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Accurate quantification of dimethylamine (DMA) in human urine by gas chromatography–mass spectrometry as pentafluorobenzamide derivative: Evaluation of the relationship between DMA and its precursor asymmetric dimethylarginine (ADMA) in health and disease☆

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Abstract

Dimethylamine [DMA, $(CH_3)_2NH$)] is abundantly present in human urine. Main sources of urinary DMA have been reported to include trimethylamine *N*-oxide, a common food component, and asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide (NO) synthesis. ADMA is excreted in the urine in part unmetabolized and in part after hydrolysis to DMA by dimethylarginine dimethylaminohydrolase (DDAH). Here we describe a GC–MS method for the accurate and rapid quantification of DMA in human urine. The method involves use of $(CD_3)_2NH$ as internal standard, simultaneous derivatization with pentafluorobenzoyl chloride and extraction in toluene, and selected-ion monitoring of *m/z* 239 for DMA and *m/z* 245 for $(CD_3)_2NH$ in the electron ionization mode. GC–MS analysis of urine samples from 10 healthy volunteers revealed a DMA concentration of 264 ± 173 μM equivalent to 10.1 ± 1.64 μmol/mmol creatinine. GC–tandem MS analysis of the same urine samples revealed an ADMA concentration of 27.3 ± 15.3 μM corresponding to 1.35 ± 1.2 μmol/mmol creatinine. In these volunteers, a positive correlation ($R = 0.83919$, $P = 0.0024$) was found between urinary DMA and ADMA, with the DMA/ADMA molar ratio being 10.8 ± 6.2. Elevated excretion rates of DMA (52.9 ± 18.5 μmol/mmol creatinine) and ADMA (3.85 ± 1.65 μmol/mmol creatinine) were found by the method in 49 patients suffering from coronary artery disease, with the DMA/ADMA molar ratio also being elevated (16.8 ± 12.8). In 12 patients suffering from end-stage liver disease, excretion rates of DMA (47.8 ± 19.7 μmol/mmol creatinine) and ADMA (5.6 ± 1.5 μmol/mmol creatinine) were found to be elevated, with the DMA/ADMA molar ratio (9.17 ± 4.2) being insignificantly lower ($P = 0.46$). Between urinary DMA and ADMA there was a positive correlation ($R = 0.6655$, $P < 0.0001$) in coronary artery disease, but no correlation ($R = 0.27339$) was found in end-stage liver disease.

Keywords: L-Arginine; Clinical study; DDAH; Nitric oxide; Quality control

1. Introduction

Dimethylamine [DMA, $(CH_3)_2NH$)] is the most abundant short-chain aliphatic amine present in human urine. The daily urinary excretion of DMA in healthy humans has been reported to be of the order of 20 mg corresponding to 444 μmol [1–6]. DMA is also present in other biological fluids including blood, saliva, gastric juice and vaginal secretions [7,8]. Origin and significance of DMA in humans are of particular importance. DMA received considerable attention at first because it is an important precursor of the potent carcinogen *N*-nitrosodimethylamine and originates from dietary chemicals. Thus, common food components such as trimethylamine *N*-oxide [3], lecithin, choline and trimethylamine may elicit increases in the levels of urinary DMA when given orally [9]. Besides exogenous sources,

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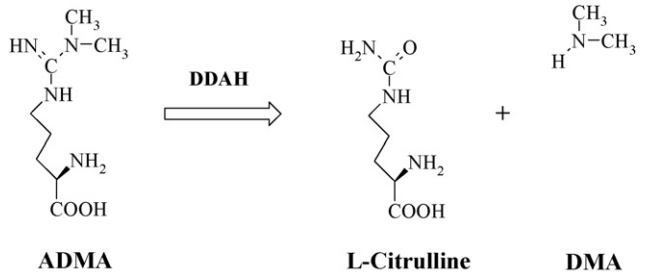

Fig. 1. Biosynthesis of dimethylamine (DMA) and L-citrulline from hydrolysis of asymmetric dimethylarginine (ADMA) by dimethylarginine dimethylaminohydrolase (DDAH).

endogenous biosynthetic pathways have also been proposed to produce DMA, such as the *N*-methylation of monomethylamine which is metabolically derived from sarcosine or glycine [9,10]. More recently, special attention has been paid to DMA since its identification as the metabolic product of N^G,N^G-dimethyl-L-arginine (asymmetric dimethylarginine, ADMA) (reviewed in ref. [11]), which is an endogenous inhibitor of nitric oxide (NO) synthesis from L-arginine (reviewed in ref. [12]). The enzyme N^G,N^G-dimethyl-L-arginine dimethylaminohydrolase (DDAH) specifically hydrolyzes ADMA to yield DMA and L-citrulline [13] (Fig. 1). ADMA has been shown to be actively metabolized to DMA by DDAH in humans in vivo [6]. Thus, intravenous administration of ADMA to humans led to a significant increase of the creatinine-corrected excretion of DMA, suggesting that endogenous ADMA may be a major endogenous source for urinary DMA [6]. The relative contribution of exogenous and endogenous sources to DMA, including food components and ADMA, and the pathophysiological significance of urinary DMA are two challenging tasks.

In this context, reliable analytical methods for the accurate quantitative determination of DMA in human urine and other biological samples are required. Reported analytical methods for DMA in human body fluids include colorimetric assays [6], head-space GC [2], HPLC with fluorescence detection [5], and GC–MS [1]. daCosta et al. reported on a GC–MS method for the quantitative determination of DMA and other amines in various biological matrices including human urine [1]. In this method *p*-toluenesulfonyl chloride was used to form the tosylamide derivative of DMA and quantification was performed in the selected-ion monitoring (SIM) mode after electron ionization (EI). This method has been reported to be suitable for metabolic studies in humans using isotopically labelled methylamines [1]. Ripley et al. described a GC method with N–P detection for the analysis of DMA and other primary and secondary amines in foodstuffs [14]. Ripley et al. used pentafluorobenzoyl chloride in benzene to prepare the pentafluorobenzamide derivatives of the amines by shaking aqueous carbonate-alkalinized acidic distillates of foodstuffs [14].

In the present work, we report validated GC–MS methods for the quantitative determination of DMA in human urine in the SIM mode using negative-ion chemical ionization (NICI) or EI. Two sample procedures using pentafluorobenzoyl chloride as the derivatization agent have been worked out. The newly developed method (referred to as *sample procedure A*) uses a 10-μl aliquot of urine, and DMA is derivatized after evaporation of the urine samples to dryness. The second sample preparation (referred to as *sample procedure B*) was lent out from Ripley et al. [14], adapted and validated for urine samples and uses routinely 100-μl urine volumes. Both sample procedures allow for accurate quantification of DMA in human urine by GC–MS in both ionization modes. The GC–MS method involving sample procedure B possesses a better practical feasibility and enables high-throughput analysis. This method operating in the EI mode was applied to quantify DMA in urine of healthy humans and of patients suffering from coronary artery disease (CAD) or end-stage liver disease as well as in quality control (QC) samples.

2. Experimental

2.1. Materials and chemicals

2,3,4,5,6-Pentafluorobenzoyl chloride and the hydrochloride salts of unlabelled dimethylamine (d_0-DMA) and hexadeuterodimethylamine (d_6-DMA; declared as 99 atom% at ^2H) were obtained from Aldrich (Steinheim, Germany). For quantitative measurements stock solutions (each 100 mM) of d_0-DMA and d_6-DMA were prepared in distilled water and were stored in stoppered flasks in a refrigerator (8 °C). The pH value of freshly prepared stock solutions was about 5.5 and did not change upon storage for at least one year. Toluene, acetonitrile, and ethyl acetate were purchased from Baker (Deventer, The Netherlands). Sodium carbonate was from Merck (Darmstadt, Germany).

2.2. Urine collection and sample preparation procedures

Because of the volatility of DMA and its potential instability in biological samples special precautions for sample collection and storage are usually taken. For DMA analysis in urine collected for 24 h, urine collection in bottles containing hydrochloric acid has been reported [1–4]. In the studies reported in the present work urine was obtained from spontaneous micturition. For this reason and in consideration of the sample procedure B which best operates at unchanged physiological pH values of urine, urine was collected in polypropylene containers without addition of hydrochloric acid or other preservatives. Immediately after urine collection the containers were closed and put on ice, and urine was proportioned into 1-ml aliquots which were analyzed immediately or were frozen and strored at −20 °C. Where this proceeding was not feasible, collected urine was frozen immediately and stored at −20 °C. Frozen urine samples were allowed to thaw on ice until proportioning into 1-ml aliquots and/or derivatization. In quantitative analyses ice-cold urine samples (1000-μl aliquots) were spiked with d_6-DMA (10 μl or 5 μl, 100 mM) to achieve a final concentration of 1000 or 500 μM. In both procedures described below in detail stock solutions of the internal standard d_6-DMA and of d_0-DMA were stored on ice during sample treatment.

2.2.1. Sample procedure A

An aliquot (10 μl) of spiked urine was transferred into a 1.3-ml conus glass vial and treated with HCl (10 μl, 10 mM) to avoid loss of the volatile. The sample was then evaporated to dryness under a stream of nitrogen gas. The residue was treated with pentafluorobenzoyl chloride (50 μl) and the sample was incubated at 60 °C for 5 min. After cooling to room temperature, reagent excess was evaporated to dryness under a nitrogen stream. The residue was treated with ethyl acetate (750 μl) and the sample was vortexed for 60 s, whereby most solid residue remained virtually undissolved. An aliquot (700 μl) of the organic phase was transferred into a clean 1.5-ml autosampler glass vial for GC–MS analysis.

2.2.2. Sample procedure B

An aliquot (100 μl) of spiked urine was transferred into a 1.5-ml polypropylene tube. Toluene (1000 μl), Na_2CO_3 (10 μl, 20 mM) and pentafluorobenzoyl chloride (10 μl, 10 wt.% in acetonitrile) were added, and the sample was mixed by vortexing for 1 min. After centrifugation (5 min, 800 × *g*, 4 °C) an aliquot (700 μl) of the organic phase was transferred into a clean 1.5-ml autosampler glass vial for GC–MS analysis.

2.3. Gas chromatography–mass spectrometry

A Hewlett-Packard MS engine 5890 connected directly to a gas chromatograph 5890 series II equipped with an autosampler (sample tray capacity for 100 vials) Hewlett-Packard model 7673 (Waldbronn, Germany) was used for GC–MS analyses. The gas chromatograph was equipped with a fused-silica capillary column Optima 17 (15 m × 0.25 mm i.d., 0.25-μm film thickness) from Macherey-Nagel (Düren, Germany). Aliquots (1 μl) were injected in the splitless mode by the autosampler. The following oven temperature program was used with helium (50 kPa) as the carrier gas: 1 min at 70 °C, then increased to 280 °C at a rate of 30 °C/min, and to 300 °C at a rate of 10 °C/min. Interface, injector and ion source were kept at 280, 250 and 180 °C, respectively. Electron energy and electron current were set to 200 eV and 300 μA, respectively, for NICI with methane (200 Pa) as the reagent gas. Electron energy was 70 eV in the EI mode. Routinely, electron multiplier voltage was set to 1.8 kV in both ionization modes. Quantification by GC–MS in the NICI as well as in the EI mode was performed by SIM of the ions at *m/z* 239.15 for d_0-DMA and *m/z* 245.15 for d_6-DMA using a dwell-time of 50 ms for each ion in both methods of ionization.

2.4. Quantitative determination of ADMA and creatinine in urine

In theory, pentafluorobenzoyl chloride should be useful to prepare pentafluorobenzoyl and pentafluorobenzamide derivatives of ADMA that would enable simultaneous analysis of DMA and ADMA by GC–MS or GC–tandem MS. However, neither sample procedure A nor sample procedure B led to formation of suitable derivatives of ADMA. For this reason and because no stable-isotope labeled analogs of ADMA are commercially available, ADMA in urine samples was quantified separately by GC–tandem MS exactly as described previously [15]. GC–tandem MS analyses were performed on the triple-stage quadrupole mass spectrometer model ThermoQuest TSQ 7000 (Finnigan MAT, San Jose, CA). Urinary DMA and ADMA levels were corrected for creatinine excretion and are expressed as μmol of DMA or ADMA per mmol of creatinine (i.e., μmol/mmol). Creatinine was quantified in urine samples by HPLC with UV absorbance detection at 236 nm as described previously [16].

2.5. Quality control for DMA

For use as QC samples, urine was collected by a healthy male volunteer for 24 h in a 2000-ml canister made of polypropylene and stored in the cold (8 °C) during collection. After completion of the collection, urine was portioned (40 ml) and centrifuged in the cold (4 °C). After centrifugation 1000-μl urine aliquots were accurately pipetted into 1.5-ml autosampler glass vials which were then immediately frozen at −20 °C and stored at this temperature until analysis. Urine samples from clinical studies were processed by the same way. In 10 not yet frozen QC samples DMA was quantitated by GC–MS (sample procedure B, EI mode). The DMA concentration was determined to be 296 ± 13 μM, i.e. with an imprecision (R.S.D.) of 4.4%. In the frame of clinical studies, one QC sample was analyzed alongside about 10 study samples. The mean level of 296 μM for DMA measured at the beginning was used as a reference value for QC samples. Thus, method accuracy (recovery, %) with which DMA concentration was measured in studies samples was calculated by dividing the DMA concentration measured in the respective QC sample by 296 μM and by multiplying this ratio by 100. Usually, at least two QC samples were co-processed within a run so that the precision of the method could also be determined. Results were considered when recovery in QC samples was 100 ± 20%.

2.6. Ethics

Approval from the local Ethics committees of the Medical School of Hannover and of the University of Würzburg was obtained for clinical studies (described in Section 3.4), as was informed written consent from patients. In studies, in which basal levels of DMA in urine samples had been determined or in which exclusively authors of the present article had been involved (described in Section 3.3), no approval from the local Ethics committee had been obtained.

3. Results and discussion

3.1. GC–MS identification of d_0-DMA and d_6-DMA derivatives and mode of quantification

GC–MS full scan analysis of separate reaction mixtures of synthetic d_0-DMA and d_6-DMA with the derivatizating agent 2,3,4,5,6-pentafluorobenzoyl chloride using sample procedure A showed several GC peaks. The NICI and EI mass

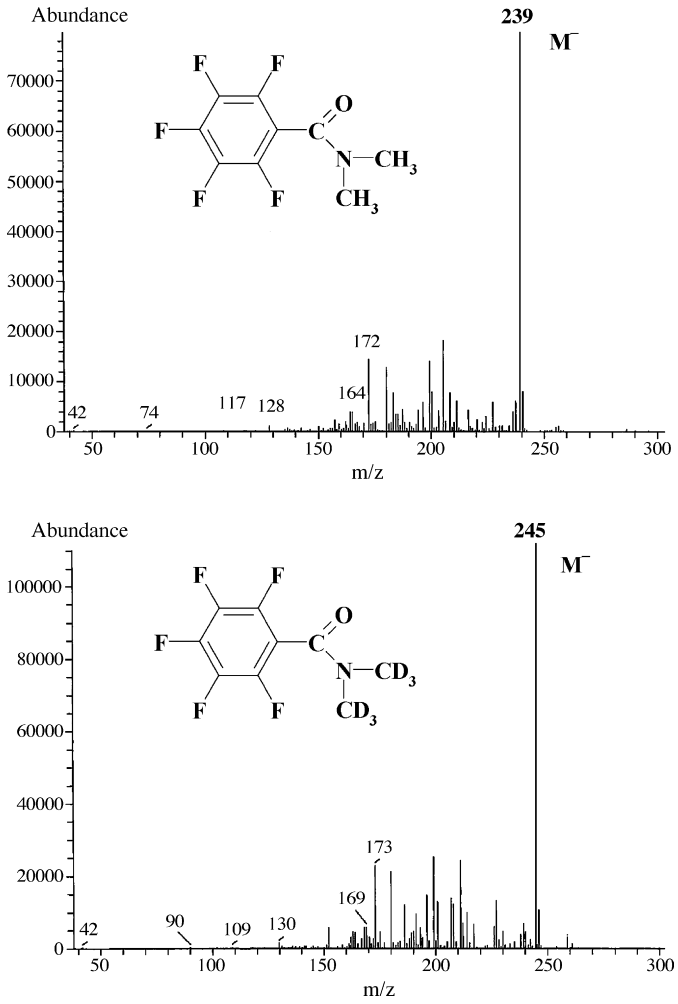

Fig. 2. Mass spectra of unlabelled DMA (upper panel) and hexadeutero-DMA (lower panel) as pentafluorobenzamide derivatives in the NICI mode. Insertions show the structure of the pentafluorobenzamide derivatives and the ion assignment.

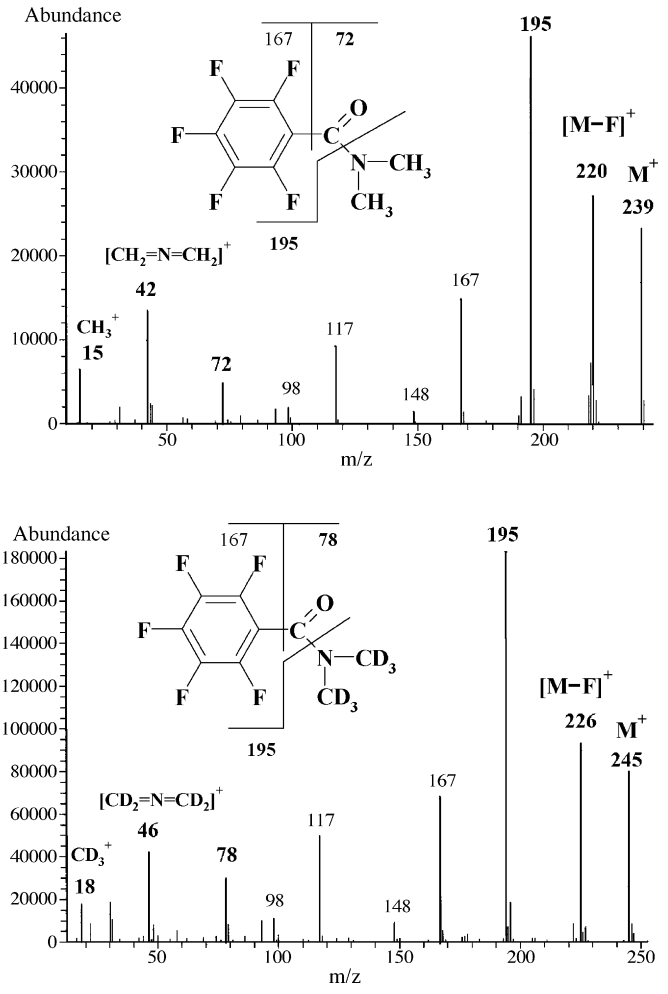

Fig. 3. Mass spectra of unlabelled DMA (upper panel) and hexadeutero-DMA (lower panel) as pentafluorobenzamide derivatives in the EI mode. Insertions show the structure of the pentafluorobenzamide derivatives and the ion assignment.

spectra of the reaction products emerging from the GC column at approximately 3.1 min are shown in Figs. 2 and 3, respectively. The mass spectra clearly identify these derivatives as *N*,*N*-dimethyl-2,3,4,5,6-pentafluorobenzamide from d_0-DMA and *N*,*N*-[$^2H_6$]dimethyl-2,3,4,5,6-pentafluorobenzamide from d_6-DMA. The most intense ions in the NICI mass spectra of the d_0-DMA and d_6-DMA derivatives were observed at *m/z* 239 and *m/z* 245, respectively, and correspond to the molecular anions ($[M]^-$) formed by electron-capture (Fig. 2). The EI mass spectra of these derivatives showed intense molecular cations ($[M]^{+\bullet}$) at *m/z* 239 and *m/z* 245, respectively (Fig. 3). Other pairs of characteristic mass fragments in the EI mass spectra were at *m/z* 72 ($[(CH_3)_2N\text{-}CO]^+$) and *m/z* 78 ($[(CD_3)_2N\text{-}CO]^+$), and at *m/z* 42 ($[CH_2{=}N{=}CH_2]^+$) and *m/z* 46 ($[CD_2{=}N{=}CD_2]^+$) (Fig. 3). The most intense ions at *m/z* 195 in the EI mass spectra of d_0-DMA and d_6-DMA are due to the pentafluorobenzoyl cations (Fig. 3). The EI mass spectrum of the pentafluorobenzamide derivative of d_0-DMA shown in Fig. 3 (upper panel) is very similar to that reported by Ripley et al. [14].

The exact *m/z* values of 239.15 and 245.15 obtained in the mass spectra were set in quantitative analyses in the SIM mode. The highly volatile pentafluorobenzamide derivatives of d_0-DMA and d_6-DMA have a relatively short retention time and elute as symmetric narrow GC peaks with a peak width at baseline of the order of 2 s (Fig. 4). Nevertheless, the pentafluorobenzamide derivatives of d_0-DMA and d_6-DMA are partially separated by GC, with the peak maxima differing by approximately 1 s. For accurate quantification in the SIM mode the dwell-time was set to 50 ms for each ion.

In both ionization modes quantitative GC–MS analysis of urinary DMA by SIM of *m/z* 239 and *m/z* 245 provided very similar chromatograms containing only two peaks corresponding to endogenous DMA and the internal standard d_6-DMA within a relevant retention time window (see Fig. 4 for EI; not shown for NICI). We did not test the possibility of interferences by other endogenous and exogenous amines. Ripley et al. showed by GC with N–P detection that the pentafluorobenzamide derivative of DMA is baseline-separated from the corresponding derivatives of 15 other amines on three different packed GC columns [14]. These findings of Ripley et al. [14], the results of the present study (see validation data below) and the fact that other biogenic amines are present in urine at much lower concentrations

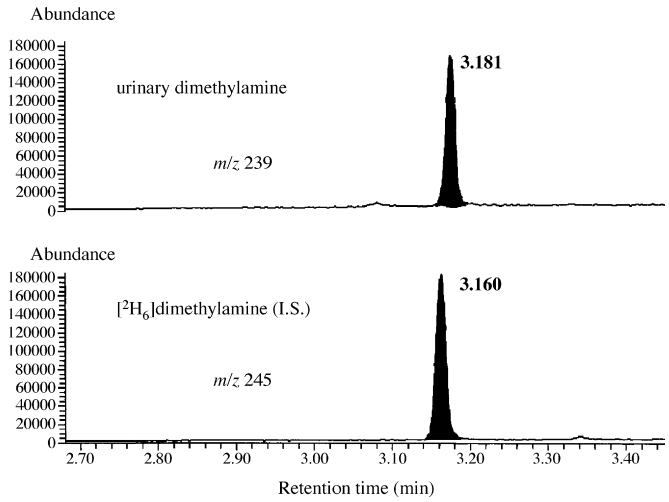

Fig. 4. Partial GC–MS chromatogram from the quantitative determination of DMA in urine of a healthy volunteer by selected-ion monitoring of *m/z* 239 for endogenous DMA and *m/z* 245 for the internal standard (d_6-DMA) which was added to the urine sample at a final concentration of 500 μM. Sample procedure B and EI were used.

strongly suggest that interferences by endogenous and exogenous amines are highly unlikely in our GC–MS methods.

3.2. Derivatization procedures

Because of the relatively low limit of detection (LOD) of the method, i.e. 195 ± 32 fmol of d_6-DMA in the SIM mode (at a S/N value of 3:1 for *m/z* 245) in EI, and the expected relatively high concentration of DMA in human urine, usually ranging in the upper μM-range [1–6], we decided to quantify DMA in small volumes of urine, i.e. 10 μl in sample procedure A and 100 μl in sample procedure B for practical reasons. In order to add the internal standard accurately to urine samples, urine volumes of 1000 μl were spiked with 10 or 5 μl of a 100 mM solution of d_6-DMA in distilled water. The LOD value of the method was not determined in the NICI mode. Since quantification in EI and NICI was performed using the same electron multiplier voltage and analyses of the same samples showed peaks of comparable abundance, we assume that NICI and EI provide comparable sensitivity in DMA GC–MS analysis. Below the analytical characteristics of the sample procedures applied to measure DMA concentrations in human urine samples are described in detail.

3.2.1. Sample procedure A

At room temperature DMA is a gas. Loss of DMA during evaporation of the urine sample (10 μl) is minimized by the addition of a 10-μl aliquot of 10 mM HCl to a 10-μl urine aliquot pre-spiked with d_6-DMA. Complete evaporation of the acidified urine sample is necessary to ensure maximum derivatization yield and to avoid hydrolysis of the pentafluorobenzamide derivatives as well as of the pentafluorobenzoyl chloride agent. Although derivatization of DMA with pentafluorobenzoyl chloride occurs rapidly at room temperature, derivatization by heating at 60 °C for 5 min is essential to ensure maximum derivatization yield. This procedure is considerably shorter than that involving *p*-toluenesulfonyl chloride [1]. Pentafluorobenzoyl chloride is a volatile compound, and reagent excess is readily removed at room temperature by means of gentle nitrogen stream. Reaction products are extracted by short vortexing with dry (molecular sieve) ethyl acetate. Virtually, residue is insoluble in ethyl acetate and remains in the bottom of the glass vial. Removal of impurities present in ethyl acetate is possible by solvent extraction with aqueous buffers such as 0.4 M borate buffer of pH 8.5. However, this additional step does not improve accuracy and may be associated with considerable hydrolysis of the pentafluorobenzamide derivatives during the extraction procedure, e.g. vortexing for 60 s (data not shown).

The data on the accuracy and precision of the GC–MS method involving sample procedure A for DMA added to urine samples from two healthy volunteers at relevant concentrations are presented in Table 1. Recovery and intra-assay imprecision ranged between 92 and 117%, and between 1 and 11%, respectively. Linear regression analysis between measured (y) and added (x) DMA concentrations revealed straight lines with the regression equations $y = 453 + 0.98x$ ($R = 0.99942$, $P < 0.0001$) for urine #1, and $y = 332 + 1.01x$ ($R = 0.99928$, $P < 0.0001$) for urine #2. The slopes of the regression equations are close to 1.0 indicating complete recovery of DMA added to the urine samples. The differences in the y-axis intercepts of the regression equations are due to the use of urine samples containing DMA at different basal levels.

3.2.2. Sample procedure B

In the GC–MS method involving sample procedure B quantitative analyses of urinary DMA were performed in the EI mode.

Table 1
Intra-day accuracy (recovery) and imprecision (R.S.D.) of the GC–MS method (NICI mode) for DMA in urine of two healthy volunteers using sample procedure A

Added (μM)	Measured (μM) (mean ± S.D., $n = 3$)		Recovery (%)[a]		Imprecision (%)	
	Urine #1	Urine #2	Urine #1	Urine #2	Urine #1	Urine #2
0	446 ± 21	337 ± 17	N.A.	N.A.	4.6	5.0
100	541 ± 30	429 ± 15	95.0	92.0	5.6	3.4
200	665 ± 18	570 ± 63	109	117	2.7	11.0
400	847 ± 35	731 ± 22	100	98.5	4.2	3.0
600	1011 ± 25	922 ± 33	94.1	97.6	2.5	3.6
800	1257 ± 37	1165 ± 38	101	104	2.9	3.3
1000	1436 ± 13	1347 ± 63	98.9	101	0.9	4.7

N.A.: not applicable.
[a] Recovery (%) = [(measured value − basal value):added value] × 100.

Table 2
Intra-day accuracy (recovery) and imprecision (R.S.D.) of the GC–MS method (EI mode) for DMA in urine a healthy volunteer using sample procedure B

Added (μM)	Measured (μM) (mean ± S.D., $n = 3$)	Recovery (%)[a]	Imprecision (%)
0	190 ± 7	N.A.	3.6
100	289 ± 6	99.0	2.0
200	379 ± 11	94.5	3.0
400	599 ± 29	102	4.9
600	799 ± 17	102	2.5
800	1076 ± 34	111	3.1
1000	1247 ± 31	106	1.7

N.A.: not applicable.
[a] Recovery (%) = [(measured value − basal value):added value] × 100.

The data on the intra-day (1 day) and inter-day (5 days) accuracy and precision of this GC–MS method for DMA added to urine samples from two healthy volunteers at relevant concentrations are presented in Tables 2 and 3, respectively. Intra-day recovery and imprecision ranged between 95 and 111%, and between 2 and 5%, respectively. Inter-day recovery and precision ranged between 94 and 120%, and between 0.3 and 7%, respectively. Linear regression analysis between measured (y) and added (x) DMA concentrations revealed straight lines with the regression equations $y = 185 + 1.05x$ ($R = 0.99892$, $P < 0.0001$) for intra-day validation (Table 2) and $y = 376 + 1.00x$ ($R = 0.99877$, $P < 0.0001$) for inter-day validation (Table 3). The slopes of the regression equations are close to 1.0 indicating complete recovery of DMA added to the urine samples. The differences in the y-axis intercepts of the regression equations result from the use of urine samples from two healthy volunteers with different basal levels of DMA.

A representative chromatogram from the quantitative GC–MS analysis of endogenous DMA in urine of a healthy human is shown in Fig. 4. With the exception of the peaks corresponding to endogenous DMA (i.e., d_0-DMA, upper trace) and the internal standard d_6-DMA (lower trace) no other peaks with m/z 239 and m/z 245 were detected within the relevant retention time window of 2.7 to 3.4 min.

In the method reported by Ripley et al. [14], the acidic distillate of foodstuffs (125 ml) was treated with 9 ml of benzene, 5 ml of 2 M K_2CO_3, and 1 ml of a pentafluorobenzoyl chloride solution in benzene (2 vol.%). Then, the mixture was stoppered and shaken for 10 min on a wrist-action shaker, and transferred into a separatory funnel. After phase separation the organic phase was analyzed by GC. We adapted and modified this derivatization procedure to the microanalysis of urinary DMA. Major modifications include use of toluene instead of benzene, reduction of carbonate concentration, and use of a vortex-mixer instead of a shaker which shortened the derivatization/extraction time 10-fold. Because of potential emulsion formation due to intensive mixing, phase separation is best achieved by short centrifugation in the cold (e.g., at 4 °C). Sample procedure B involves a single step for simultaneous derivatization of DMA and extraction of the pentafluorobenzamide derivative. Because of its easy feasibility, rapidity and extendibility to plasma samples (see below) we decided to use procedure B for routine quantitative determination of DMA in human urine in the frame of clinical studies. Both NICI (sample procedure A, Table 1) and EI (sample procedure B, Tables 2 and 3) were found to provide comparable accuracy, precision and sensitivity for urinary DMA. Because operation of our GC–MS instrument in the EI mode is less expensive due to renunciation of the reagent gas (i.e. methane) and due to the lower consumption of filaments (cathodes), we performed

Table 3
Inter-day accuracy (recovery) and imprecision (R.S.D.) of the GC–MS method (EI mode) for DMA in urine of a healthy volunteer using sample procedure B

Day	DMA (μM)								
	0			100			200		
	Mean ± S.D.[a] (μM)	Recovery[b] (%)	Imprecision (%)	Mean ± S.D. (μM)	Recovery (%)	Imprecision (%)	Mean ± S.D. (μM)	Recovery (%)	Imprecision (%)
A	349 ± 9	N.A.	2.7	463 ± 16	114	3.5	570 ± 9	111	1.6
B	359 ± 7	N.A.	1.9	475 ± 12	116	2.5	577 ± 22	109	3.8
C	384 ± 11	N.A.	2.9	501 ± 18	117	3.6	572 ± 15	94	2.6
D	380 ± 6	N.A.	1.6	501 ± 32	117	6.4	595 ± 3	108	0.5
E	398 ± 27	N.A.	6.8	518 ± 29	120	5.6	605 ± 32	104	5.3

Day	DMA (μM)								
	400			600			1000		
	Mean ± S.D.[a] (μM)	Recovery (%)	Imprecision (%)	Mean ± S.D. (μM)	Recovery (%)	Imprecision (%)	Mean ± S.D. (μM)	Recovery (%)	Imprecision (%)
A	758 ± 8	102	1.0	996 ± 10	108	1.0	1360 ± 16	101	1.2
B	769 ± 10	103	1.3	1004 ± 24	108	2.4	1365 ± 23	101	1.7
C	769 ± 10	96.3	1.3	1022 ± 13	106	1.3	1379 ± 4	99.5	0.3
D	768 ± 16	97	2.1	1040 ± 30	110	2.9	1389 ± 31	101	2.2
E	781 ± 11	95.8	1.4	1044 ± 25	108	2.4	1396 ± 54	99.8	3.9

All analyses (each 18 samples per day) were performed in triplicate. N.A.: not applicable.
[a] Measured concentration of DMA.
[b] Recovery (%) = [(measured value − basal value):added value] × 100.

Table 4
Measured basal levels of DMA and ADMA and calculated DMA/ADMA molar ratio in urine of 10 healthy humans

Subject	Sex	Age (years)	Creatinine (mM)	DMA		ADMA		DMA/ADMA ratio
				μM	μmol/mmol creatinine	μM	μmol/mmol creatinine	
1	Female	33	12.8	117	9.14	10.2	0.797	11.5
2	Female	41	9.53	103	10.8	4.03	0.423	25.5
3	Female	35	15.7	133	8.47	15.2	0.968	8.75
4	Female	43	3.38	33.8	10.0	15.7	4.645	2.15
5	Female	28	39.5	360	9.11	41.0	1.038	8.78
6	Female	26	26.2	242	9.24	38.3	1.462	6.32
7	Male	43	25.5	304	11.9	38.0	1.490	7.99
8	Male	29	43.4	456	10.5	45.6	1.050	10.0
9	Male	46	42.9	583	13.6	41.9	0.977	13.9
10	Male	41	36.1	305	8.45	22.6	0.626	13.5
Mean ± S.D.		36.5 ± 7.2	25.5 ± 14.7	264 ± 173	10.1 ± 1.64	27.3 ± 15.3	1.348 ± 1.204	10.8 ± 6.2

GC–MS analyses of DMA in the EI mode. Routinely, 100 urine samples can be worked up by a person per day and can be analyzed daily by this method with our GC–MS instrument which has a sample tray capacity for 100 vials. The pentafluorobenzamide derivatives of urinary DMA and d_6-DMA in toluene were found to be stable for several weeks, when stored at 4 °C (data not shown).

Sample procedure B, but not sample procedure A, was found to be extendable to human plasma. Preliminary investigations using 100-μl aliquots of plasma from five healthy humans and d_6-DMA added at 50 μM to plasma samples resulted in a plasma DMA concentration of 2.5 ± 1.4 μM. This order of plasma DMA concentration is supported by the findings of Teerlink et al. [5], who measured DMA at 3.3 ± 1.5 μM in plasma of healthy humans by HPLC with fluorescence detection [5].

3.3. Urinary excretion rates of DMA and ADMA in health

3.3.1. Basal levels of urinary DMA in healthy volunteers and relationship to ADMA

The present GC–MS method was applied to measure DMA in urine of healthy volunteers without history of hepatic, renal, cardiovascular or other diseases. Because of the potential association between DMA and ADMA we also measured ADMA in the same urine samples by GC–tandem MS. Table 4 summarizes the DMA and ADMA levels measured in urine of ten healthy volunteers and the calculated molar ratio of DMA to ADMA, i.e. DMA/ADMA. DMA concentration ranged between 34 and 583 μM, with the mean value being 264 μM. This range for urinary DMA concentrations in healthy humans has also been reported by other groups [1–6]. Considering a mean creatinine excretion rate of 9–14 mmol per day, our findings suggest that healthy humans excrete 90–140 μmol of DMA per day. This range for daily DMA excretion in healthy humans is lower than that reported by other groups [1,2,6]. The range of creatinine-corrected DMA levels was considerably more closely, with the mean value amounting to 10.1 μmol/mmol creatinine. ADMA concentration in the urine samples of the same volunteers ranged between 4 and 46 μM, with the mean value being 27 μM. The range of creatinine-corrected ADMA levels was also more closely, with the mean value amounting to 1.35 μmol/mmol creatinine. The DMA/ADMA molar ratio ranged between 2 and 26, with the mean value being approximately 11, indicating that DMA levels in urine are in average about 11 times higher than those of ADMA (Table 4). Linear regression analysis between DMA and ADMA levels in the urine of these subjects revealed a statistically significant positive correlation (Fig. 5). To the best of our knowledge, the present work reports for the first time on a correlation between DMA and ADMA in human urine.

3.3.2. Diurnal variation of urinary DMA and ADMA excretion in healthy humans

To investigate diurnal variation of DMA and ADMA, two healthy male volunteers (A and B) being on unrestricted diet collected urine on different days. In the urine samples DMA was determined by the present GC–MS method (sample procedure B, EI) and ADMA was quantified by GC–tandem MS [15]. Creatinine-corrected excretion of DMA and ADMA was found to moderately vary during the working day (data not shown). Because of the positive correlation between urinary DMA and ADMA (Fig. 5) and the relatively constant molar ratio of DMA to ADMA observed in healthy volunteers (Table 4), the

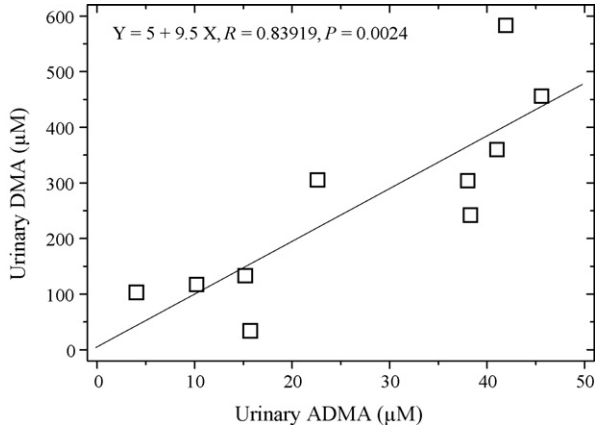

Fig. 5. Correlation between DMA and ADMA in urine of ten healthy volunteers. Figure was constructed by using the data of Table 4.

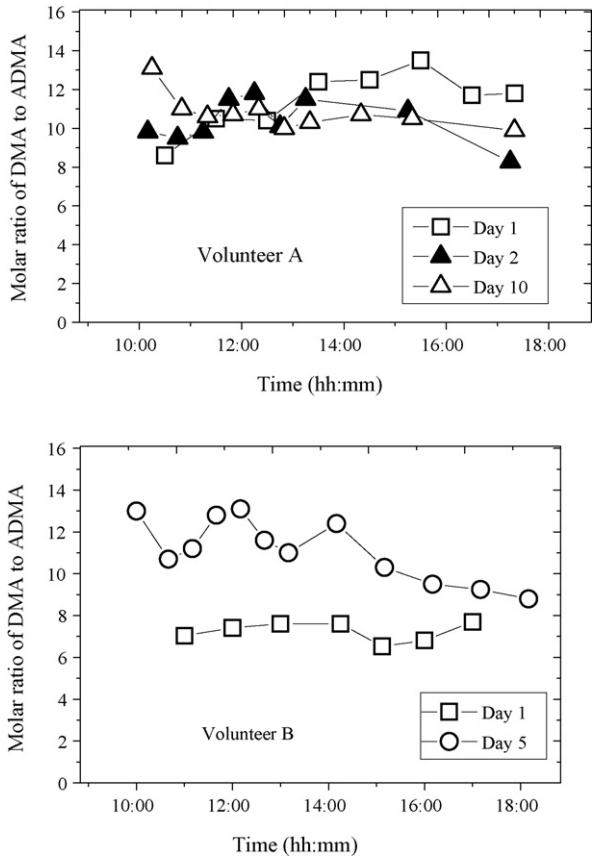

Fig. 6. Diurnal variation of the DMA/ADMA molar ratio in urine of two healthy volunteers on different days. Upper panel: volunteer A on days 1, 2 and 10. Lower panel: volunteer B on days 1 and 5.

DMA/ADMA molar ratio was calculated and plotted versus time (Fig. 6). In volunteer A, the DMA/ADMA molar ratio ranged between 8 and 14 on three days, with the variation (R.S.D.) being 13.4, 11.1 and 8.3%, respectively. In volunteer B, the DMA/ADMA molar ratio varied by 6.3% on day 1 and by 13.4% on day 5. Interestingly, on day 5 (volunteer B) the DMA/ADMA molar ratio decreased almost constantly from 13 to 9 during the working day. This decrease was caused by constantly decreasing urinary excretion of DMA (data not shown). Because certain foods may contribute to DMA in blood and urine, we tested for this potential contribution. Thus, volunteer A ate canned fish in the evening at 07:20, and urine samples from spontaneous micturition were collected 2 h before and 12 h afterwards. DMA concentrations in these urine samples were determined to be 168 and 1135 μM corresponding to an approximate seven-fold increase, whereas the respective ADMA levels amounted to 17 and 35 μM corresponding to a two-fold increase. These data indicate an increase of the DMA/ADMA molar ratio from 9.9 before to 32.4 after fish consumption. The most likely explanation for the elevation of the DMA/ADMA molar ratio in urine is that the consumed canned fish contained DMA. In the literature various foodstuffs including fish have been reported to contain considerable amounts of DMA and other methylamines or trimethylamine *N*-oxide [1,17,18], the consumption of which may considerably increase urinary excretion of DMA [3]. Thus, analogous to nitrite and nitrate measurement in biological fluids as indices of NO synthesis [19], proper attention has also to be paid to diet with regard to DMA and precautions should be taken to minimize intake of DMA by foods, notably fish.

3.3.3. Effect of the diuretic acetazolamide on DMA and ADMA excretion

The proximally acting diuretic acetazolamide reduces excretion of ammonium (NH_4^+) and has been shown to increase nitrite + nitrate excretion in vivo in the rat [20]. Therefore and because DMA (pK_b 3.3) is also present as an ammonium ion in urine, we investigated the effect of this diuretic agent on the urinary excretion of DMA in humans. For this, two healthy volunteers collected urine from about 2 h before and up to 12 h after oral administration of acetazolamide. In the collected urine samples DMA, ADMA, other biochemical parameters and pH were measured. In agreement with previous study in the rat [20], we found that acetazolamide increased approximately two-fold urinary excretion of nitrate in both volunteers 30 min after administration (data not shown), indicating that both nitrate and nitrite are extensively reabsorbed in the proximal tubule in humans. By contrast, the concentration both of DMA and ADMA did not considerably change upon acetazolamide (data not shown). Also, Fig. 7 shows that the DMA/ADMA molar ratio did not considerably change in the urine of both volunteers upon drug administration. In the investigated time interval of about 14 h, the DMA/ADMA molar ratio amounted to 12.7 ± 1.2 in volunteer A and to 7.2 ± 0.7 in volunteer C, with the variation being each about 10%. Unlike in volunteer A, in volunteer C a continuous decrease in the DMA/ADMA molar ratio was seen from 1 h to 4 h, with maximum fall amounting to approximately 25% (Fig. 7). Upon acetazolamide administration, urine pH values increased from 5.4 up to 7.8. Neither DMA nor ADMA concentration correlated with urine pH (data not shown), suggesting that urinary excretion of DMA and ADMA is almost independent of urine pH within the physiological range.

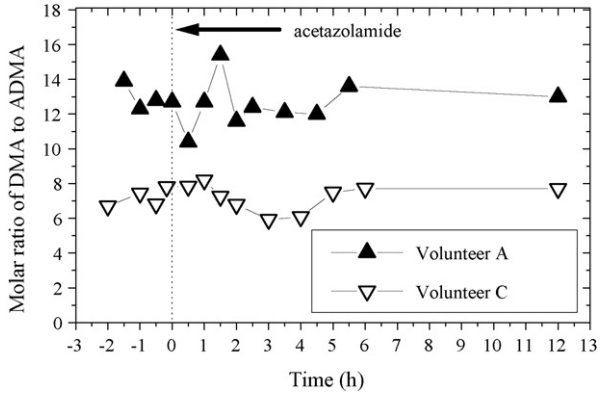

Fig. 7. Time profile of the DMA/ADMA molar ratio in urine of two healthy volunteers before and after oral administration of the drug acetazolamide at a dose of 5 mg/kg body weight by volunteer A and of 5.6 mg/kg body weight by volunteer C. The drug was taken at time "0" as indicated by the vertical line and the arrow.

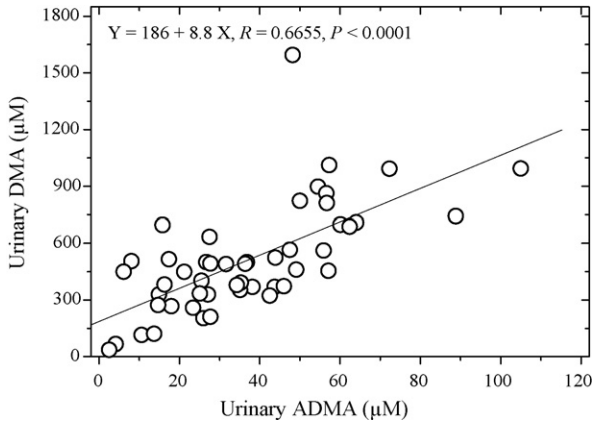

Fig. 8. Correlation between DMA and ADMA in urine of 49 patients suffering from coronary artery disease. The characteristics of the patients have been reported elsewhere [21].

Table 5
Creatinine-corrected DMA and ADMA excretion rates and DMA/ADMA molar ratio in urine of end-stage liver disease patients

Patient	DMA (μmol/mmol) Mean (R.S.D.)[a]	ADMA (μmol/mmol)	DMA/ ADMA
A	51.4 (3.9)	3.79	13.6
B	47.6 (0.4)	7.69	6.18
C	37.1 (8.4)	4.88	7.6
D	48.8 (5.4)	5.75	8.49
F	39.3 (6.9)	2.19	17.9
G	41.0 (2.0)	6.63	6.18
H	47.9 (2.7)	6.03	7.94
I	43.6 (1.4)	5.8	7.52
J	108 (0.7)	6.77	15.9
K	37.3 (0.4)	5.04	7.4
L	41.6 (0.8)	7.37	5.64
M	30.3 (2.0)	5.34	5.67
Mean ± S.D.	47.8 ± 19.7	5.61 ± 1.54	9.17 ± 4.20

[a] All samples were analyzed in duplicate using sample procedure B.

3.4. Urinary levels of DMA and ADMA in disease

3.4.1. Coronary artery disease

DMA and ADMA were measured in urine of 49 patients (age 67 ± 10 years) suffering from coronary artery disease (CAD) as diagnosed by coronary angiography; patient characteristics have been described by us recently [21]. Urinary concentrations ranges were 36–1600 μM for DMA and 2.5–105 μM for ADMA (Fig. 8), with the corresponding mean values being 510 ± 287 μM and 36.9 ± 21.8 μM. The creatinine-corrected excretion rates were calculated to be 52.9 ± 18.5 μmol/mmol for DMA and 3.85 ± 1.65 μmol/mmol for ADMA. The DMA/ADMA molar ratio was determined to be 16.8 ± 12.8. In comparison to healthy humans (Table 4), CAD patients had statistically significantly elevated excretion rates of DMA ($P = 1.2 \times 10^{-9}$, unpaired t test) and of ADMA ($P = 2.9 \times 10^{-5}$, unpaired t test), and a borderline DMA/ADMA molar ratio ($P = 0.1558$, unpaired t test). A positive correlation ($R = 0.6655$, $P < 0.0001$) was found between urinary concentrations of DMA and ADMA in patients suffering from CAD (Fig. 8), with the correlation coefficient being slightly lower compared to that in healthy humans (Fig. 5).

These findings suggest that ADMA synthesis is elevated in CAD. Because ADMA plasma levels in the CAD patients were only slightly elevated (range 470–580 nM) [21] as compared to healthy humans [15], our present results suggest that excretion of unchanged ADMA as well as hepatic and renal elimination of ADMA via the DDAH pathway are sufficiently efficient to compensate for greatly elevated ADMA synthesis in CAD. Thus, determination of circulating ADMA levels alone may not satisfactorily describe the actual status of the ADMA pathway.

3.4.2. End-stage liver disease

In previous study we found a statistically significantly elevated urinary excretion of ADMA in twelve patients (age 47 ± 13 years) suffering from end-stage liver disease immediately prior to orthotopic liver transplantation as compared to healthy controls [22]. It was therefore of interest to investigate DMA excretion in this disease. By means of the present GC–MS method we quantified DMA in the same urine samples previously analyzed for ADMA. The urine samples had been stored at −20 °C. All samples were analyzed for DMA in duplicate. Urinary DMA concentration in the patients ranged between 140 and 1100 μM. Imprecision (R.S.D.) of the measurement ranged between 0.4 and 8.4%. The creatinine-corrected excretion rates of DMA and ADMA as well as their molar ratios are summarized in Table 5. In comparison to healthy humans (Table 4), end-stage liver disease patients show statistically significantly higher excretion rates of DMA ($P = 7.9 \times 10^{-6}$, unpaired t test) and ADMA ($P = 6.7 \times 10^{-7}$, unpaired t test). By contrast, the DMA/ADMA molar ratio in end-stage liver disease and health did not differ significantly ($P = 0.46$). Also in contrast to healthy humans (Fig. 5), no correlation ($R = 0.27339$, $P = 0.3899$) was found between urinary DMA and ADMA in patients suffering from end-stage liver disease. In the present study we measured by GC–tandem MS in plasma samples of these patients preoperatively statistically significantly ($P < 0.05$, unpaired t test) elevated ADMA levels as compared to healthy subjects, i.e. 600 ± 105 nM versus 493 ± 84 nM. These levels are in good conformity with ADMA plasma levels recently reported for patients with chronic hepatic failure (690 nM, preoperatively) and healthy volunteers (410 nM) as measured by HPLC with fluorescence detection [23].

Our present findings suggest that ADMA synthesis is elevated in end-stage liver disease, with hepatic and renal elimination via the DDAH metabolic pathway being apparently insufficient to compensate for ADMA overproduction. It is noteworthy that DDAH is highly expressed in the liver but is also present in the kidney. Our results support recent findings suggesting that both organs are of particular importance for the effective elimination of ADMA from the circulation via the DDAH metabolic pathway [24].

Table 6
Results of the quality control (QC) for urinary DMA in various clinical studies

QC-No.	DMA (μM)[a]	Recovery (%)[b]	Imprecision (%)
Study #1, August 2004, 166 samples			
1	285	96.3	N.A.
2, 3, 4	281, 280, 290	94.9, 94.6, 98	1.9
5, 6	308, 305	104, 103	0.7
7, 8, 9	301, 307, 312	102, 104, 105	1.8
10, 11	287, 294	97.0, 99.3	1.7
12, 13, 14	293, 303, 285	99.0, 102, 96.3	3.1
Mean ± S.D.		99.7 ± 3.6	1.8 ± 0.9
Study #2, November 2004, 113 samples			
1, 2	282, 288	95.3, 97.3	1.5
3, 4	341, 305	115, 103	7.9
5, 6, 7, 8, 9	314, 327, 326, 297, 316	106, 110, 110, 100, 107	3.8
10, 11	310, 315	105, 106	1.1
Mean ± S.D.		105 ± 6	3.6 ± 3.1
Study #3, August 2005, 40 samples			
1, 2, 3, 4	278, 283, 274, 292	93.9, 95.6, 92.6, 98.6	2.8
Mean ± S.D.		95.2 ± 2.6	N.A.
Study #4, September 2005, 28 samples			
1, 2, 3, 4	274, 286, 266, 296	92.6, 96.6, 89.9, 100	4.7
Mean ± S.D.		94.8 ± 4.4	N.A.
Study #5, September 2005, 51 samples			
1, 2	283, 297	95.6, 100	3.4
3, 4	256, 269	86.4, 90.9	3.5
5, 6	276, 299	93.2, 101	5.7
7, 8	328, 306	111, 103	4.9
Mean ± S.D.		97.6 ± 7.7	4.4 ± 1.1
Study #6, January 2006, 87 samples			
1–9	289, 301, 291, 294, 309	97.6, 102, 98.3, 99.3, 104	2.8
	298, 284, 313, 297	101, 95.9, 106, 100	4.0
Mean ± S.D.		100 ± 3	3.4 ± 0.8
Study #7, February 2006, 48 samples			
1–6	278, 294, 300, 290, 310	93.9, 99.3, 101, 98, 105	4.0
	297	100	N.A.
Mean ± S.D.		99.4 ± 4.1	N.A.
Mean ± S.D. over the entirety		100 ± 6	3.2 ± 2.0

N.A.: not applicable.
[a] The concentration of the internal standard d_6-DMA was 500 μM in all urine samples. In this period, the stock solution of d_6-DMA (100 mM in distilled water) was prepared twice. The first stock solution was used in studies #1 to #5, the second stock solution was used in studies #6 and #7.
[b] Recovery was calculated considering a mean DMA concentration of 296 μM (see Section 2.5). Sample procedure B and EI were used in all studies.

3.5. Quality control

The quality control for urinary DMA started in August 2004 and is continued to date. In this 18-month covering period, seven clinical studies on NO have been performed in our institute. In these studies urinary DMA was also included as a biochemical parameter and was measured by GC–MS (sample procedure B, EI). The current status of the QC for urinary DMA is summarized in Table 6. In all studies accuracy and precision of the method were within acceptable ranges underscoring the reliability of the GC–MS method for the quantitative determination of DMA in urine of healthy and ill humans. Considering all QC samples mean recovery was very close to 100%, whereas mean imprecision was 3%, with the highest imprecision value not exceeding 8%. This type of QC may serve as a model for QC systems involving endogenous substances.

4. Conclusions

The GC–MS method involving sample procedure B for the simultaneous derivatization and extraction of DMA from human urine is accurate, precise, and rapid. The pentafluorobenzamide derivatives of endogenous DMA and the hexadeutero-DMA internal standard are stable in toluene. These characteristics allow for automated and high-throughput quantitative determination of DMA in human urine. The method is a useful analytical tool in clinical studies. Elevated DMA excretion rates were found in patients suffering from coronary artery disease or end-stage liver disease, who, however, also showed elevated ADMA excretion rates as compared to healthy humans. The present study suggests that investigations on synthesis, metabolism and elimination of ADMA should include quantitative analysis both of DMA and of ADMA in the urine. The molar ratio of DMA to

ADMA in urine may be an additional useful measure to evaluate the effectiveness of hepatic and renal elimination of ADMA in humans via the DDAH pathway. In this context, determination of the relative contribution of ADMA and other alternative pathways to DMA is of particular importance. Dietary DMA should be considered as a potential abundant exogenous source for DMA, and appropriate precautions should be taken to minimize its input in clinical studies. By contrast, drugs, such as the proximally acting diuretic acetazolamide which may inhibit renal excretion of ammonium, seem not to affect considerably DMA excretion in healthy humans.

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References

[1] K.-A. daCosta, J.J. Vrbanac, S.H. Zeisel, Anal. Biochem. 187 (1990) 234.
[2] A.Q. Zhang, S.C. Mitchell, R. Ayesh, R.L. Smith, J. Chromatogr. 584 (1992) 141.
[3] A.Q. Zhang, S.C. Mitchell, R. Ayesh, R.L. Smith, Biochem. Pharmacol. 45 (1993) 2185.
[4] A.Q. Zhang, S.C. Mitchell, R.L. Smith, Clin. Chim. Acta 233 (1995) 81.
[5] T. Teerlink, M.W.T. Hennekes, C. Mulder, H.F.H. Brulez, J. Chromatogr. B 691 (1997) 269.
[6] V. Achan, M. Broadhead, M. Malaki, G.S.J. Whitley, L. Leiper, R. MacAllister, P. Vallance, Arterioscler. Thromb. Vasc. Biol. 23 (2003) 1455.
[7] S.H. Zeisel, K.-A. daCosta, Cancer Res. 46 (1986) 6136.
[8] S.H. Zeisel, K.-A. daCosta, J.T. La Mont, Carcinogenesis 9 (1988) 179.
[9] A.M. Asator, M.L. Simenhoff, Biochim. Biophys. Acta 111 (1965) 384.
[10] S.H. Zeisel, K.-A. daCosta, J.G. Fox, Biochem. J. 232 (1985) 403.
[11] J.M. Leiper, P. Vallance, Eur. J. Clin. Pharmacol. 62 (2006) 33.
[12] D. Tsikas, R.H. Böger, J. Sandmann, S.M. Bode-Böger, J.C. Frölich, FEBS Lett. 478 (2000) 1.
[13] T. Ogawa, M. Kimoto, K. Sasaoka, J. Biol. Chem. 264 (1989) 10205.
[14] B.D. Ripley, B.J. French, L.V. Edgington, J. Assoc. Off. Anal. Chem. 65 (1982) 1066.
[15] D. Tsikas, B. Schubert, F.-M. Gutzki, J. Sandmann, J.C. Frölich, J. Chromatogr. B 898 (2003) 87.
[16] D. Tsikas, A. Wolf, J.C. Frölich, Clin. Chem. 50 (2004) 201.
[17] W. Fiddler, R.C. Doerr, R.A. Gates, J. Assoc. Off. Anal. Chem. 74 (1991) 400.
[18] L.B. Fay, C.D. Leaf, E. Gremaud, J.-M. Aeschlimann, C. Steen, D.E.G. Shuker, R.J. Turesky, Carcinogenesis 18 (1997) 1039.
[19] D. Tsikas, Free Radic. Res. 39 (2005) 797.
[20] T. Sütö, G. Losonczy, C. Qiu, C. Hill, L. Samsell, J. Ruby, N. Charon, R. Venuto, C. Baylis, Kidney Int. 48 (1995) 1272.
[21] T. Thum, D. Tsikas, S. Stein, M. Schultheiss, M. Eigenthaler, S.D. Anker, P.A. Poole-Wilson, G. Ertl, J. Bauersachs, J. Am. Coll. Cardiol. 46 (2005) 1693.
[22] D. Tsikas, I. Rode, T. Becker, B. Nashan, J. Klempnauer, J.C. Frölich, Hepatology 38 (2003) 1063.
[23] M.P.C. Siroen, J.R.M. van der Sijp, T. Teerlink, C. va Schaik, R.J. Nijeveldt, P.A.M. van Leeuwen, Hepatology 41 (2005) 559.
[24] M.P.C. Siroen, M.C. Warlé, T. Teerlink, R.J. Nijveldt, E.J. Kuipers, H.J. Metselaar, H.W. Tilanus, D.J. Kuik, J.R.M. van der Sijp, S. Meijer, B. van der Hoven, P.A.M. Leeuwen, Liver Transpl. 10 (2004) 1524.