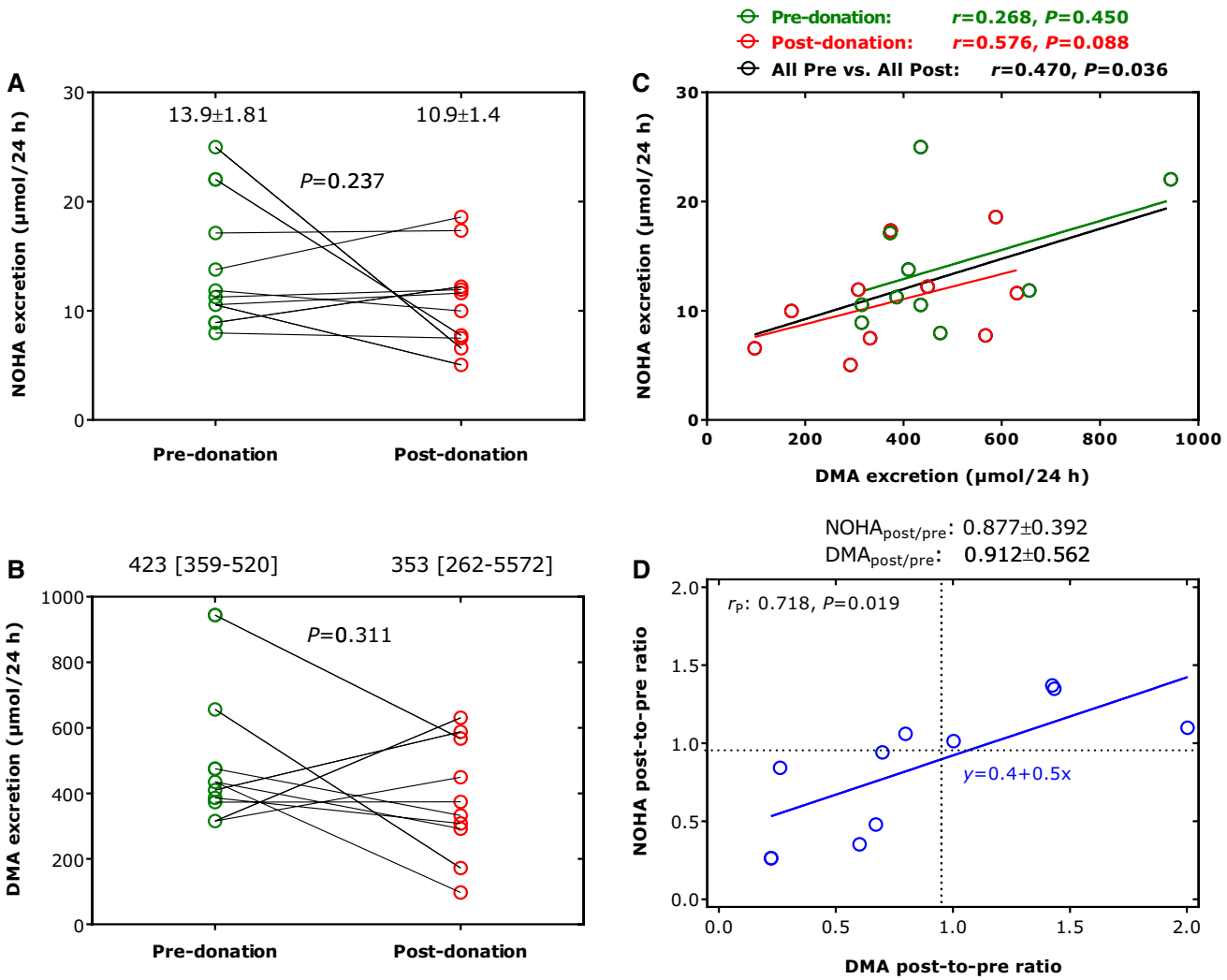

Fig. 6 Urinary excretion rates of **a** L-N^G-hydroxy-arginine (NOHA) and **b** dimethylamine (DMA) by healthy subjects before and after unilateral nephrectomy and their relationships (**c**, **d**). DMA was measured in 10-µL aliquots of urine by GC–MS as described elsewhere (Tsikas et al. 2007) with some modifications

with nitric acid from acidified nitrate, which is abundantly present in human urine. This shortcoming could be overcome using NOHA labelled with ^{15}N preferably in its guanidine group or with ^{13}C or ^{2}H in the side chain of NOHA to correct for loss of NOHA. The present GC–MS method can be further improved using larger volumes of serum, plasma and urine (e.g., 1 mL) in combination with solid-phase extraction using cation exchangers to isolate and concentrate endogenous NOHA.

Compared to other Arg metabolites related to the Arg/NOS pathway, notably ADMA and SDMA which are produced by post-translational N^G-dimethylation of Arg residues and are established risk factors in the renal and cardiovascular systems, NOHA's two synergistic biological activities, i.e., its utility as substrate for NOS and as inhibitor of arginase activity, are known for at least two decades. Reliable analytical methods for the accurate quantification of NOHA in biological samples, including blood and urine are indispensable for uncovering biological roles of NOHA and its potential pharmacological use. NOHA is physiologically present in human urine and the kidney is a considerable origin of urinary NOHA. The physiological roles of NOHA in the kidney remain to be established. The positive correlation between the urinary excretion rates of NOHA and DMA may suggest that NOHA is associated with DDAH expression and activity. The present GC–MS method should be useful in these areas. Yet, NOHA remains a challenging analyte for GC–MS.

Compliance with ethical standards

Conflicts of interest All authors report no conflicts of interest.

Ethical statement The Institutional Review Board approved the study protocol (METc 2008/186) which was in adherence to the Declaration of Helsinki.

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