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Quantitative determination of circulating and urinary asymmetric dimethylarginine (ADMA) in humans by gas chromatography–tandem mass spectrometry as methyl ester tri(*N*-pentafluoropropionyl) derivative

Dimitrios Tsikas*, Bibiana Schubert, Frank-Mathias Gutzki, Jörg Sandmann, Jürgen C. Frölich

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

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Abstract

Asymmetric dimethylarginine (ADMA; N^G,N^G-dimethyl-L-arginine) is the most important endogenous inhibitor of nitric oxide synthase and a potential risk factor for cardiovascular diseases. This article describes a gas chromatographic–tandem mass spectrometric (GC–tandem MS) method for the accurate quantification of ADMA in human plasma or serum and urine using de novo synthesized [$^2H_3$]-methyl ester ADMA (d_3Me-ADMA) as the internal standard. Aliquots (100 μl) of plasma/serum ultrafiltrate or native urine and of aqueous solutions of synthetic ADMA (1 μM for plasma and serum; 20 μM for urine) are evaporated to dryness. The residue from plasma/serum ultrafiltrate or urine is treated with a 100 μl aliquot of 2 M HCl in methanol, whereas the residue of the ADMA solution is treated with a 100 μl aliquot of 2 M HCl in tetradeuterated methanol. Methyl esters are prepared by heating for 60 min at 80 °C. After cooling to room temperature, the plasma or urine sample is combined with the d_3Me-ADMA sample, the mixture is evaporated to dryness, the residue treated with a solution of pentafluoropropionic (PFP) anhydride in ethyl acetate (1:4, v/v) and the sample is incubated for 30 min at 65 °C. Solvent and reagents are evaporated under a stream of nitrogen gas, the residue is treated with a 200 μl aliquot of 0.4 M borate buffer, pH 8.5, and toluene (0.2 ml for plasma, 1 ml for urine). Reaction products are extracted by vortexing for 1 min, the toluene phase is decanted, and a 1 μl aliquot is injected into the GC–tandem MS instrument. Quantitation is performed by selected reaction monitoring (SRM) of the common product ion at *m/z* 378 which is produced by collision-induced dissociation of the ions at *m/z* 634 for endogenous ADMA and *m/z* 637 for d_3Me-ADMA. In plasma and urine of healthy humans ADMA was measured at concentrations of 0.39±0.06 μM ($n = 12$) and 3.4±1.1 μmol/mmol creatinine ($n = 9$), respectively. The limits of detection and quantitation of the method are approximately 10 amol and 320 pM of d_3Me-ADMA, respectively.

Keywords: Derivatization, GC; Asymmetric dimethylarginine

1. Introduction

Nitric oxide synthases (NOSs; EC 1.14.13.39) are a family of enzymes which catalyse the oxidation of the imino group of the guanidino moiety of L-arginine to nitric oxide (NO), with L-citrulline being the second reaction product [1]. The L-arginine/NO biosynthetic pathway is involved in many physiological and pathophysiological processes. They include vasodilation, inhibition of platelet aggregation and adhesion, immune function, neurotransmission, inflammation, atherosclerosis, cytotoxicity, cell proliferation and apoptosis. Exogenous and endogenous N^G-substituted L-arginine analogues, i.e. N^G-nitro-L-arginine (NNA), N^G-monomethyl-L-arginine (MMA) and N^G,N^G-dimethyl-L-arginine (ADMA; 1 asymmetric dimethylarginine), are inhibitors of various NOS isoforms [2,3]. ADMA has been suggested as a potent risk factor for the development of NO-associated endothelial dysfunction and cardiovascular diseases as well as in chronic renal failure in man [4–6]. NO synthesis and endothelial function in animals and humans negatively correlate with ADMA concentration in plasma or serum [3]. Inhibition of NO production in vivo by elevated circulating ADMA concentrations in NO-associated diseases could be the basic responsible mechanism. In haemodialysis patients, circulating ADMA could be a strong and independent predictor of overall

* 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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mortality and cardiovascular outcome [4]. Accumulation of ADMA in chronic renal failure could be an important risk factor for cardiovascular disease [5].

The interest in this potentially fundamental role of ADMA led to the development of different analytical methods among which HPLC with fluorescence detection using *o*-phthaldialdehyde derivatization is currently the most frequently used and most efficient analytical approach for ADMA, other methylated L-arginine analogues such as symmetric dimethylarginine (SDMA) and MMA as well as L-arginine [7–13]. Quantitative determination by HPLC with UV absorbance detection at 205 nm of non-derivatized ADMA, SDMA, MMA and L-arginine from plasma or serum has also been reported [2]. These techniques revealed circulating ADMA levels in healthy humans of the order of 0.5 μM. Recently, L-arginine and methylated arginines including ADMA, SDMA and MMA have been quantitated in human plasma by liquid chromatography–tandem mass spectrometry (LC–tandem MS) without previous derivatization [14]. Quantitation by LC–tandem MS revealed ADMA plasma concentrations in healthy humans of the order of 0.1 μM, i.e. clearly below the levels obtained by HPLC with fluorescence or UV absorbance detection. The applicability of this LC–tandem MS method to urinary ADMA has not been reported so far [14]. In 1992, Vallance et al. reported that ADMA is present in human plasma and it is excreted into the urine as found by HPLC with UV absorbance detection and/or capillary electrophoresis (CE), with urinary excretion rate being estimated approximately 65 μmol/24 h [2]. Unfortunately, these HPLC and CE methods have not been described in detail. To the best of our knowledge, there are no other reports on urinary levels of ADMA in health and disease.

The objective of the present study was to develop an analytical method permitting reliable quantitative determination of ADMA in plasma or serum and urine of humans. The HPLC method previously reported by our group for the measurement of ADMA, SDMA and L-arginine in the circulation has found wide application in experimental and clinical studies (reviewed in [15]), but it was insufficiently reliable to quantitate these substances in human urine (unpublished data). In previous work concerning quantitative determination of 3-nitro-L-tyrosine, a further member of the L-arginine/NO pathway, we found that GC–tandem MS allows for highly accurate and sensitive quantitation of this amino acid derivative in human plasma in the low nM-range after chemical conversion to its *n*-propyl ester *N*-pentafluoropropionyl derivative [16]. However, the current lack of stable isotope-labelled ADMA analogues is a handicap in MS-based analytical methods. One possibility to overcome this difficulty is the use of commercially available stable isotope-labelled analogues of L-arginine, e.g. L-[guanidino-$^{15}N_2$]-arginine or L-[$^{13}C_6$]-arginine, as has been managed by Vishwanathan et al. in their LC–tandem MS method [14]. Because of different chromatographic and mass spectrometric behaviour and also due to highly differing concentrations of L-arginine and ADMA in plasma (e.g. 50 and 0.5 μM or even below, respectively), use of L-[guanidino-$^{15}N_2$]-arginine as an internal standard for ADMA was found by us inappropriate in GC–MS or GC–tandem MS analysis of ADMA (see Section 4). In the present work we describe a novel alternative approach which involves methylation of the carboxylic group of endogenous ADMA by using HCl in methanol and of the carboxylic group of synthetic ADMA by using HCl in tetradeuterated methanol (CD_3OD). We show that this derivatization procedure solves the problems associated with the lack of stable isotope-labelled analogues of ADMA and allows for reliable quantitative determination of ADMA in plasma, serum and urine. Moreover, this approach is extendable to SDMA, MMA and L-arginine and in principle to every amino acid and amino acid derivative.

2. Experimental

2.1. Materials and chemicals

L-Arginine, asymmetric dimethylarginine (N^G,N^G-dimethyl-L-arginine; ADMA) hydrochloride, symmetric dimethylarginine (N^G,$N^{G'}$-dimethyl-L-arginine; SDMA) hydrochloride, and monomethylarginine (N^G-monomethyl-L-arginine; MMA) were purchased from Sigma (Deisenhofen, Germany). Pentafluoropropionic anhydride (PFPA) was obtained from Pierce (Rockford, IL, USA). Amicon Ultra Millipore ultrafiltration cartridges (cut-off, 10 kDa) were supplied by Millipore Corporation (Bedford, MA, USA). L-[Guanidino-$^{15}N_2$]-arginine hydrochloride (98% at both ^{15}N atoms) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Tetradeuterated methanol (CD_3OD, 99.8% at D) was supplied by Aldrich (Steinheim, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany).

2.2. Procedure for the quantification of ADMA in the plasma and urine of humans

2.2.1. Biological samples—recovery of blood, plasma and ultrafiltrate

Blood (5 ml) was drawn from antecubital veins of healthy volunteers using syringes containing EDTA and put immediately on ice. Blood samples were centrifuged at 1500 × *g* and 2 °C for 15 min. The plasma generated was used immediately or stored at −78 °C until further analysis. Ultrafiltrates (approximately 0.7–0.8 ml) from 1 ml aliquots of plasma samples were obtained by centrifugation at 7500 × *g* and 20 °C for 20 min using the Amicon Ultra Millipore ultrafiltration cartridges.

2.2.2. Derivatization procedure

Aliquots (100 μl) of plasma ultrafiltrate or urine and of aqueous solutions of ADMA (1 μM for plasma; 20 μM for

urine) were evaporated to dryness by means of a nitrogen stream. The residues of plasma and urine were treated with 100 μl aliquots of a 2 M HCl solution in CH_3OH. The residue of the standard ADMA solution was treated with a 100 μl aliquot of a 2 M HCl solution in CD_3OD. Amino acids were methylated by heating the samples separately for 60 min at 80 °C. After cooling to room temperature, the methanolic solution of the plasma or urine sample was combined with the methanolic solution of synthetic ADMA. To ensure quantitative transfer, each residue was taken up with a 100 μl aliquot of CH_3OH and solvents and reagents of the combined solutions were evaporated to dryness by a stream of nitrogen gas. The residue was treated with a 100 μl aliquot of a solution of PFPA in ethyl acetate (1:4, v/v) and the sample was heated for 30 min at 65 °C. This procedure has been originally described for 3-nitro-L-tyrosine [16,17]. After cooling to room temperature, the sample was evaporated to dryness, the residue was reconstituted with a 200 μl aliquot of 0.4 M borate buffer, pH 8.5, and the sample was vortexed for 60 s with toluene (200 μl for plasma, 1000 μl for urine samples). Aliquots (100–800 μl) of the organic layer were taken and transferred into glass vials, from which 1 μl aliquots were analysed by GC–MS or GC–tandem MS.

2.3. Validation of the method, limits of detection and quantitation

The accuracy and precision of the method were determined using freshly obtained pooled plasma and urine from a healthy volunteer. Each 100 μl aliquot of plasma ultrafiltrate and urine samples was treated in triplicate. Synthetic ADMA serving as the internal standard was used at 1 μM for plasma and 20 μM for urine (each 100 μl aliquots). Derivatives were extracted with toluene (1 ml) from which 1 μl aliquots were analysed by GC–tandem MS as described further. Instrumental precision was determined by repeated GC–tandem MS analysis of unspiked plasma and urine samples. Intra- and interday precision was determined by working up in triplicate unspiked pooled plasma and unspiked urine samples on five consecutive days.

The limit of detection (LOD) of the method was determined using a stock solution of authentic ADMA (100 pmol). For this purpose, ADMA was converted to its d_3Me-PFP derivative as described earlier, extracted with 1000 μl of toluene which was further diluted with toluene. Each 1 μl aliquot was analysed by GC–tandem MS.

The limit of quantitation (LOQ) of the method was estimated by analyzing in triplicate a basal plasma sample (100 μl) using 100 pmol of synthetic ADMA as internal standard. The Me-PFP derivative of endogenous ADMA and the d_3Me-PFP derivative of synthetic ADMA were extracted with toluene (1000 μl) from which 1 μl aliquots were analysed by GC–tandem MS.

2.4. Determination of ADMA basal levels in plasma and urine of healthy humans

Blood and urine samples were obtained from healthy volunteers with consent. The group consisted of five males, aged 27–64 years (mean: 33 years), and seven females, aged 24–43 years (mean: 31 years). Plasma ultrafiltrate and urine samples (each 100 μl aliquots) were treated as described earlier. Synthetic ADMA serving as the internal standard was used at 1 μM for plasma and 20 μM for urine samples. Samples were analysed by GC–tandem MS as described further.

2.5. Quantitative determination of L-arginine in human plasma

Plasma (1 ml) spiked with L-[guanidino-$^{15}N_2$]-arginine (final concentration, 50 μM) was ultrafiltered as described earlier, a 10 μl aliquot of the ultrafiltrate was evaporated to dryness, and derivatization and further sample treatment was performed as described earlier for ADMA using 2 M HCl in methanol followed by PFPA. GC–MS analysis in the negative-ion chemical ionization (NICI) mode was carried out by selected ion monitoring (SIM) of *m/z* 586 for L-arginine and *m/z* 588 for the internal standard L-[guanidino-$^{15}N_2$]-arginine. GC–tandem MS analysis in the NICI mode was carried out by selected reaction monitoring (SRM) of the product ions at *m/z* 293 for L-arginine and *m/z* 295 for L-[guanidino-$^{15}N_2$]-arginine, which were produced from the corresponding parent ions at *m/z* 586 and 588 (see Section 3 for details).

2.6. GC–MS and GC–tandem MS conditions

GC–MS and GC–tandem MS analyses in the NICI mode were performed on a triple-stage quadrupole mass spectrometer 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 17 (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 of 1 ml/min) as the carrier gas: 2 min at 90 °C, then increased to 340 °C at a rate of 20 °C/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 (0.13 Pa collision pressure) were used as reagent and collision gases, respectively. Collision energy was set to 15 eV. Electron multiplier voltage was set to 2–2.5 kV. Aliquots (1 μl) were injected in the splitless mode by means of an autosampler.

GC–MS analyses were also carried out on a Hewlett-Packard MS Engine 5989A connected directly to a gas chromatograph 5890 series II (Waldbronn, Germany). A fused-silica capillary column Optima 17 (15 m × 0.25 mm

i.d., 0.25 μm film thickness) from Macherey-Nagel was used. Helium (38 kPa) and methane (200 Pa) were used as the carrier and the reagent gases, respectively, for NICI. Oven temperature was held for 1 min at 90 °C, then increased to 340 °C at a rate of 25 °C/min. Interface, injector and ion-source were kept at 280, 250 and 180 °C, respectively. The dwell time was 50 ms for each ion.

3. Results

3.1. GC–MS and GC–tandem MS characterization of unlabelled and labelled ADMA derivatives

The mass spectra obtained from GC–MS and GC–tandem MS analyses of unlabelled and labelled L-arginine and analogues are summarized in Tables 1 and 2. These data suggest that under the derivatization conditions chosen the methyl esters of unlabelled and labelled amino acids were entirely *N*-acylated to form their pentafluoropropionyl derivatives (Scheme 1). Thus, L-arginine was converted to its methyl ester tetra(*N*-pentafluoropropionyl) derivative, and the methyl ester of ADMA formed the tri(*N*-pentafluoropropionyl) derivative. The molecular ions have not been obtained from the methyl ester *N*-pentafluoropropionyl derivatives of all compounds analysed or they had weak intensity. In the NICI mass spectrum of the ADMA derivative, the most intense ion was *m*/*z* 634 (Fig. 1, upper panel) which results from neutral loss of HF (20 Da) from the molecular anion of *m*/*z* 654 of the entirely derivatized molecule ($M = 654$). In the NICI mass spectrum of the d_3Me-ADMA derivative the most intense ion was *m*/*z* 637 (Fig. 1, lower panel) which results from neutral loss of HF (20 Da) from the molecular anion of *m*/*z* 657 of the entirely derivatized molecule ($M = 657$).

Collision-activated dissociation (CAD) of the parent anions $[M - HF]^-$ of ADMA and d_3Me-ADMA, i.e. *m*/*z* 634 and 637, respectively, resulted in the generation of numerous product ions of the same *m*/*z* value due to the loss of the trideuteromethyl group, but also of product ions differing by 3 Da due to the presence of the trideuteromethyl moiety in these ions (Fig. 2). For quantification of ADMA as methyl ester *N*-perfluoroacyl derivative in the SRM mode the most intense and common product ions at *m*/*z* 378 were used.

3.2. Recovery of the methylation reaction

The yield of ADMA methyl ester from the methylation reaction was determined by HPLC analysis [11] of five

Table 1
Electron impact ionization GC–MS mass spectra of the methyl ester *N*-pentafluoropropionyl derivatives of unlabelled and labelled L-arginine and methylated L-arginine analogues

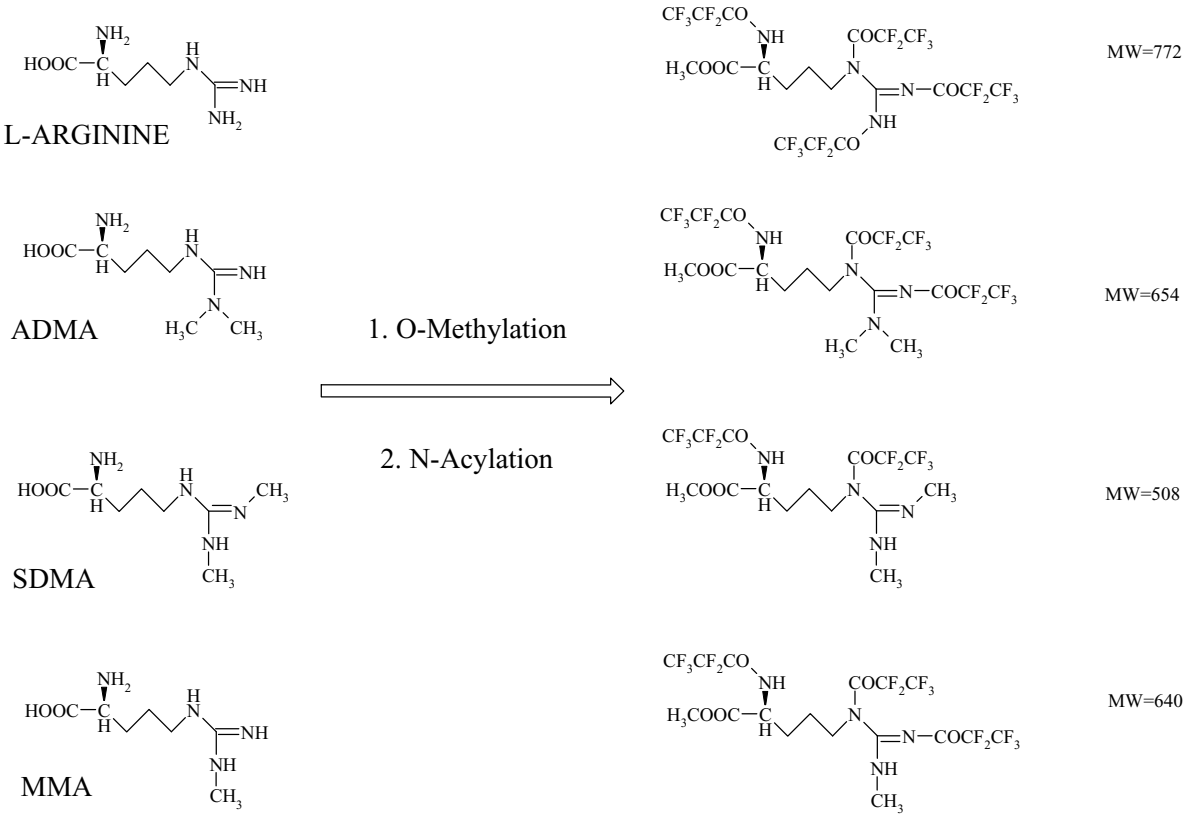
L-Arginine	L-[Guanidino-$^{15}N_2$]-arginine *m*/*z* (intensity, %)	ADMA	d_3-ADMA	MMA	d_3-MMA
216 (100)	216 (100)	216 (100)	216 (100)	216 (18)	216 (20)
–	234 (12)	234 (13)			
–	248 (15)	–			
260 (15)	262 (26)	260 (23)	260 (23)	260 (10)	260 (10)
276 (37)	276 (48)	276 (86)	279 (74)	276 (12)	279 (10)
284 (48)	286 (59)	–	–	–	
365 (20)	367 (19)	–	–	–	
378 (16)	380 (14)			375 (100)	378 (100)
392 (50)	394 (46)	–	–	–	
404 (23)	406 (25)	–	–	–	
507 (80)	509 (82)	–	–	–	
–	–	535 (47)	538 (17)		
626 (10)	628 (10)	–	–	–	

Mass spectra were generated on the Hewlett-Packard GC–MS instrument. Listed are ions with *m*/*z* ≥200 and intensity greater than 10%.

Table 2
Negative-ion chemical ionization GC–MS mass spectra of the methyl ester *N*-pentafluoropropionyl derivatives of unlabelled and labelled L-arginine and methylated L-arginine analogues

L-Arginine	L-[Guanidino-$^{15}N_2$]-arginine *m*/*z* (intensity, %)	ADMA	d_3-ADMA	MMA	d_3-MMA
–	–	233 (17)	236 (15)	–	–
462 (10)	464 (12)	–	–	474 (100)	477 (100)
566 (20)	568 (25)	–	–	–	–
586 (100)	588 (100)	–	–	–	–
606 (15)	608 (15)	616 (16)	619 (14)	–	–
–	–	634 (100)	637 (100)	–	–

Mass spectra were generated on the Hewlett-Packard GC–MS instrument. Listed are ions with *m*/*z* ≥200 and intensity ≥10%.



Scheme 1. Chemical structures of L-arginine, asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA) and monomethylarginine (MMA) (left panel), and of their methyl ester *N*-pentafluoropropionyl derivatives (right panel). Methyl esters are prepared using 2 M HCl in methanol. Subsequently, *N*-pentafluoropropionyl derivatives are prepared using pentafluoropropionic anhydride. For more details, see Section 2.

aliquots of a 1 μM aqueous solution of ADMA before and after methylation. These measurements revealed that 13.6% of ADMA remained underivatized, suggesting that 86.4 ± 0.95% of ADMA were converted into the methyl ester.

In addition, the methylation reaction of ADMA was investigated by GC–tandem MS as follows. Each two 100 μl aliquots of a 1 μM aqueous solution of ADMA were derivatized by using 2 M HCl in (A) CH_3OH, (B) CD_3OD, and in mixtures of CH_3OH–CD_3OD containing (C) 25%, (D) 50%, and (E) 75% (v/v) of CD_3OD. GC–tandem MS analysis of entirely derivatized ADMA was performed. The peak area ratio of *m/z* 378 from *m/z* 637 to *m/z* 378 from *m/z* 634 of the ADMA derivatives was determined to be $2.54 \pm 0.23 \times 10^{-3}$ in (A), 835 ± 0.1 in (B), 0.361 ± 0.003 in (C), 1.115 ± 0.008 in (D), and 3.278 ± 0.013 in (E). Linear regression analysis between the peak area ratio measured (*Y*) and the molar ratio of CD_3OD to CH_3OH (*X*), with the exception of sample (B), resulted in a straight line with the regression equation: $Y = 0.006 + 1.092X$, $R = 0.9997$. This result indicates that the methylation of the carboxylic group of ADMA with CD_3OD and CH_3OH proceeds with very similar yields.

The methylation reaction of L-arginine was also investigated by the present GC–tandem MS method. Each five 1 ml aliquots of human plasma and human urine were spiked with L-[guanidino-$^{15}N_2$]-arginine to achieve a final concentration of 10 μM each. Aliquots (100 μl) of the plasma ultrafiltrate samples and of urine samples were evaporated to dryness. Subsequently, methylation was performed by using 2 M HCl in unlabelled methanol. Again, samples were evaporated to dryness and methylation was repeated using 2 M HCl in CD_3OD. PFPA derivatization and further sample treatment was performed as described for ADMA. GC–tandem MS analysis was carried out by SRM of the product ions at *m/z* 295 for the methyl (CH_3) ester PFP derivative of L-[guanidino-$^{15}N_2$]-arginine and at *m/z* 298 for the trideuteromethyl (CD_3) ester PFP derivative of L-[guanidino-$^{15}N_2$]-arginine. These product ions were produced from the corresponding parent ions at *m/z* 588 and 591. The peak area ratio of *m/z* 295 to *m/z* 298 of the L-[guanidino-$^{15}N_2$]-arginine derivatives was determined to be 16.5 ± 1.6 in the plasma and 12.3 ± 0.8 in the urine samples. These findings suggest that the methylation yield of L-arginine from the plasma and urine samples is of the order of 90%. The higher portion of the CD_3 ester PFP derivative of L-[guanidino-$^{15}N_2$]-arginine in the urine sample is most likely due to the higher portion of L-[guanidino-$^{15}N_2$]-arginine in the urine as compared with the plasma.

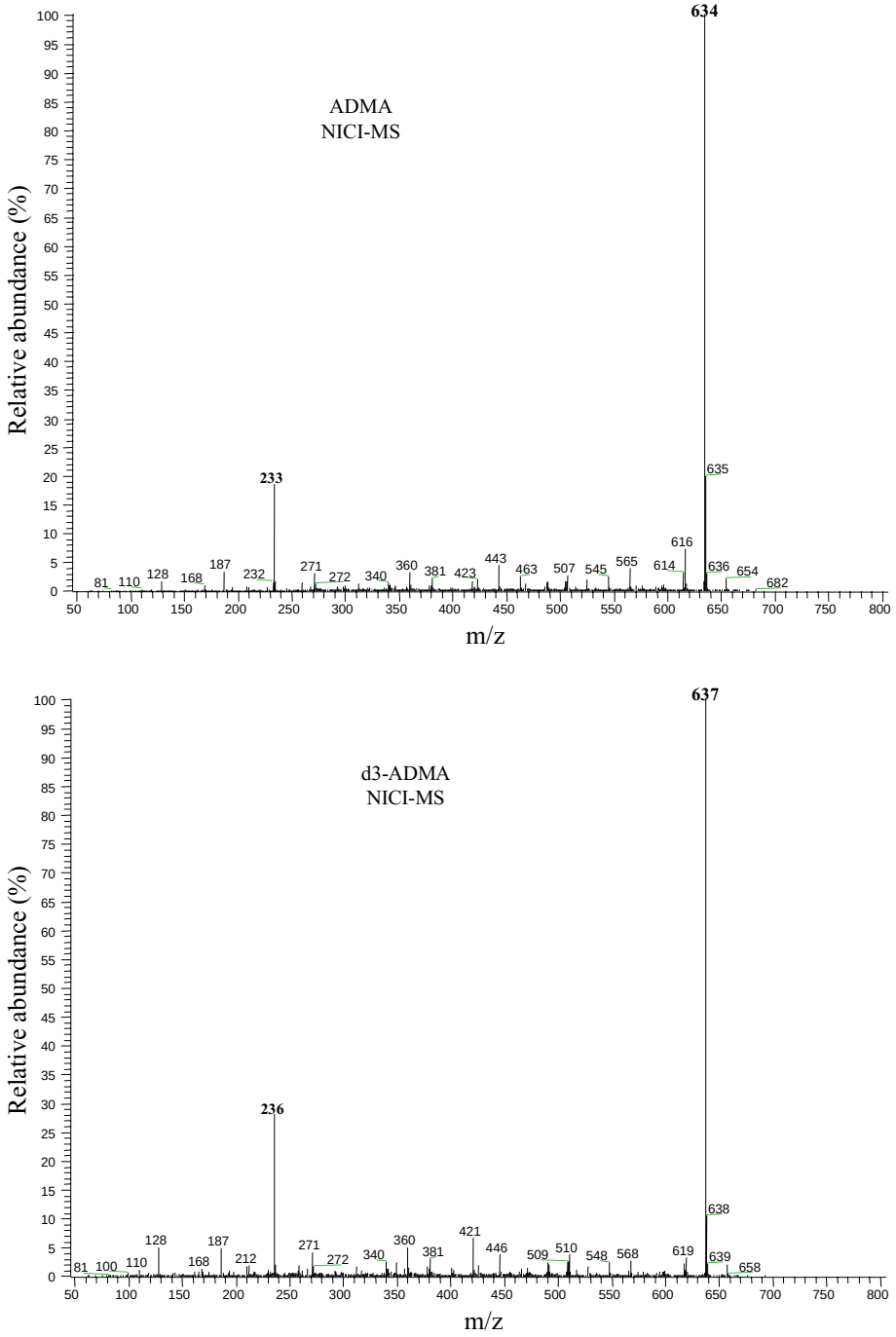


Fig. 1. Negative-ion chemical ionization mass spectra of the tri(*N*-pentafluoropropionyl) derivatives of ADMA methyl ester (upper panel) and ADMA trideuteromethyl ester (d_3-ADMA; lower panel).

3.3. Accuracy, precision, and limits of detection and quantitation of the method

The data from the validation of the method for circulating and excretory ADMA are summarized in Tables 3 and 4, respectively. In both matrices, ADMA was quantitated by GC–tandem MS with high accuracy and precision. In the pooled plasma used in these experiments the ADMA plasma concentration amounted to 0.455 μM. The lowest ADMA concentration added to plasma, i.e. 0.1 μM, was quantitated with high accuracy and high precision, suggesting that even lower concentrations of ADMA externally added to plasma would be quantitated with satisfactory accuracy and precision (Table 3). In the pooled urine sample used for method validation, endogenous ADMA was measured at 20 μM, and the creatinine concentration amounted to 10 mM. The

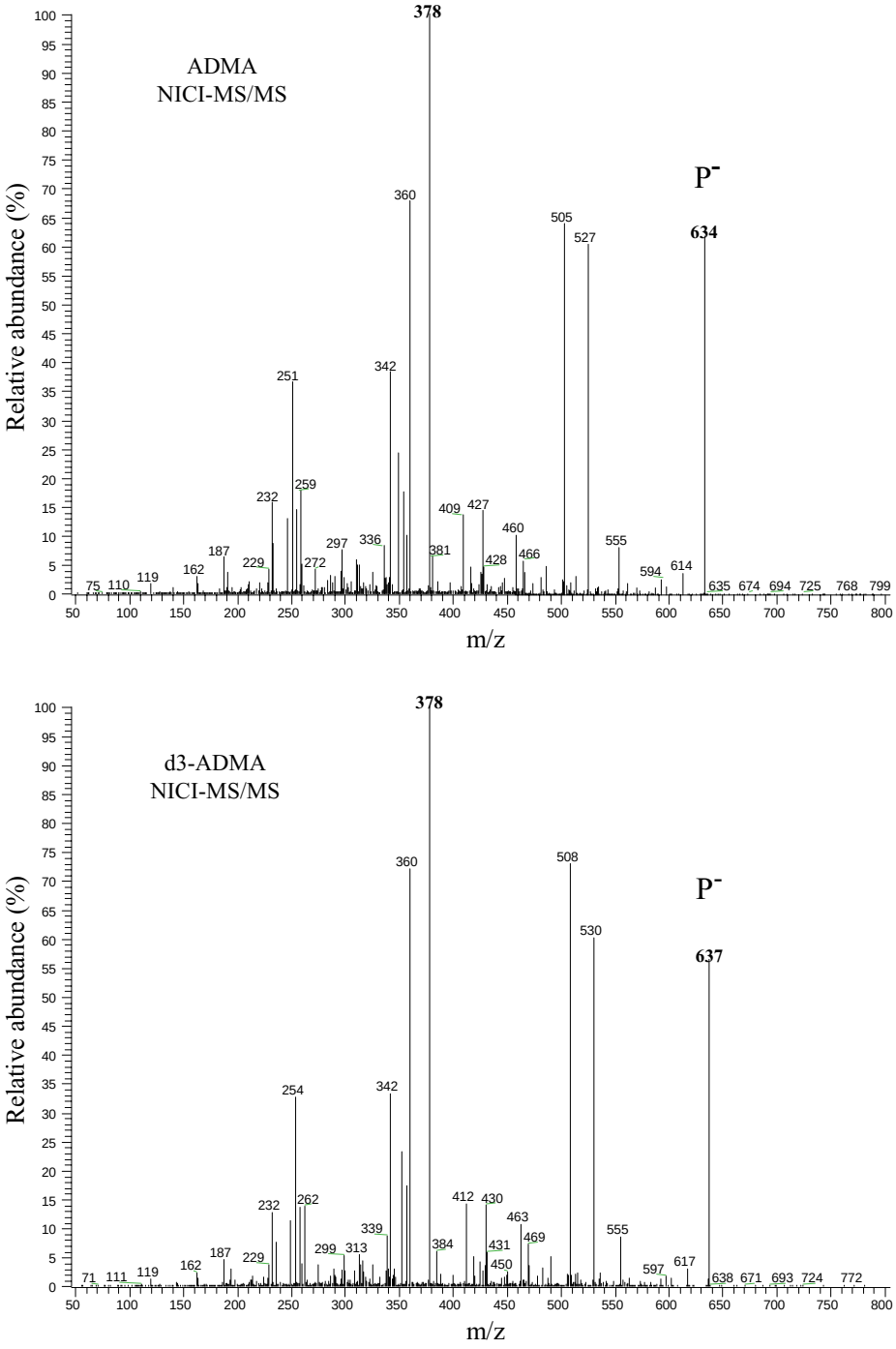


Fig. 2. Product ion mass spectra in the negative-ion chemical ionization mode of the tri(*N*-pentafluoropropionyl) derivatives of ADMA methyl ester (upper panel) and ADMA trideuteromethyl ester (d_3-ADMA; lower panel). The parent ions at *m*/*z* 634 and 637 (P^-) were subjected to collision-activated dissociation (CAD) with argon.

lowest concentration of ADMA externally added to the urine sample, that could be measured with recovery and precision values ≥80%, was 3 μM, i.e. 15% of the basal level. Linear regression analysis between found (*Y*) and added (*X*) ADMA concentrations revealed straight lines with the regression equations: $Y = 0.45 + 0.922X$, $R = 0.99812$, for plasma, and $Y = 20.4 + 1.02X$, $R = 0.99735$, for urine. Typical GC–tandem MS chromatograms from analyses of ADMA in plasma and urine of humans are shown in Figs. 3 and 4, respectively. With the exception of the peaks corresponding to the derivatives of unlabelled and labelled ADMA, no other peaks appear in the chromatograms, indicating that no other substances interfere with the GC–tandem MS measurement of ADMA in human plasma and urine by the present method.

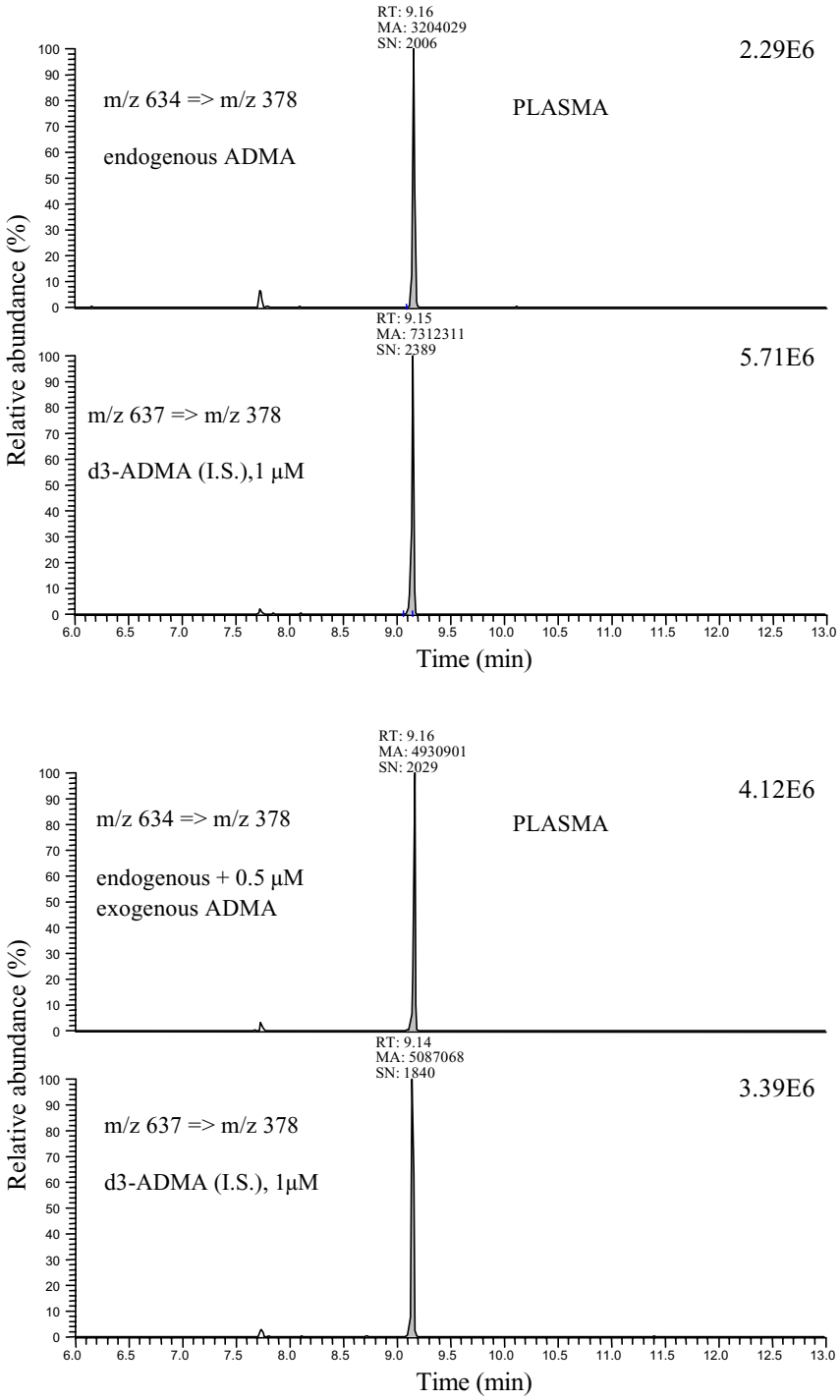


Fig. 3. Partial chromatograms from the GC–tandem MS analysis of a plasma sample from a healthy volunteer before (upper panel) and after spiking (lower panel) with synthetic ADMA at 0.5 μM. Selected reaction monitoring of *m/z* 378 from *m/z* 634 for endogenous ADMA (upper traces) and *m/z* 378 from *m/z* 637 (lower traces) for the internal standard d_3-ADMA of the methyl ester *N*-pentafluoropropionyl derivatives was performed.

The data on intra- and interday precision of the method for circulating and excretory ADMA are summarized in Table 5 and indicate the high precision of the method both for plasma and urinary ADMA.

GC–tandem MS analysis in the SRM mode of 1 fmol of d_3Me-ADMA from an aqueous solution resulted in a peak with a signal-to-noise (S/N) ratio of 343 ± 30 (mean $\pm$ S.D., $n = 3$), suggesting an LOD value of approximately 10 amol of ADMA. GC–tandem MS analysis in the SRM mode of 1 fmol of d_3Me-ADMA from the treatment of an uspiked plasma sample (see Table 3), which contained endogenous ADMA at 455 ± 6 nM, resulted in a d_3Me-ADMA peak

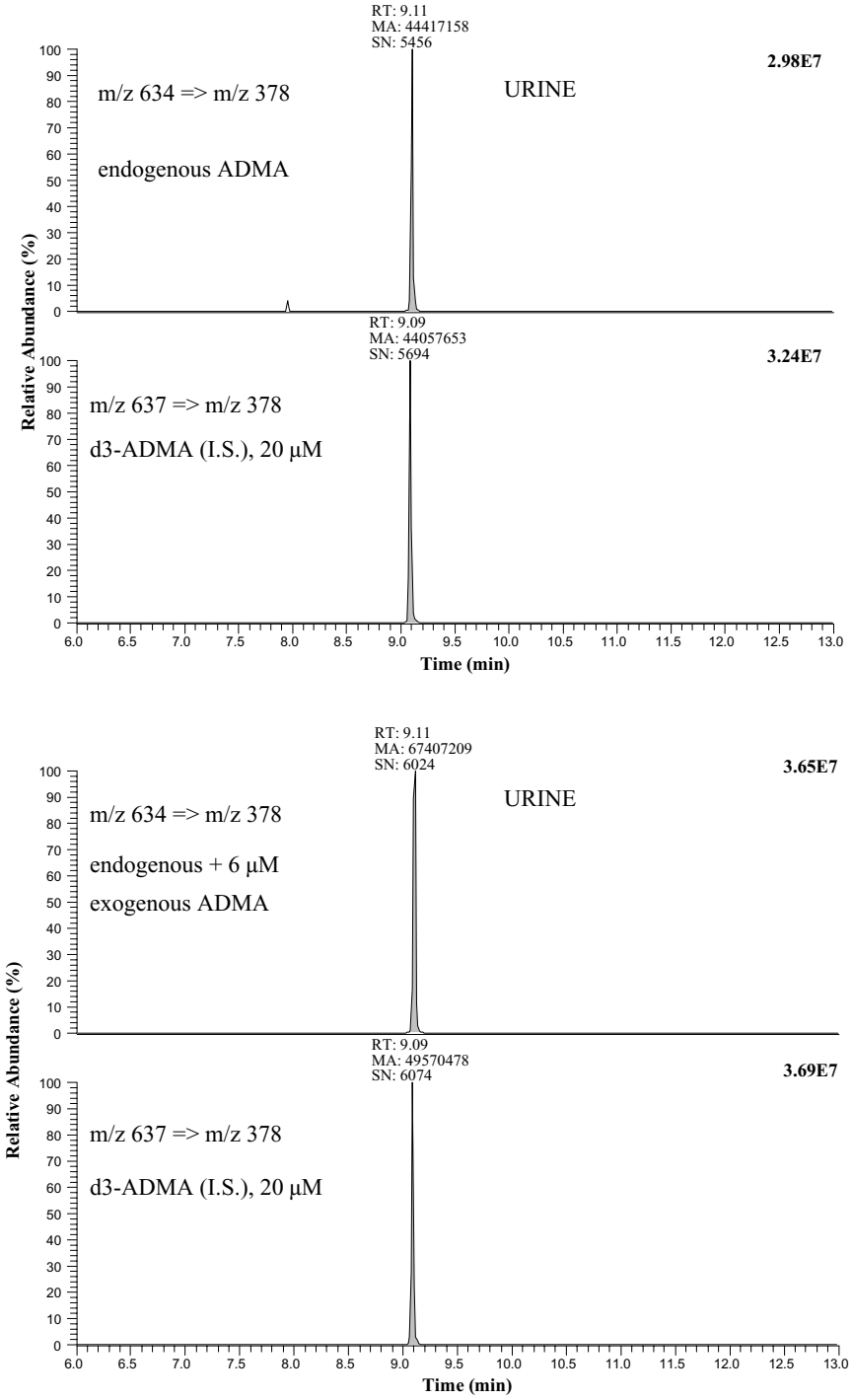


Fig. 4. Partial chromatograms from the GC–tandem MS analysis of a urine sample from a healthy volunteer before (upper panel) and after spiking (lower panel) with synthetic ADMA at 6 μM. Selected reaction monitoring of *m*/*z* 378 from *m*/*z* 634 for endogenous ADMA (upper traces) and *m*/*z* 378 from *m*/*z* 637 (lower traces) for the internal standard d_3-ADMA of the methyl ester *N*-pentafluoropropionyl derivatives was performed.

with a S/N ratio of 310 ± 27 (mean ± S.D., $n = 3$). This value confirms the LOD value that was obtained from the analysis of an aqueous solution of ADMA and indicates no matrix effects. Defining the LOQ as the concentration giving a S/N ratio of 10, the LOQ of the method for ADMA is approximately 320 pM.

3.4. ADMA basal levels in plasma and urine of healthy humans

The identity of circulating and excretory ADMA was demonstrated by generating product ion mass spectra from CAD of *m*/*z* 634. Analysis of ADMA in plasma resulted in

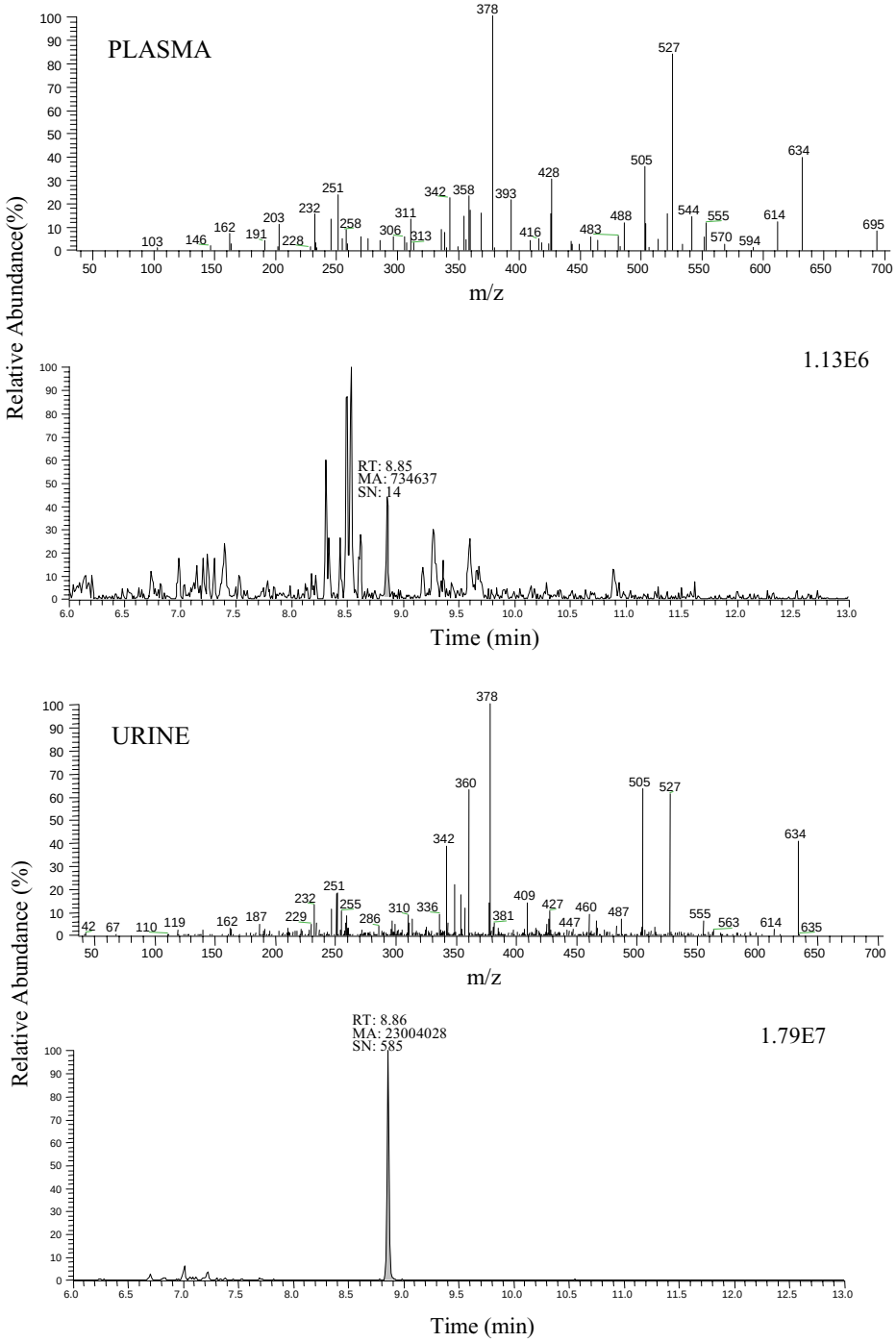


Fig. 5. GC–tandem MS mass spectra (upper traces) of the methyl ester pentafluoropropionyl derivatives of endogenous ADMA from plasma (upper panel) and urine (lower panel) with the retention time of synthetic ADMA derivative eluting at 8.85 min (see Fig. 2, upper panel) after collision-activated dissociation of the parent ion *m*/*z* 634.

many GC peaks (Fig. 5, upper panel), whereas analysis of urinary ADMA yielded a major GC peak eluting with the retention time of synthetic ADMA derivative (Fig. 5, lower panel). The product ion mass spectra of the GC peaks eluting at 8.85 min (Fig. 5) contain all the product ions of *m*/*z* 634 of authentic ADMA derivative (Fig. 2, upper panel), underlining the identity of endogenous ADMA in plasma and urine.

In the plasma of 12 healthy humans, ADMA was measured at a concentration of 390 ± 62 nM in the range of 294–504 nM (Table 6). In a subset of nine subjects of the same group ADMA was measured in urine at 25 ± 18 μM, which is 64 times higher than the mean plasma level (Table 6). With respect to urinary creatinine, ADMA excretion rate amounted to 3.4 ± 1 μmol/mmol creatinine.

Table 3
Precision and accuracy of the method for plasma ADMA

ADMA added (μM)	ADMA measured (μM)[a]	Precision (R.S.D., %)	Recovery (%)
0.0	0.455 ± 0.006	1.37	NA
0.1	0.550 ± 0.009	1.59	94.8
0.2	0.664 ± 0.018	2.74	105
0.4	0.801 ± 0.007	0.91	86.5
0.6	1.039 ± 0.025	2.42	97.3
0.8	1.163 ± 0.029	2.48	88.5
1.0	1.482 ± 0.038	2.59	103
1.3	1.714 ± 0.022	1.28	96.8
2.0	2.539 ± 0.173	6.81	104

NA: not applicable.
[a] The concentration of d_3Me-ADMA (internal standard) was 1.0 μM; values are given as mean ± S.D., $n = 3$.

Table 4
Precision and accuracy of the method for urinary ADMA

ADMA added (μM)	ADMA measured (μM)[a]	Precision (R.S.D., %)	Recovery (%)
0.0	20.0 ± 1.3	6.7	NA
1.0	21.4 ± 0.3	1.4	140
2.0	22.5 ± 0.2	0.7	122
3.0	22.7 ± 0.9	3.9	90.1
6.0	26.6 ± 2.6	9.9	110
8.0	28.9 ± 0.3	1.0	111
10	30.5 ± 0.8	2.7	104
15	35.4 ± 0.5	1.5	103
20	40.5 ± 3.5	8.5	102

NA: not applicable.
[a] The concentration of d_3Me-ADMA (internal standard) was 20.0 μM; values are given as mean ± S.D., $n = 3$.

3.5. Measurement of ADMA basal levels in plasma and urine of humans by GC–MS and GC–tandem MS

In plasma and urine samples of ten children suffering from various diseases, ADMA was quantified both by GC–tandem MS in the SRM mode and by GC–MS in the SIM mode, i.e. by monitoring the ions at *m*/*z* 634 for endogenous ADMA and *m*/*z* 637 for the internal standard d_3Me-ADMA, after routine work up of the samples. Comparison of the data on an individual basis reveals good agreement between the ADMA levels in both matrices measured by GC–MS and GC–tandem MS (Table 7). These data suggest that ADMA can be accurately measured in plasma and urine by simple GC–MS.

Table 5
Intra- and interday precision of the method for circulating and urinary ADMA[a]

Day	Plasma		Urine	
	ADMA (nM)	Precision (R.S.D., %)	ADMA (μM)	Precision (R.S.D., %)
A	438 ± 28	6.5	24.9 ± 1.3	5.1
B	439 ± 8	1.7	24.5 ± 0.5	1.9
C	431 ± 7	1.5	23.0 ± 1.9	8.3
D	393 ± 16	4.2	26.4 ± 3.3	12.4
E	397 ± 8	1.9	27.6 ± 2.5	8.9
Mean ± S.D.	420 ± 23	3.2 ± 2.1	25 ± 2	7.3 ± 4.0
Mean ± S.D.[b]	421 ± 24	5.8	25 ± 2	9.8

[a] The concentration of d_3-ADMA (internal standard) was 1.0 μM in plasma and 20.0 μM in urine; all analyses were performed in triplicate.
[b] All values were considered to calculate mean, S.D., and R.S.D. in plasma and urine for interday precision.

Table 6
Basal levels of ADMA in plasma and urine of healthy volunteers

Subject/sex	Age (years)	Plasma (μM)	Urine (μM)	Creatinine (μmol/mmol)
A/female	38	0.340	8.40	2.89
B/female	33	0.294	10.5	4.61
C/female	41	0.373	46.7	4.13
D/female	26	0.341	7.76	4.38
E/female	33	0.412	NM	NM
F/female	31	0.440	NM	NM
G/female	26	0.356	NM	NM
H/male	44	0.352	23.6	2.27
I/male	26	0.375	54.3	1.70
K/male	42	0.391	41.6	3.50
L/male	23	0.487	26.2	4.25
M/male	64	0.504	7.34	2.57
Mean ± S.D.	35.6 ± 11.4	0.389 ± 0.062	25.2 ± 18.4	3.37 ± 1.05

NM: not measured.

3.6. Quantitative determination of L-arginine in human plasma

L-Arginine added to human plasma in the concentration range 0–90 μM was determined by GC–MS with a recovery (mean ± S.D.) of 101.6 ± 10.3% ($n = 8$) and an imprecision of 2.52 ± 1.99% ($n = 9$) (Fig. 6). The lowest added concentration of L-arginine of 2 μM was determined in plasma with recovery and precision values of 115 and 99.6%, respectively. The basal L-arginine concentration in that plasma sample was 75.4 μM. Instrumental precision for the analysis of basal L-arginine plasma concentration was 0.58% (Hewlett-Packard instrument) and 1.0% (TSQ 7000 instrument operating in the SRM mode). The ratio of the L-arginine concentrations measured in the plasma samples by GC–MS to those measured by GC–tandem MS was (mean ± S.D., $n = 9$) 1.024 ± 0.018, indicating excellent agreement between these techniques. Intraday precision (R.S.D.) from six-fold GC–MS analysis of L-arginine in unspiked plasma for five consecutive days ranged between 0.80 and 2.65%, whereas interday imprecision (R.S.D.) was calculated to be 5.14% in this experiment.

4. Discussion

The present study reports on the first GC–tandem MS method for the quantitative determination in human plasma

Table 7
Comparison between GC–tandem MS and GC–MS with regard to ADMA levels in human urine (μM) and plasma (nM)

Urine		Plasma		Ratio of GC–MS to GC–tandem MS	
GC–tandem MS	GC–MS	GC–tandem MS	GC–MS	Urine	Plasma
36.0	39.4	1130	1167	1.094	1.033
12.6	14.0	587	606	1.111	1.032
17.5	20.2	706	725	1.154	1.027
7.8	8.9	432	461	1.141	1.067
3.2	3.9	518	515	1.219	0.994
2.0	2.5	384	398	1.250	1.036
0.5	0.7	781	823	1.400	1.054
39.5	44.3	471	450	1.122	0.955
4.9	5.7	444	462	1.163	1.040
8.4	9.0	949	932	1.071	0.982
Mean ± S.D.				1.173 ± 0.097	1.022 ± 0.034

and urine of ADMA, an endogenous inhibitor of NOS activity and an independent risk factor of cardiovascular diseases. In healthy non-smoking volunteers, ADMA was measured in plasma at a mean concentration of 0.38 μM. The mean ADMA concentration measured in plasma of healthy humans by GC–tandem MS is similar to that measured by means of HPLC by our group in many clinical studies (range: 0.52–1.25 μM) [11,18–20] and other groups [7–10,12,13], and approximately three times higher than that measured by LC–tandem MS (i.e. 0.123 μM) [14]. The variation in the ADMA basal plasma levels measured by us could be due to the heterogeneity of the recruited control groups which included pregnant healthy women [18], and young [20] and elderly [19] healthy female and male persons. In the present study, we measured by the HPLC method [11] in eight young healthy volunteers (four females and four males) basal ADMA plasma levels of 0.476 ± 0.211 μM. ADMA added to these plasma samples at 1.1 and 2.2 μM was recovered by 96 and 105%, respectively.

In urine from spontaneous micturition of our volunteers, ADMA was measured at concentrations ranging between 7 and 54 μM. With respect to urinary creatinine, ADMA was measured at a mean value of 3.4 μmol/mmol creatinine. The first report on the excretion by humans of ADMA into the urine stems from Vallance et al. [2], who found an approximate urinary excretion of 65 μmol ADMA per day. Considering a daily mean excretion rate of 1.5 g (13 mmol) of creatinine [21], the ADMA excretion rate estimated by Vallance et al. would correspond to approximately 5 μmol ADMA per mmol creatinine ($n = 6$), which is similar to that measured by GC–tandem MS in the present study (i.e. 3.4 μmol/mmol creatinine, $n = 9$).

Accurate quantitative determination of a certain analyte by MS-based approaches, such as GC–tandem MS and LC–tandem MS, is best achieved by using stable isotope-labelled analogues of the analyte. Unlike L-arginine, currently no such analogues are commercially available for ADMA. Our study shows that this analytical

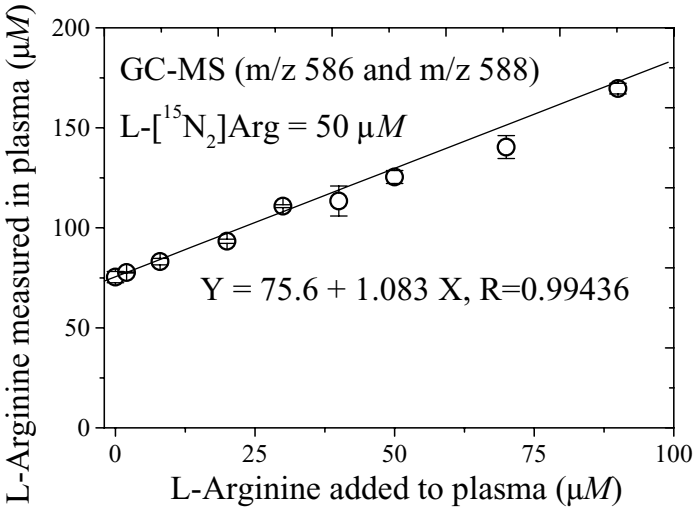


Fig. 6. A GC–MS standard curve for L-arginine in human plasma using L-[guanidino-$^{15}N_2$]-arginine as internal standard externally added at a final concentration of 50 μM. Selected-ion monitoring in the negative-ion chemical ionization mode of *m/z* 586 for L-arginine and *m/z* 588 for L-[guanidino-$^{15}N_2$]-arginine as their methyl ester tetra(*N*-pentafluoropropionyl) derivatives was performed. For more details, see text.

problem can be satisfactorily overcome by de novo synthesis of the trideuteromethyl ester of synthetic ADMA, i.e. d_3Me-ADMA, and its use as an internal standard for endogenous ADMA. Interestingly, this approach is not limited to ADMA, but it can be extended in a similar manner to SDMA, MMA, and in principle to any other amino acid, all of which are commercially available in their unlabelled forms (Scheme 1, left panel). That this approach is feasible to ADMA, is due to the facts that the HCl-catalysed esterification of the carboxylic groups of endogenous and synthetic ADMA by CH_3OH and CD_3OD, respectively, proceeds with very similar yields, and that Me-ADMA and d_3Me-ADMA behave almost identically through the subsequent derivatization step, i.e. *N*-acylation by pentafluoropropionic anhydride. Under the conditions used in the present study, *N*-acylation by pentafluoropropionic anhydride yields amino acid derivatives of varying number of *N*-pentafluoropropionyl moieties. For quantitative analysis of ADMA, we have chosen the entirely *N*-acylated, i.e. the *N*-perfluoroacylated derivative (Scheme 1, right panel), because this derivative possesses more suitable GC and MS properties than partially *N*-acylated derivatives.

The use of commercially available stable isotope-labelled L-arginine analogues, such as L-[guanidino-$^{15}N_2$]-arginine and L-[$^{13}C_6$]-arginine, is an alternative practicable way. L-[$^{13}C_6$]-Arginine has been found to be suitable as an internal standard for ADMA, SDMA and MMA in a LC–tandem MS method [14]. However, our investigations with L-[guanidino-$^{15}N_2$]-arginine revealed that the use of this analogue allows only a less accurate quantitation of ADMA. Unlabelled L-arginine (50 μM) was derivatized and analysed by SIM of *m/z* 588 and 586. This measurement revealed that unlabelled L-arginine contributes to L-[guanidino-$^{15}N_2$]-arginine by 3.8 ± 0.2% (mean ± S.D., $n = 3$). Thus, L-arginine at physiological plasma concentrations of 50–100 μM will contribute to the internal standard L-[guanidino-$^{15}N_2$]-arginine by approximately 2–4 μM. In order to minimise this contribution, L-[guanidino-$^{15}N_2$]-arginine must be used at a concentration of at least 50 μM, which is more than 100 times higher than that of ADMA. By using L-[guanidino-$^{15}N_2$]-arginine (50 μM) as an internal standard for the quantitative determination of ADMA and L-arginine in human plasma we observed by SIM of *m/z* 634 for ADMA, *m/z* 588 for L-[guanidino-$^{15}N_2$]-arginine and *m/z* 586 for L-arginine peak area ratios of ≤1:100 for ADMA and approximately 1:1.4 for L-arginine. As shown here for L-arginine, the use of L-[guanidino-$^{15}N_2$]-arginine at 50 μM allows for the accurate determination of L-arginine in human plasma. Under such conditions, however, ADMA could not be determined by analytically acceptable values for recovery and imprecision. For ADMA spiked to human plasma up to 10 μM, we measured ADMA with a recovery of only ≤65% and an imprecision of ≥30%. Particularly in plasma, where the molar ratio of L-arginine to ADMA may vary greatly, e.g. from 10 to 200 [3], the use of L-[guanidino-$^{15}N_2$]-arginine as an internal standard for ADMA in GC–MS is not recommended.

Measurement of ADMA in plasma and urine of humans with high accuracy and high precision by the GC–tandem MS method described here, offers the possibility to investigate the unique significance of ADMA in NO-related dysfunctions including cardiovascular and renal diseases associated with accumulation of ADMA in the circulation and most likely in NO-producing cells, notably in endothelial cells. Determination of the activity of enzymes responsible for formation and metabolism of ADMA, i.e. the protein-L-arginine methyl transferases and the dimethylarginine dimethylaminohydrolases, respectively, and of other factors primarily responsible for the accumulation/elimination of ADMA would be helpful in taking appropriate measures in order to shift the status of the L-arginine/NO pathway in favour of NO.

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