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Determination of N^G,N^G-dimethyl-L-arginine, an endogenous NO synthase inhibitor, by gas chromatography–mass spectrometry

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Abstract

A fully validated gas chromatographic–mass spectrometric (GC–MS) method for the accurate and precise quantification of N^G,N^G-dimethyl-L-arginine (asymmetric dimethylarginine, ADMA), an endogenous inhibitor of the NO synthase, in cell culture supernatants and in small volumes of plasma is described. ADMA was concentrated by solid phase extraction and converted to its methyl ester pentafluoropropionic amide derivative. The derivatives were analyzed without any further purification. Using gas chromatography–chemical ionization mass spectrometry, fragment ions at *m*/*z* 634 and *m*/*z* 640 were obtained for ADMA and for N^G,N^G-[$^2H_6$]-dimethyl-L-arginine ([$^2H_6$]-ADMA) as internal standard, respectively. [$^2H_6$]-ADMA was synthesized by reaction of L-ornithine fastened at bromcyan-agarose with dimethylamine. The limit of detection of the method was 2 fmol, while the limit of quantitation for cell culture supernatants was 0.05 μM. The method was validated in a concentration range of 0–1.2 μM in cell culture medium and 0–2 μM in 50 μl aliquots of human plasma. The precision was ≥97% and the accuracy was determined to be ≥94%. This method is fast, rugged and an alternative to high performance liquid chromatography (HPLC) analysis of ADMA in cell culture supernatants and small volumes of human plasma.

Keywords: N^G,N^G-Dimethyl-L-arginine

1. Introduction

Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthase [1]. It has been suggested as a novel independent risk factor for endothelial dysfunction and coronary heart disease [2,3]. Plasma levels of ADMA are elevated, e.g. in patients with hypertriglyceridemia, hypertension and occlusive vascular disease [4–6]. This elevation is associated with reduced NO production and impaired endothelium-dependent vasodilation [7–9]. The biosynthesis and metabolism of dimethylarginines are not completely understood. ADMA is most probably formed posttranslationally by methylation of arginine incorporated into proteins [9] and metabolised by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) to L-citrulline and dimethylamine [10,11]. Further investigations concerning the biological effects of ADMA have been performed in cell culture, e.g. in endothelial cells [12]. Thus far, high performance liquid chromatography (HPLC) has been applied to determine ADMA in biological samples [13]. However, measurement of ADMA in cell culture supernatants is quite difficult due to the high concentration of other interfering amino acids. In addition, small amounts of plasma cannot be analyzed with the HPLC method.

Therefore, we developed a method for the quantitation of ADMA in biological samples applying gas chromatography–mass spectrometry (GC–MS). For this purpose N^G,N^G-[3,3,4,4,5,5-$^2H_6$]-dimethyl-L-arginine ([$^2H_6$]-ADMA) was synthesized and utilized as internal standard. The high selectivity and sensitivity of this GC–MS method facilitates the measurement of ADMA in small sample volumes and difficult matrices.

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2. Experimental

2.1. Chemical reagents

Pentafluoropropionic anhydride (PFPA) was obtained from Pierce (Rockford, IL, USA). L-[3,3,4,4,5,5-$^2H_6$]-Ornithine was from Euriso-top (>98%, atom%, ^2H isotopic purity, Saint Aubin, France). L-[Guanidino-$^{15}N_2$]-arginine hydrochloride (98%, atom%, ^{15}N isotopic purity) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). N^G,N^G-Dimethyl-L-arginine hydrochloride (ADMA), dimethylamine and bromcyan-agarose were supplied by Sigma (Deisenhofen, Germany). All other solvents and reagents of analytical grade were from Merck (Darmstadt, Germany). Carboxy acid (CBA) cartridges (500 mg) were supplied from Macherey–Nagel (Düren, Germany). Dulbecco's Modified Eagle Medium was from Invitrogen (Karlsruhe, Germany).

2.2. Chemical synthesis of N^G,N^G-[$^2H_6$]-dimethyl-L-arginine ([$^2H_6$]-ADMA)

Synthesis of [$^2H_6$]-ADMA was performed according to a modification of the procedure published by Pundak and Wilchek [14]. In brief, bromcyan-agarose (0.4 g) was suspended in 10 ml of 1 mM HCl in methanol and incubated for 30 min. After incubation, the mixture was filtered and repeatedly washed with 5 ml of 1 mM HCl and 5 ml of water. To prepare a copper–ornithine-complex, 0.125 g [$^2H_6$]-ornithine–HCl was solved in 1.25 ml of water. Small amounts of $CuCO_3$ were added until a blue colour was obtained. After filtration of the solution the filtrate was brought to pH 10 with 10 M KOH. The washed bromcyan-agarose was suspended in 1.25 ml of the copper–ornithine-complex and was shaken over night at 4 °C. The next morning the suspension was filtered and washed one time with 1 M HCl and with an appropriate amount of water for several times. The [$^2H_6$]-ornithine–bromcyan-agarose was suspended in a 20% solution of dimethylamine in water and stirred for 24 h at 50 °C. The suspension was filtered and washed two times with 10 ml water. The filtrate together with the washings was concentrated to dryness in vacuum. The residue was solved in water again and concentrated to dryness in vacuum for a second time. The residue was dissolved in water to yield a stock solution which was stored at −20 °C.

2.3. Extractions and derivatization procedures

[$^2H_6$]-ADMA was added for use as internal standard to 500 μl aliquots of Dulbecco's Modified Eagle Medium (DMEM) resulting in a final concentration of 0.375 μM. The samples were diluted with 500 μl water and supplied to CBA-cartridges preconditioned with 3 ml methanol and 3 ml water. Cartridges were washed twice with water, and compounds were eluted with 1 ml 1 M HCOOH. After evaporation to dryness, analytes were converted to their methylester derivatives adding 100 μl 2 M HCl in methanol at 80 °C for 1 h. After evaporation a volume of 100 μl reagent solution (PFPA in ethylacetate, 1:4 (v/v)) was added and the samples were heated for 1 h at 100 °C. The reagent solvent was evaporated and the derivatives were extracted with toluene and borate buffer (0.2 M, pH 8.5). After vortexing and centrifugation, the toluene phase was supplied to a 0.3 ml microinsert in an autosampler vial and stored at 4 °C until analysis.

The plasma samples were treated the same way for derivatization, but they were not extracted on CBA-cartridges. A volume of 50 μl of a freshly prepared plasma sample was spiked with [$^2H_6$]-ADMA and L-[guanidino-$^{15}N_2$]-arginine to reach a final concentration of 2.4 and 80 μM. Acetone was added for protein precipitation to a final volume of 150 μl, and the supernatant obtained after centrifugation was evaporated under nitrogen. The residue was treated with 2 M HCl in methanol and PFPA as described above without previous solid phase extraction (CBA-cartridges).

2.4. Experiments on precision, accuracy and validation of the method

Standard curves were calculated for DMEM with 10% FBS (Foetal Bovine Serum) and for human plasma. For this purpose, 500 μl of medium or 50 μl of plasma were spiked with appropriate amounts of ADMA. For plasma samples, the L-arginine to ADMA ratio is of interest. Therefore, we also calculated a standard curve for L-arginine using L-[guanidino-$^{15}N_2$]-arginine as an internal standard. For calibration, weighted linear regression analysis was applied. Inter-day precision was determined in DMEM with 10% FBS and in human plasma by repeating sample preparation and GC–MS analysis of one sample at each concentration level throughout the analytical sequence on five consecutive days. Intra-day precision was determined by analyzing five samples at each concentration level throughout the analytical sequence within one day. The data from inter- and intra-day precision measurements were used to calculate the accuracy. For determining instrumental precision and long term stability, five spiked samples were analyzed at different concentration levels immediately after work-up, after standing for 24 h in the autosampler at room temperature, and after 4 weeks of storage time at 4 °C. Concentrations were calculated and the means were compared. The limit of detection (LOD) of the method was determined using dilutions of a stock solution of authentic ADMA. For this purpose, ADMA was converted to its methylester-*N*-pentafluoropropionyl (PFP) derivative as described above and analyzed by GC–MS. The limit of quantification (LOQ) was determined using 500 μl DMEM without FBS (containing no ADMA) spiked with different concentrations of ADMA and [$^2H_6$]-ADMA as internal standard.

2.5. GC–MS

A CP-3800 GC coupled to a 1200 Quadrupole MS was used (Varian, Walnut Creek, USA). The GC was fitted with a fused-silica capillary column Optima-17 (30 m × 0.25 mm i.d., 0.25 μm film thickness, Macherey–Nagel). The injector was operated in the split/splitless mode at 260 °C. Helium was used as a carrier gas at a constant flow-rate of 1 ml/min. Initial column temperature was 80 °C for 2 min, followed by an increase of 25 °C/min to 300 °C. The mass spectrometer transfer line was kept to 280 °C. CI was performed at an electron energy of 70 eV and an emission current of 300 μA. Electron multiplier voltage was set to 1.8 kV for ADMA and 1.1 kV for the measurement of L-arginine. Aliquots of 1.0 μl of cell culture or plasma samples were injected. During single ion monitoring (SIM), *m/z* 634.3 and *m/z* 640.3 were determined for target and internal standard, respectively.

3. Results

3.1. Synthesis of N^G,N^G-[$^2H_6$]-dimethyl-L-arginine ([$^2H_6$]-ADMA)

The synthesis of ADMA by methylation of L-arginine results in monomethylated, symmetrically dimethylated and asymmetrically dimethylated L-arginine derivatives. To overcome subsequent purification steps, the synthesis of deuterated ADMA from deuterated L-ornithine revealed a feasible alternative (Fig. 1). For this synthesis, a solution of copper-[$^2H_6$]–ornithine-complex was bound to bromcyan-agarose. The washed [$^2H_6$]-ornithine–bromcyan-agarose was treated with dimethylamine in water. This resulted in the release of deuterated ADMA from the bromcyan-agarose. The obtained solution was evaporated to dryness and reconstituted with water to eliminate dimethylamine excess. No traces of SDMA (symmetric dimethylarginine, N^G,N'^G-dimethyl-L-arginine), monomethylated L-arginine, L-arginine or ornithine were present as confirmed by HPLC analysis according to Tsikas et al. [13]. The isotopic purity of the deuterated ADMA was found to be 99.0% confirmed by GC–MS. The overall recovery of the synthesis was 0.59% or 0.736 mg of [$^2H_6$]-ADMA quantified by GC–MS. However, the unconverted [$^2H_6$]-ornithine could be recycled several times.

3.2. Sample preparation

The extraction procedure described under section 2.3 has been applied for the analysis of ADMA in cell culture supernatant. Proteins and non-polar amino acids were separated by this procedure. After extraction with borate buffer, the methylester–PFP derivative in the toluene phase was stable for at least 4 weeks at 4 °C and no degradation occurred for 24 h at room temperature.

3.3. Mass spectrometry

For the detection of ADMA negative-ion chemical ionization (NICI) mass spectrometry of the PFPA derivative was used. The NICI mass spectrum of the methylester–PFP derivative of unlabelled and labelled ADMA with fragment ions at *m/z* 634.3 and *m/z* 640.3, for ADMA and [$^2H_6$]-ADMA, respectively, is shown in Fig. 2. These fragment ions were used for quantification. A typical mass chromatogram of a cell supernatant sample is given in Fig. 3A. For plasma samples, the L-arginine concentration and the L-arginine/ADMA ratio were obtained. Therefore, we used L-[guanidino-$^{15}N_2$]–arginine as an internal standard and detected the fragment ions at *m/z* 586 and *m/z* 588 within the same SIM experiment (Fig. 3B).

The mass spectrum of SDMA shows a major fragment ion at *m/z* 593.9 (Table 1). The retention times were 6.7 min for SDMA and of 8.4 min for ADMA and [$^2H_6$]-ADMA. No interference with SDMA was observed for the measurement of ADMA. The NICI GC–MS spectra of the methylester–PFP derivatives of unlabelled and labelled L-arginine and methylated L-arginine analogues are shown in Table 1.

3.4. Analytical method validation

The standard curves were linear within the range of 0–0.8 μM in water and cell culture medium using 0.375 μM [$^2H_6$]-ADMA as internal standard, and within the range of 0–2 μM plasma using 2.4 μM [$^2H_6$]-ADMA. The standard curves with the statistical data are shown in Fig. 4A and B. The standard curves for ADMA and L-arginine in human plasma samples are shown in Fig. 5A and B. The coefficients of inter- and intra-day variations and accuracy of the spiked samples are given in Table 2 for cell culture medium

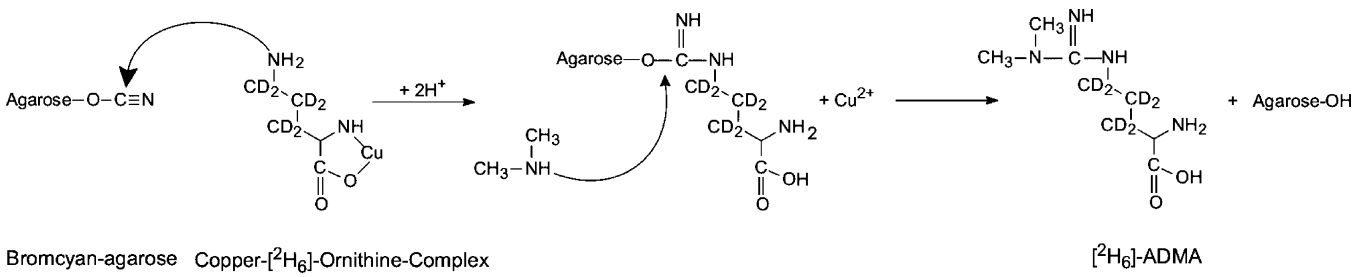

Fig. 1. Synthesis of [$^2H_6$]-ADMA using a copper–ornithine-complex and bromcyan-agarose. For more details, see text.

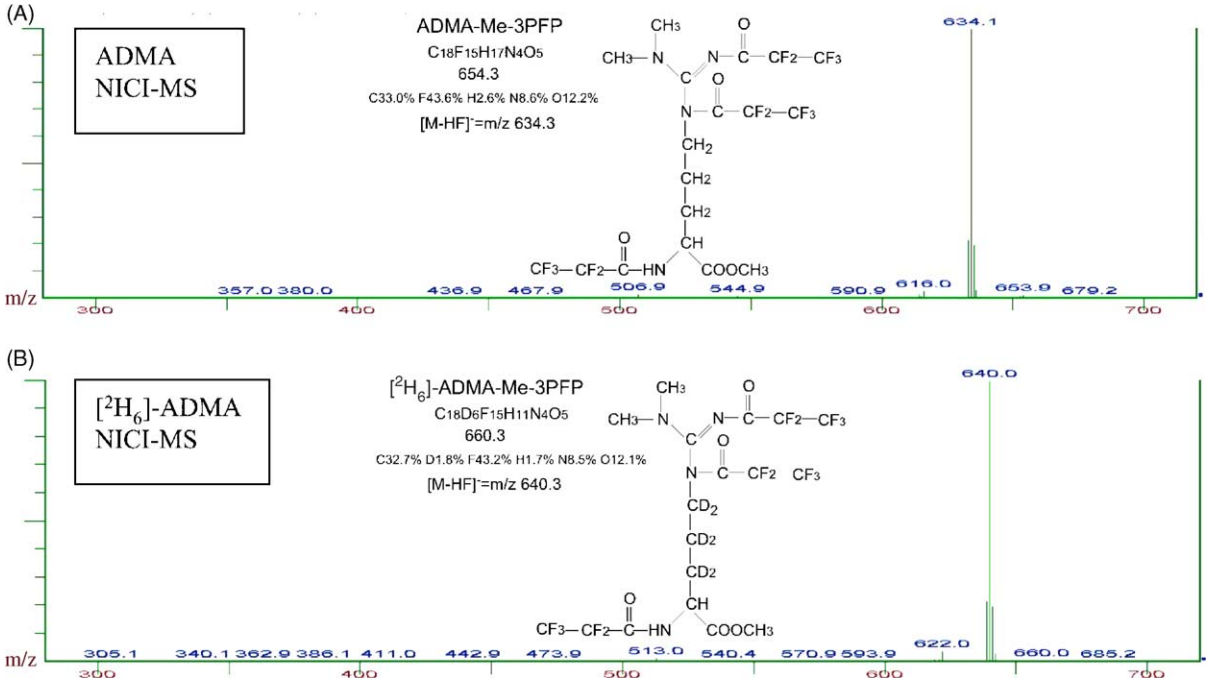

Fig. 2. GC–MS NICI mass spectra of the methylester–PFP derivatives of ADMA and [$^2H_6$]-ADMA are shown. Ions were observed at *m/z* 634 for ADMA (A) and at *m/z* 640 for [$^2H_6$]-ADMA (B).

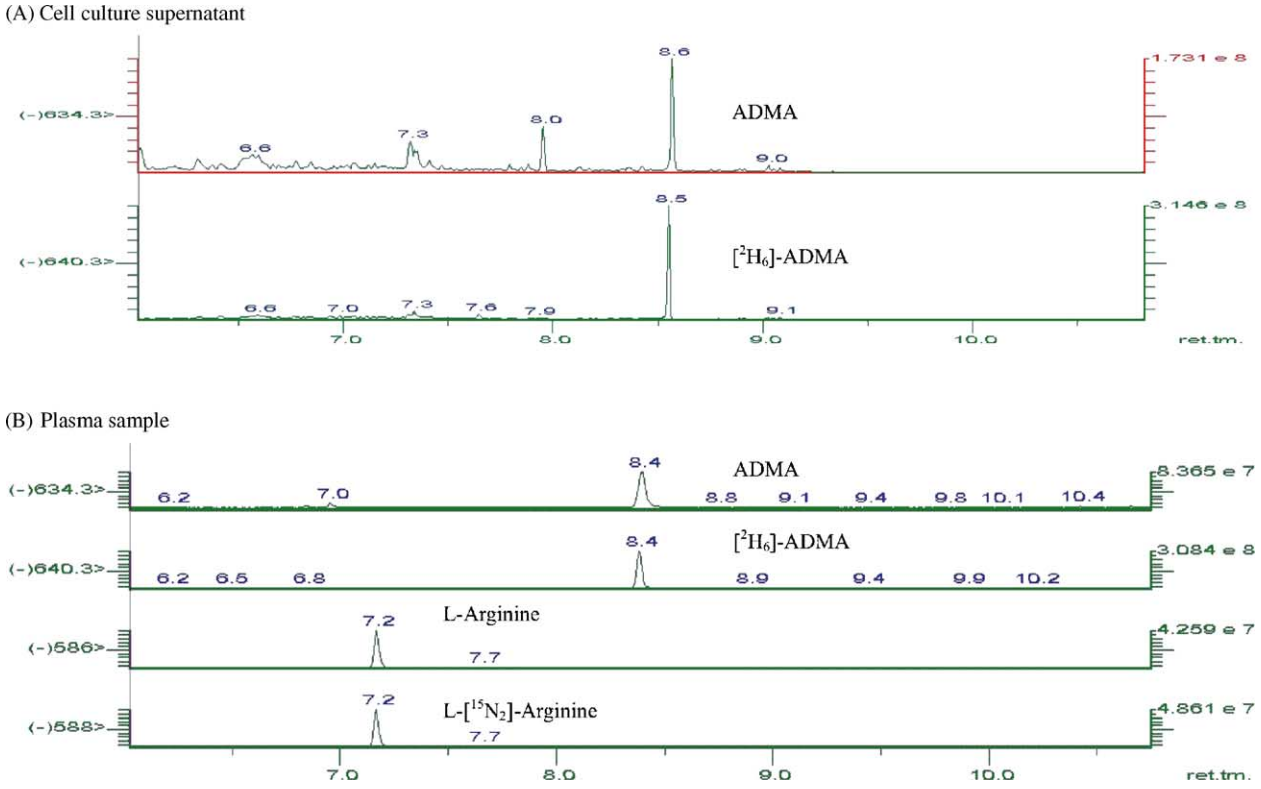

Fig. 3. Partial chromatograms from the GC–MS analysis of a (A) cell culture supernatant after spiking with [$^2H_6$]-ADMA (0.375 μM) and (B) a plasma sample after spiking with [$^2H_6$]-ADMA (2.4 μM) and L-[guanidino-$^{15}N_2$]-arginine (80 μM). The retention times are 7.2 min for L-arginine and 8.4 min for ADMA.

Table 1
NICI GC–MS mass spectra of the methylester–PFP derivatives of unlabelled and labelled L-arginine, ADMA and SDMA

Ion assignment compound	L-Arginine	L-[$^{15}N_2$]-Arginine *m/z* (intensity, %)	ADMA	[$^2H_6$]-ADMA	SDMA
[M–HF]$^-$	605.9 (15)	608.0 (12)	634.1 (100)	640.0 (100)	
[M–2HF]$^-$	586.0 (100)	588.1 (100)			613.9 (17)
[M–3HF]$^-$	565.9 (27)	567.9 (30)			593.9 (100)
[M–3HF–PFP]$^-$					449.9 (21)

Listed are ions with $m/z \geq 200$ and intensity $\geq 10\%$.

with 10% FBS and in Table 3 for plasma samples. The difference seen in Table 2 between 0.0 μM ADMA added and the concentration found can be explained by the content of ADMA in FBS. Comparing analytical results for extracted samples at three concentrations with unextracted standards, the recovery was determined to be 92.5 ± 2.4%. The lowest ADMA concentration added to cell culture medium without FBS, i.e. 0.05 μM, was quantitated with an accuracy of 110.6 ± 10.7% (mean ± S.D.) and a R.S.D. of 9.7% ($n = 5$). GC–MS analysis of 2 fmol ADMA in the SIM mode resulted in a peak with a signal-to-noise ratio of 11.8 ± 1 (mean ± S.D., $n = 3$).

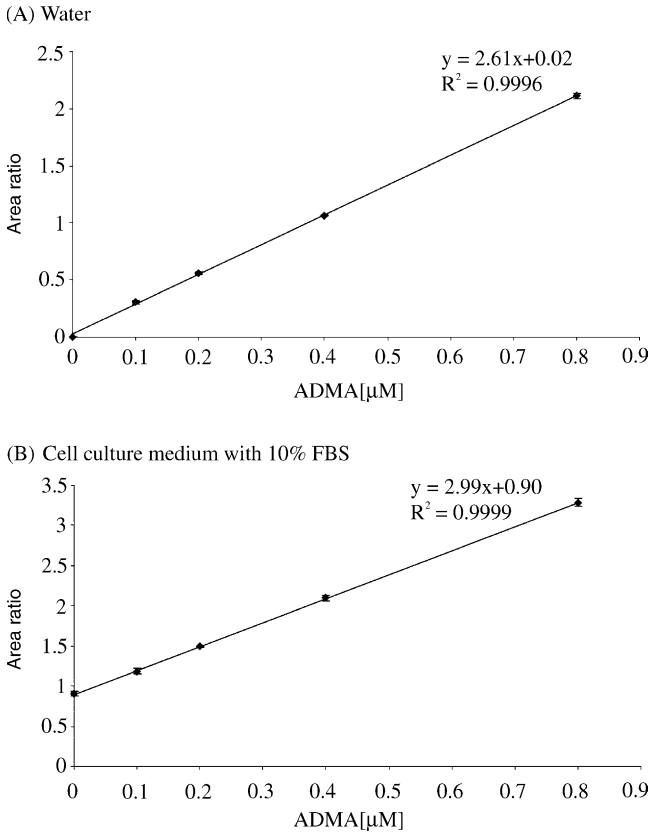

Fig. 4. Standard curve obtained after analysis of ADMA as its methylester–PFP derivative using [$^2H_6$]-ADMA as internal standard (0.375 μM) for quantification in water (A) and in cell culture medium (B) (mean ± S.D., $n = 3$).

Table 2
Intra-day (a) and inter-day (b) precision and accuracy of the method for ADMA in cell culture medium with 10% FBS

ADMA added (μM)	ADMA measured (μM)	R.S.D. (%)	Accuracy (%)
(a) Intra-day			
0.0	0.34	2.91	N.A.
0.1	0.43	4.12	94.1
0.2	0.57	2.62	114.1
0.4	0.74	4.10	99.9
0.8	1.19	2.07	105.7
1.2	1.64	1.40	108.4
Mean ± S.D.		2.87 ± 1.09	104.5 ± 7.72
(b) Inter-day			
0.0	0.33	3.52	N.A.
0.1	0.45	3.75	111.8
0.2	0.55	2.26	103.9
0.4	0.77	0.41	107.1
0.8	1.20	2.15	107.2
1.2	1.65	2.23	108.8
Mean ± S.D.		2.39 ± 1.19	107.8 ± 2.87

N.A., not applicable; 0.375 μM [$^2H_6$]-ADMA were used as internal standard; data are shown as means ($n = 5$).

Table 3
Intra-day (a) and inter-day (b) precision and accuracy of the method for ADMA in 50 μl plasma

ADMA added (μM)	ADMA measured (μM)	R.S.D. (%)	Accuracy (%)
(a) Intra-day			
0.00	0.88	4.71	N.A.
0.25	1.16	2.67	111.7
0.50	1.36	3.15	95.5
1.00	1.99	1.60	110.5
2.00	2.93	2.61	102.5
Mean ± S.D.		2.95 ± 1.14	105.1 ± 7.57
(b) Inter-day			
0.00	0.85	3.66	N.A.
0.25	1.16	1.08	121.7
0.50	1.34	0.92	98.0
1.00	1.98	1.56	112.6
2.00	2.97	0.88	105.7
Mean ± S.D.		1.62 ± 1.17	109.5 ± 10.08

N.A., not applicable; 2.4 μM [$^2H_6$]-ADMA were used as internal standard; data are shown as means ($n = 5$).

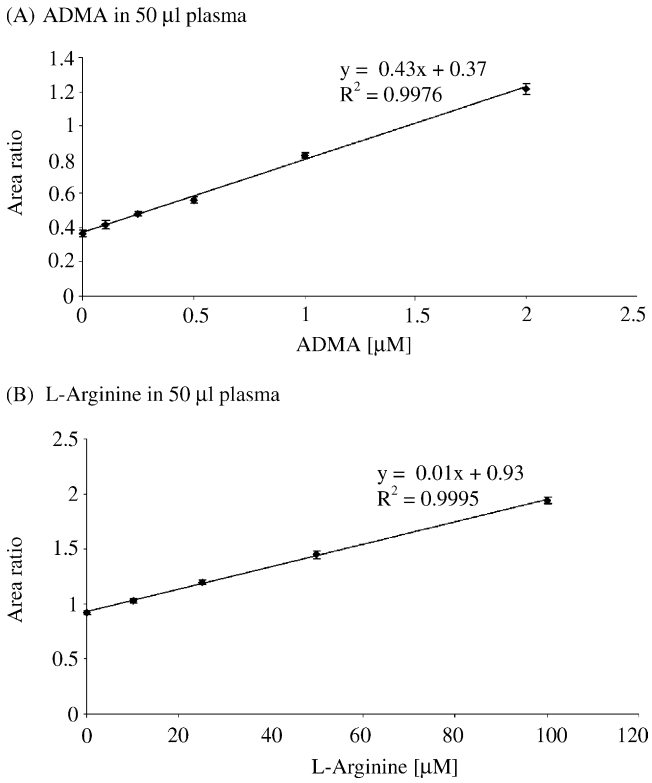

Fig. 5. Standard curve for ADMA and L-arginine in human plasma after spiking the samples with [$^2H_6$]-ADMA (2.4 μM) and L-[guanidino-$^{15}N_2$]-arginine (80 μM) as internal standards (mean ± S.D., $n = 3$).

3.5. ADMA basal levels in cell culture supernatants and in human plasma

In Table 4, data are summarized from endothelial cell culture after an incubation time of 12 and 48 h. Endothelial cells were incubated with cell culture medium at 37 °C and 5% CO_2 without any additional chemical agent. These data show that ADMA accumulates in the cell culture medium of endothelial cells over time. Mean ADMA in plasma of 10 healthy humans was found to be 0.600 ± 0.076 μM ranging from 0.48 to 0.69 μM (Table 5).

Table 4
Concentrations of ADMA in cell culture supernatants

Samples of EA.hy 926 cells in two different experiments for different incubation times	ADMA pmol/μg protein
Exp. 1 (12 h)	
Sample 1	1.83
Sample 2	2.11
Sample 3	2.25
Sample 4	1.90
Mean ± S.D.	2.02 ± 0.19
Exp. 2 (48 h)	
Sample 1	3.04
Sample 2	3.85
Sample 3	3.10
Sample 4	3.09
Mean ± S.D.	3.27± 0.39

Endothelial cells (EA.hy 926) were incubated at 37 °C and 5% CO_2 in cell culture medium for 12 and 48 h showing the accumulation of ADMA over time.

Table 5
Concentrations of ADMA in plasma of 10 healthy volunteers

Subject/gender	Age (years)	Plasma (μM)
I/female	25	0.563
II/female	27	0.693
III/female	32	0.666
IV/male	23	0.522
V/female	29	0.671
VI/female	26	0.483
VII/female	26	0.678
VIII/female	43	0.603
IX/male	33	0.609
X/male	33	0.516
Mean ± S.D.	29.7 ± 5.8	0.600 ± 0.076

4. Discussion

Mass spectrometric quantitation of ADMA was thus far hampered by the unavailability of an appropriate internal standard [15–17]. We, therefore, decided to develop a GC–MS based analytical method including newly synthesized stable isotope labelled ADMA as internal standard. Synthesis of deuterated methyl ester of ADMA by esterification of the carboxyl group with [$^2H_4$]-methanol resulting in [$^2H_3$]-methyl-ADMA seems to be another possibility to get a feasible internal standard [15]. However, the reaction is not complete so that the content of unlabelled ADMA must be considered for quantitation. Using [$^2H_6$]-ADMA, we demonstrated that our method can accurately quantify a concentration of 0.05 μM ADMA with a limit of detection of 2 fmol. Our GC–MS method is characterized by high specificity and sensitivity, and by good accuracy of intra- and inter-day reproducibility. The total analysis cycle including cooling of the GC and equilibration time is 12 min per sample. Herein, our GC–MS method distinguishes by shorter analytical time from previous HPLC methods which take up to 45 min per sample [13].

The measurement of ADMA in plasma samples revealed a mean concentration of 0.60 μM ranging from 0.48 to 0.69 μM ($n = 10$). This mean ADMA concentration in healthy subjects is similar to that measured by HPLC in several human studies (range, 0.52–1.25 μM) [6,18,19]. They are also in accordance with previous reports based on GC–MS/MS and LC–MS, i.e. 0.39 ± 0.06 μM [15] and 0.45 ± 0.13 μM [16], respectively. However, lower plasma levels have been reported by others [17]. The quantitation of ADMA in cell culture supernatants is quite difficult due to the high concentration of other interfering amino acids.

We, therefore, decided to include a solid phase extraction step with an anionic exchange resign, i.e. CBA, in the sample preparation procedure for cell culture supernatants. Interference of other amino acids with the measurement of ADMA was thus eliminated and we were able to show a time dependent enrichment of ADMA in cell culture supernatants (Table 4).

The second endogenous L-arginine analogue, i.e. N^G,N'^G-dimethyl-L-arginine (SDMA), has no influence on NO production. Levels of SDMA have been shown to be unaltered in many settings where ADMA was found increased [2,6,20]. Thus, a discimination between SDMA and ADMA is of some interest. We, therefore, investigated the fragmentation pattern and chromatographic behaviour of SDMA. The methyl ester PFP-amide derivative of SDMA showed a prominent fragment ion at *m*/*z* 593. However, a simultaneous quantification of SDMA with [$^2H_6$]-ADMA as internal standard was not possible. The SDMA derivative shows a different retention time possibly due to the formation of another PFP derivative. Thus, the synthesis of a specific internal standard for SDMA is a challenge for future research.

5. Conclusion

These data show that our method provides a highly precise and accurate assay for ADMA in cell culture supernatant and small volumes of plasma. This can be attributed to the use of stable isotope labelled ADMA as internal standard. Mass spectrometry in combination with stable isotope dilution is a powerful tool in external quality assessment schemes and assays based on this technique can be regarded as reference procedures to validate other analytical methods.

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