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Accurate quantification of dimethylamine (DMA) in human plasma and serum by GC–MS and GC–tandem MS as pentafluorobenzamide derivative in the positive-ion chemical ionization mode☆

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Abstract

Dimethylamine (DMA) circulates in human blood and is excreted in the urine. Major precursor for endogenous DMA is asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide (NO) synthesis. ADMA is hydrolyzed to DMA and L-citrulline by dimethylarginine dimethylaminohydrolase (DDAH). In previous work, we reported a GC–MS method for the quantification of DMA in human urine. This method involves simultaneous derivatization of endogenous DMA and the internal standard $(CD_3)_2NH$ by pentafluorobenzoyl chloride (PFBoylCl) and extraction of the pentafluorobenzamide derivatives by toluene. In the present work, we optimized this derivatization/extraction procedure for the quantitative determination of DMA in human plasma. Optimized experimental parameters included vortex time and concentration of PFBoylCl, carbonate and internal standard. The GC–MS method was thoroughly validated and applied to measure DMA concentrations in human plasma and serum samples. GC–MS quantification was performed by selected-ion monitoring of the protonated molecules at *m/z* 240 for DMA and *m/z* 246 for $(CD_3)_2NH$ in the positive-ion chemical ionization mode. Circulating DMA concentration in healthy young women ($n = 18$) was determined to be 1.43 ± 0.23 μM in serum, 1.73 ± 0.17 μM in lithium heparin plasma, and 9.84 ± 1.43 μM in EDTA plasma. DMA was identified as an abundant contaminant in EDTA vacutainer tubes (9.3 ± 1.9 nmol/monovette, $n = 6$). Serum and lithium heparin vacutainer tubes contained considerably smaller amounts of DMA (0.42 ± 0.01 and 0.95 ± 0.01 nmol/monovette, respectively, each $n = 6$). Serum is recommended as the most appropriate matrix for measuring DMA in human blood. The present GC–MS method should be useful for the determination of systemic and whole body DDAH activity by measuring circulating and excretory DMA in experimental and clinical studies.

Keywords: ADMA; Contamination; DDAH; Derivatization; Nitric oxide; Validation

1. Introduction

Dimethylamine [DMA, $(CH_3)_2NH$)] is the most abundant short-chain aliphatic amine present in human urine and blood [1–6]. DMA is also present in other biological fluids including saliva, gastric juice and vaginal secretions [7,8]. Origin and significance of DMA in humans are of particular importance. Besides exogenous sources, endogenous biosynthetic pathways have 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]. The relative contribution of exogenous and endogenous sources to DMA and the pathophysiological significance of DMA are currently two challenging tasks [11].

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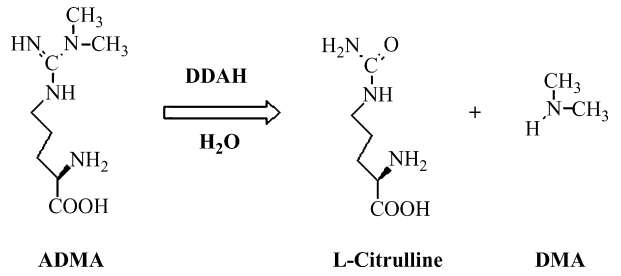

Fig. 1. Dimethylarginine dimethylaminohydrolase (DDAH)-catalyzed formation of L-citrulline and dimethylamine (DMA) from asymmetric dimethylarginine (ADMA).

Reported analytical methods for the measurement of DMA in human body fluids include colorimetric assays [6], head-space GC [2], HPLC with fluorescence detection [5], and GC–MS [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 by using a benzene solution of pentafluorobenzoyl chloride (PFBoylCl) to prepare the pentafluorobenzamide derivatives [14]. In previous work, we have adopted and modified this method for the quantitative determination of DMA in human urine [15]. Preliminary investigations had shown that this method is in principle applicable to human plasma samples [15].

In the present work, we report the optimization, thorough validation and application of a GC–MS method for the measurement of DMA in human plasma as pentafluorobenzamide derivative. Optimized parameters include vortex time, concentration of PFBoylCl, carbonate and internal standard $(CD_3)_2NH$. Instead of electron ionization (EI) used in previous work [15], positive-ion chemical ionization (PICI) was used in the present method. GC–MS quantification was performed by selected-ion monitoring (SIM) of the protonated molecules ($[M+H]^+$) at m/z 240 for endogenous DMA and m/z 246 for the internal standard $(CD_3)_2NH$. In selected plasma samples, quantification was also carried out by GC–tandem MS in the selected-reaction monitoring (SRM) mode.

Preliminary investigations revealed considerably higher DMA concentrations in EDTA plasma as compared to heparinized plasma. The reason for this could be presence of DMA as a contaminant in EDTA-containing vacutainer tubes. Theoretically, DMA in EDTA monovettes could originate from various sources including (1) DMA-contaminated ethylenediamine which is used in the synthesis of EDTA, (2) thermally decomposed EDTA chelates in aqueous solution at high temperatures [16], (3) γ irradiation which is used for sterilization of vacutainer tubes, and (4) EDTA-mediated formation/release of DMA from endogenous sources in blood. Therefore, the potential DMA contamination of vacutainer tubes and of the chemical EDTA was also investigated in the present study.

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 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). Toluene and acetonitrile were purchased from Baker (Deventer, The Netherlands). Sodium carbonate and EDTA disodium salt were from Merck (Darmstadt, Germany). All vacutainer tubes used for blood sampling and generation of plasma or serum were obtained from Sarstedt and were sterilized by γ irradiation as declared by the manufacturer (Nümbrecht, Germany).

2.2. Procedures for plasma and serum collection and sample derivatisation—optimization of derivatization/extraction conditions

Because urine and plasma or serum greatly differ with respect to experimentally relevant parameters such as pH, buffer capacity, protein and DMA concentration [5], and emulsion formation susceptibility, the derivatization/extraction procedure was thoroughly investigated and optimized for plasma samples.

2.2.1. Plasma and serum generation, sample preparation and standard solutions

DMA is volatile and potentially unstable in biological samples, and therefore special precautions for sample collection and storage have to be taken. In the present study, plasma and serum were collected without addition of HCl or other preservatives. Immediately after plasma or serum generation by centrifugation (10 min, 800 × g, 4 °C) of blood the vacutainer tubes were closed and put on ice, and plasma/serum was proportioned into 1-ml aliquots which were analyzed immediately or were frozen and stored at −20 °C. Frozen plasma/serum samples were allowed to thaw on ice until proportioning into 100-μl aliquots and/or derivatization/extraction. In quantitative analyses, ice-cold plasma/serum samples (100-μl aliquots each) were spiked with d_6-DMA (10 μl, 100 μM) to achieve a final concentration of 10 μM (see below). Stock solutions and dilutions of the internal standard d_6-DMA and of d_0-DMA were prepared in 50 mM HCl and stored on ice during sample treatment.

2.2.2. Optimization of derivatization/extraction conditions

Investigated and optimized parameters are described in detail in the respective experiments below. All analyses were performed in duplicate using human pooled CPD plasma (citrate phosphate dextrose, CPD). Samples were vortex-mixed manually using a Heidolph vortex mixer model Reax 2000 (Schwabach, Germany) at the highest vortex speed (i.e., stage 9). The following general procedure was used in all optimization experiments. Aliquots (100 μl) of ice-cold plasma were transferred into 1.5-ml polypropylene tubes and spiked with d_6-DMA, mixed by vortexing for 5 s and put in ice for 10 min. Then toluene (constantly at 1000 μl), Na_2CO_3 and PFBoylCl were added in this order, and samples were mixed by vortexing. After centrifugation (5 min, 800 × g, 4 °C), an aliquot (constantly at 700 μl) of the organic phase was transferred into a

1.5-ml autosampler glass vial for GC–MS analysis. The peak area of the internal standard added to the plasma samples or the concentration of endogenous DMA measured in the plasma samples were used to evaluate the effects of the variables.

2.2.2.1. The effect of pentafluorobenzoylchloride concentration. To investigate the effect of PFBoylCl on DMA derivatization, 10-μl aliquots of a 10-μM solution of d_6-DMA were added to the plasma samples to achieve a final concentration of 1 μM. Na_2CO_3 was added as 10-μl aliquots of a 20-mM aqueous solution. PFBoylCl was added as 5-, 10-, 20- and 40-μl aliquots of a 10 vol% solution in acetonitrile. The vortex time was constant at 1 min.

2.2.2.2. The effect of carbonate concentration. In this set of experiments, 10-μl aliquots of a 10-μM solution of d_6-DMA were added to plasma samples to achieve a final concentration of 1 μM. Na_2CO_3 was added as 10-μl aliquots of 0, 20, 40, 80 or 160 mM aqueous solutions. PFBoylCl was added as 10-μl aliquots of a 10 vol% solution in acetonitrile. The vortex time was constant at 1 min.

2.2.2.3. The effect of vortex time. In these experiments, 10-μl aliquots of a 10-μM solution of d_6-DMA were added to plasma samples to achieve a final concentration of 1 μM. Sample volume and concentration of the other reagents added were 10 μl of 20 mM for Na_2CO_3 and 10 μl of 10 vol% of PFBoylCl in acetonitrile. Samples were vortex-mixed for 1, 2, 3, 4 or 5 min.

2.2.2.4. The effect of internal standard concentration. The internal standard d_6-DMA (2–20-μl aliquots of a 50 μM solution in 50 mM HCl) was added to plasma samples at the following final concentrations: 1, 2, 4, 6, 8, or 10 μM. Na_2CO_3 was added as 10-μl aliquots of a 20-mM aqueous solution. PFBoylCl was added as 10-μl aliquots of a 10 vol% solution in acetonitrile. The vortex time was kept constant at 5 min.

2.2.2.5. Optimum derivatization/extraction conditions for quantitative analysis. On the basis of the results from optimization experiments (see Section 3) the following specific conditions in the derivatization/extraction procedure were chosen and used for quantitative analyses. Ice-cold plasma/serum (100-μl aliquots) was pipetted into 1.5-ml polypropylene tubes. The internal standard (10 μl, 100 μM), toluene (1000 μl), Na_2CO_3 (10 μl, 20 mM) and PFBoylCl (10 μl, 10 vol% in acetonitrile) were added to plasma/serum samples in this order, and samples were put in an ice-bath for 1 min. Then samples were mixed manually in duplicate by vortexing for 5 min. After centrifugation (5 min, 800 × *g*, 4 °C), an aliquot (700 μl) of the organic phase was transferred into 1.5-ml autosampler glass vials for GC–MS analysis.

2.3. Validation of the method

A series of experiments was performed to determine intra-assay (two experiments) and inter-assay (one experiment repeated on five days) accuracy (recovery, %) and imprecision (RSD, %) of the method for DMA in human pooled CPD plasma using the optimum experimental conditions and an added final concentration of 10 μM for the internal standard in all experiments. The concentration of endogenous basal DMA in the CPD plasma was determined to be 1.84 ± 0.16 μM ($n = 10$). DMA was added at potentially relevant final concentrations in the range 0–10 μM [5], i.e. at 0, 2, 4, 6, 8, and 10 μM. In addition, the method was validated for the narrow range of added DMA concentrations, i.e. between 0 and 2.4 μM, in particular at 0, 100, 300, 600, 1200, 1600 and 2400 nM. In all validation experiments, unspiked plasma samples were analyzed in quadruplicate, whereas spiked plasma samples were analyzed in duplicate. Recovery was calculated for each added concentration as follows: recovery (%) = [(measured DMA concentration *minus* basal DMA concentration): added DMA concentration)] × 100.

2.4. Determination of the DMA content of vacutainer tubes and of the chemical EDTA

For simplicity the vacutainer tubes (monovettes) used are named for their declared volume and anticoagulation agent. Thus, each six monovettes of 3-ml citrate, 9-ml EDTA, 7.5-ml serum, 9-ml lithium heparin, and 4.5-ml ammonium heparin were analyzed for their DMA content as follows. The monovettes were filled with 3, 9, 7, 9 and 4 ml of potassium phosphate buffered saline (PBS, 100 mM potassium phosphate, 500 mM NaCl, pH 7.4), respectively, shaken gently and allowed to stand for 10 min. Each two 100-μl aliquots of the supernatants and six 100-μl aliquots of PBS were spiked each with 10 μM of d_6-DMA, samples were derivatized/extracted using the optimum conditions reported above and analyzed by GC–MS. The DMA content of the monovettes was calculated from the respective concentrations measured and volumes of PBS added to the monovettes after subtraction of the DMA amount present in the equivalent volume of PBS.

Presence of DMA as a contaminant in the chemical EDTA (i.e., ethylenediamine tetraacetic acid disodium salt) was investigated by analyzing in duplicate freshly prepared solutions of EDTA in PBS at relevant final concentrations (0, 1, 2, 3, 4, 5, and 10 mM).

2.5. GC–MS and GC–tandem MS conditions

GC–MS was performed on a ThermoElectron DSQ quadrupole mass spectrometer connected directly to a ThermoElectron Focus gas chromatograph and to an autosampler AS 3000 (ThermoElectron, Dreieich, Germany). A fused-silica capillary column Optima-17 (15 m × 0.25 mm i.d., 0.25 μm film thickness) from Macherey-Nagel (Düren, Germany) was used. Aliquots (1 μl) of the toluene extracts were injected in the splitless mode. The following oven temperature program was used with helium (constant flow of 1 ml/min) 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 260, 200 and 250 °C, respectively. Electron energy and electron current were set to 50 eV and 120 μA, respectively, for PICI with methane (2.4 ml/min) as the reagent gas. Quantification by GC–MS in the PICI mode was performed by SIM of the ions at *m/z* 240.15 for d_0-DMA and *m/z* 246.15 for d_6-DMA using a dwell-time of 50 ms and an electron multiplier voltage of 1.5 kV.

GC–tandem MS analyses in the PICI mode were performed on a triple-stage quadrupole mass spectrometer ThermoElectron 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-XLB (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: 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 250 °C, 200 °C and 180 °C, respectively. Electron energy and electron current was set to 50 eV and 120 μA, respectively. Methane (530 Pa) and argon (0.2 Pa collision pressure) were used as reagent and collision gases, respectively. Collision energy was set to 25 eV. Aliquots (1 μl) were injected in the splitless mode by means of the autosampler.

2.6. Ethics

Approval from the local Ethics Committee of the Medical School of Hannover was obtained for the clinical study (described in Section 3.4), as was informed written consent from study participants.

3. Results and discussion

3.1. PICI GC–MS and GC–tandem MS of d_0-DMA and d_6-DMA pentafluorobenzamide derivatives

The pentafluorobenzamide derivatives of unlabelled (d_0-DMA) and ^{2}H-labelled (d_6-DMA) DMA have been previously identified by GC–MS in the negative-ion chemical ionization (NICI) and EI mode as *N*,*N*-dimethyl-pentafluorobenzamide and *N*,*N*-[$^2H_6$]dimethyl-pentafluorobenzamide, respectively [15]. Quantitative determination of DMA in human urine revealed comparable accuracy and precision both in the NICI and EI mode [15]. Preliminary investigations had shown that EI is principally suitable for the GC–MS quantification of DMA in human plasma. In the present study, we investigated the utility of PICI as an alternative ionization mode for the quantitative determination of circulating DMA by GC–MS as pentafluorobenzamide derivative.

PICI GC–MS full scan analysis of separate reaction mixtures of synthetic d_0-DMA and d_6-DMA with the derivatizating agent PFBoylCl showed several GC peaks. The PICI mass spectra of the reaction products emerging from the GC column at approximately 3.5 min are shown in Fig. 2. The most intense ions in the PICI mass spectra of the d_0-DMA and d_6-DMA derivatives were observed at *m/z* 240 and *m/z* 246, respectively, and correspond to the protonated molecules ($[M+H]^+$) (Fig. 2). In the EI and NICI mass spectra of these derivatives the corresponding ions were *m/z* 239 and *m/z* 245 due to M^+ and M^-, respectively [15]. The less intense ions at *m/z* 268 and *m/z* 280 from d_0-DMA (Fig. 2A) and at *m/z* 274 and *m/z* 286 from d_6-DMA (Fig. 2B) are due to adducts of the corresponding DMA derivatives with the reagent gas methane, i.e. $[M+C_2H_5]^+$ and $[M+C_3H_5]^+$, respectively. In contrast to NICI [15], the mass spectra depicted in Fig. 2 clearly show protonation and adduct formation of the pentafluorobenzamide derivatives of d_0-DMA and d_6-DMA under PICI conditions; also unlike in NICI, no intense ions were observe at *m/z* values below 240 in PICI.

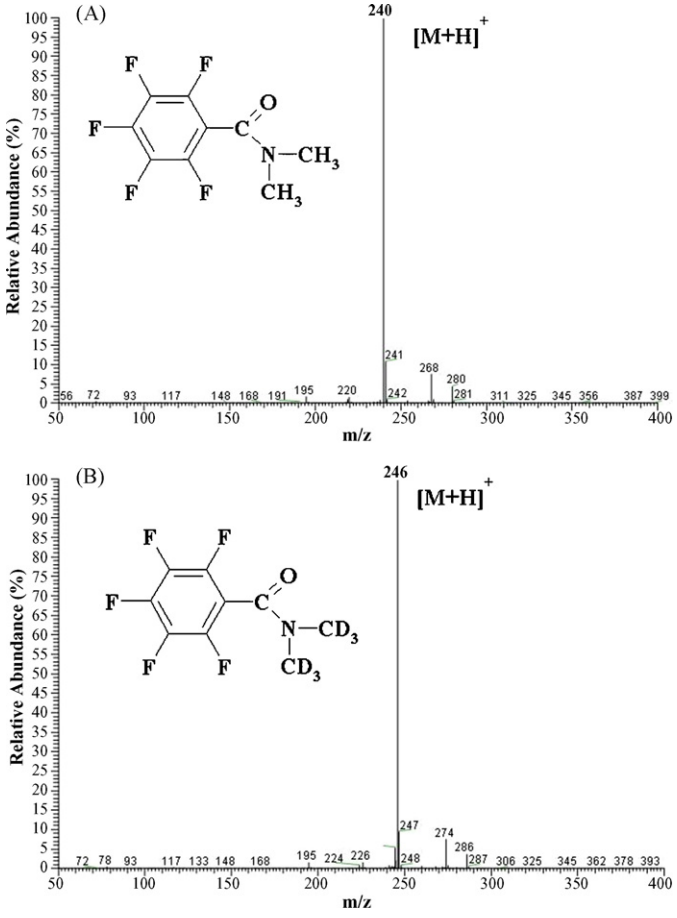

Fig. 2. PICI mass spectra of the pentafluorobenzamide derivatives of unlabelled DMA (A) and deuterated DMA (d_6-DMA) (B). Insertions show the structures of the pentafluorobenzamide derivatives.

The exact *m/z* values of 240.15 and 246.16 obtained in the PICI mass spectra were used in quantitative analyses. The pentafluorobenzamide derivatives of d_0-DMA and d_6-DMA are partially separated by GC. For accurate quantification in the SIM mode the dwell-time was set to 50 ms for each ion. Quantitative GC–MS analysis of plasma DMA by SIM of *m/z* 240 and *m/z* 246 provided chromatograms mainly containing two intense peaks corresponding to endogenous DMA and the internal standard d_6-DMA within a relevant retention time window (Fig. 3).

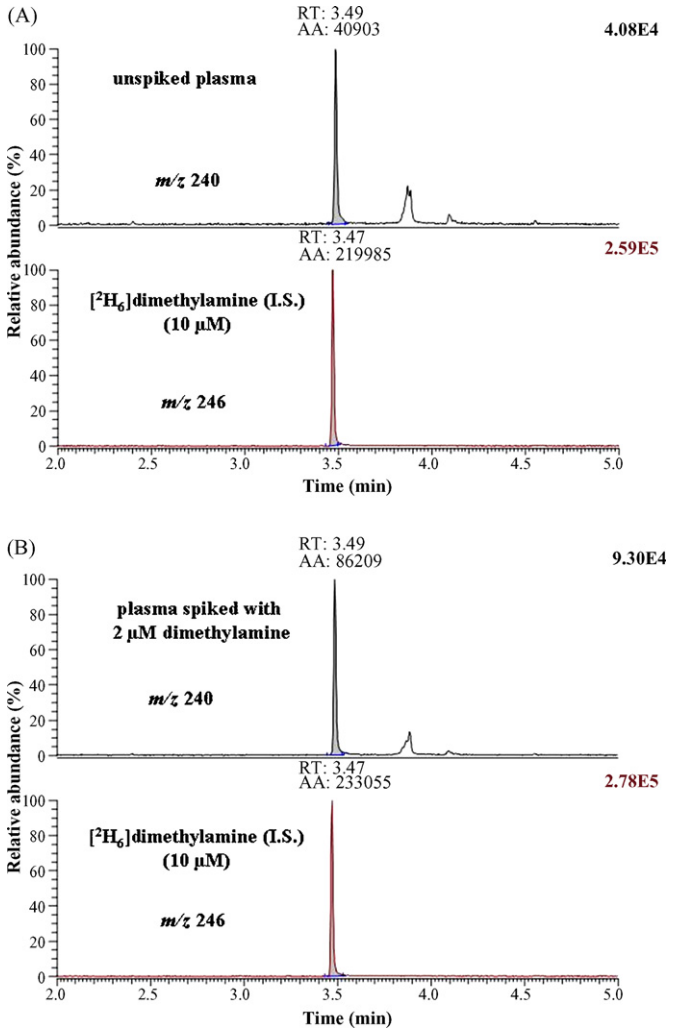

Fig. 3. Partial GC–MS chromatograms from the quantitative determination of DMA in human plasma (CPD) used in validation experiments before (A) and after (B) addition of 2 μM of d_0-DMA by selected-ion monitoring of *m/z* 240.15 for DMA (d_0-DMA, upper tracing) and *m/z* 246.15 for the internal standard (d_6-DMA, lower tracing) which was added to the plasma sample at a final concentration of 10 μM.

Under the routine experimental and instrumental conditions relatively constant peak area values were obtained for the internal standard d_6-DMA (lower tracings in Fig. 3). The signal-to-noise (S/N) ratio for the d_6-DMA peak emerging from the column at 3.47 min (lower tracings in Fig. 3A and B) usually ranged between 4200:1 and 4700:1, and was produced from the injection of 1 pmol of d_6-DMA on the assumption that derivatization and extraction were each quantitative (see below). From these data, it can be approximated that injection of about 700 amol of d_6-DMA pentafluorobenzamide derivative would produce a peak with as S/N value of 3:1. Although not directly comparable because of the use of different GC–MS instruments, i.e. the ThermoElectron DSQ in the present study and the MS Engine in previous work [15], PICI of pentafuorobenzamide derivatives of DMA seems to provide a considerably higher sensitivity than EI (LOD of 195 fmol of d_6-DMA in the SIM mode at a S/N value of 3:1 for *m/z* 245) [15]. A likely explanation for the higher sensitivity in PICI could be the almost missing fragmentation of $[M+H]^+$ as compared with the abundant fragmentation of M^+ under EI conditions [15]. The S/N value for the peak produced by endogenous DMA in plasma, e.g. 550:1 for 1.86 μM in Fig. 3A (retention time, 3.49 min, *m/z* 240), usually ranged between 500:1 and 600:1, suggesting that the noise for the ion at *m/z* 240 is higher than the noise for the ion *m/z* 246 in the plasma sample analyzed. Addition of 2 μM of DMA to the same plasma sample increased the S/N value of the peak eluting at 3.49 min (*m/z* 240) to 1200:1, i.e. by 650:1, in accordance with an approximate doubling of the peak area (Fig. 3B).

Subjection of the precursors at *m/z* 240.15 and 246.15 to collision-induced dissociation (CID) with argon in the PICI mode resulted in the formation of few product ions (Fig. 4). The product ions at *m/z* 195 and 167 were observed from d_0-DMA and d_6-DMA and are due to $[C_6F_5CO]^+$ and $[C_6F_5]^+$, respectively, i.e. they are not characteristic for DMA. Characteristic product ions were obtained at *m/z* 72 ($[(CH_3)_2NCO]^+$) from *m/z* 240 for d_0-DMA and *m/z* 78 ($[(CD_3)_2NCO]^+$) from *m/z* 246 for d_6-DMA. Quantification by GC–tandem MS in the PICI mode was performed by SRM of *m/z* 72.0 and *m/z* 78.0 using a dwell-

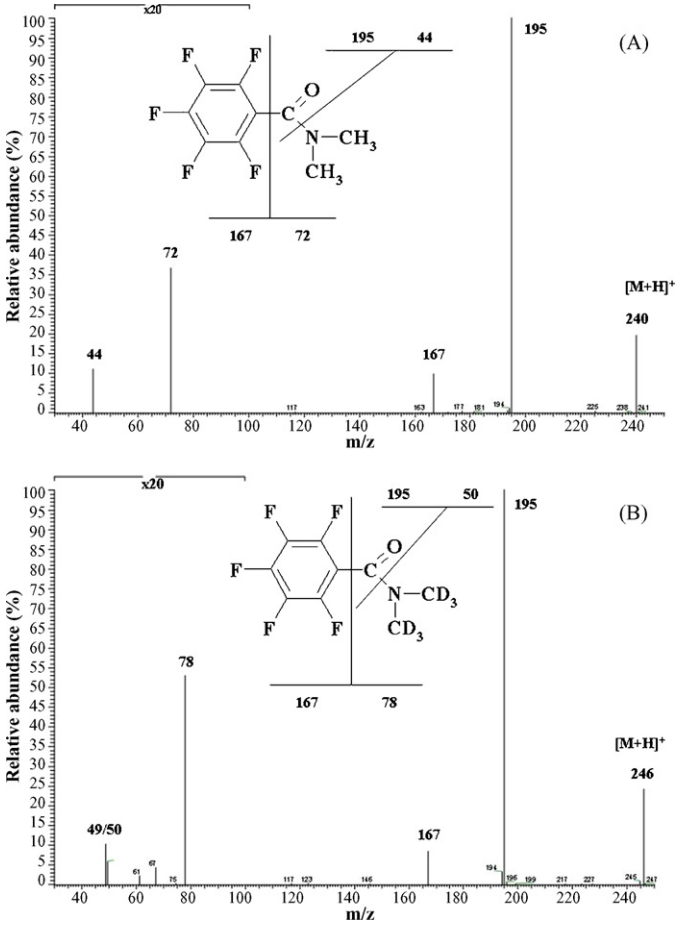

Fig. 4. PICI tandem mass spectra of the pentafluorobenzamide derivatives of unlabelled DMA (A) and of deuterated DMA (d_6-DMA) (B). Insertions show the structures of the pentafluorobenzamide derivatives and the product ions formed from subjection of the respective protonated molecular cations to collision-induced dissociation with argon at a collision energy of 25 eV. The *m/z* range 30–100 was amplified by a factor of 20.

time of 50 ms for each ion and an electron multiplier voltage of 1.9 kV.

3.2. Derivatization/extraction procedure and concentration of the internal standard d_6-DMA

The effect of the concentration of the derivatizing agent PFBoylCl added from a 10 vol% solution in acetonitrile, of carbonate and vortex time on the formation of the pentafluorobenzamide derivative of d_6-DMA is shown in Fig. 5. We assumed that increasing concentration of PFBoylCl in toluene would increase the yield of the pentafluorobenzamide derivative of d_6-DMA under otherwise comparable conditions; however, the opposite was observed (Fig. 5A). For practical reasons, we decided to routinely add to 100-μl aliquots of plasma samples 10-μl aliquots of the 10 vol% solution of PFBoylCl in acetonitrile.

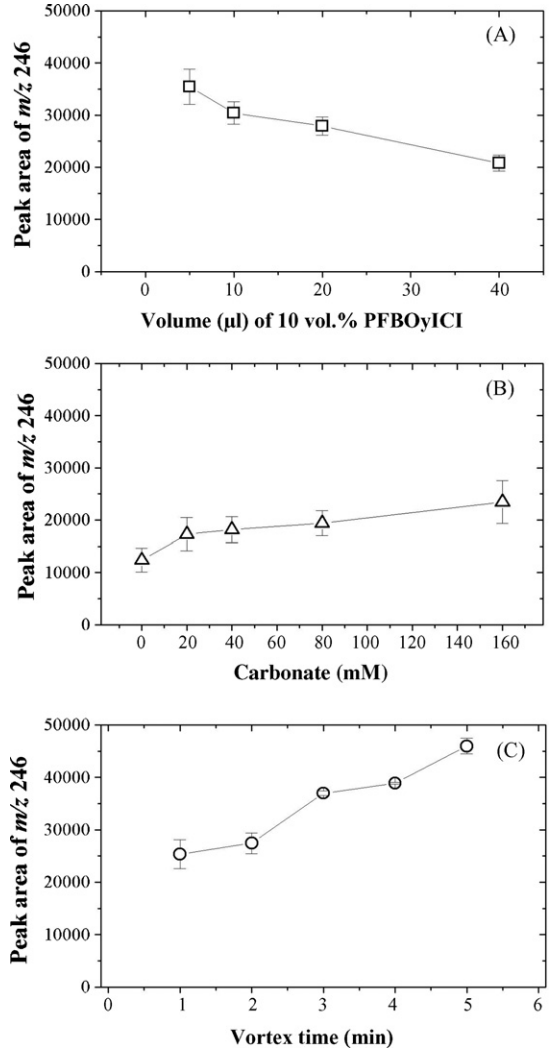

Fig. 5. Effect of the concentration of the derivatization agent pentafluorobenzoyl chloride (PFBOylCl) (A), carbonate (B), and of the vortex-mixing time (C) on the formation of the pentafluorobenzamide derivative of d_6-DMA (*m/z* 246) added to human plasma (CPD) at a final concentration of 1 μM. The area of the peak at *m/z* 246 eluting at 3.47 min was used to evaluate the extent of formation.

By contrast, the effect of carbonate was as expected. Thus, increasing concentrations of carbonate increased the yield of the pentafluorobenzamide derivative of d_6-DMA, however the effect was rather minor (Fig. 5B). Because the greatest increase was observed already with 10 μl of a 20 mM carbonate solution, use of 10-μl aliquots of a 20 mM Na_2CO_3 solution in distilled water in the derivatization/extraction procedure was considered optimum. Thus, the concentration of PFBoylCl and carbonate for plasma samples is the same as for urine samples, despite a generally constantly higher pH value in plasma samples (about pH 7.3) compared to urine samples (common pH range, 4.5–6).

Formation of the pentafluorobenzamide derivative of d_6-DMA was found to increase with vortex time (Fig. 5C). The absence of a plateau suggests that derivatization/extraction is not complete even after 5 min of manual vortex-mixing at the highest stage of the device used. Because vortex-mixing for 5 min produced double as high peak areas in comparison to 1-min vortex-mixing time, a time of 5 min for manual vortex-mixing was considered necessary and satisfactory. That vortex-mixing of plasma samples requires 4 min longer than of urine samples, might be explained by the higher viscosity of plasma and the considerably lower DMA concentration in plasma compared to urine, i.e. about 2–12 μM (present study) versus 264 μM [15].

In serum of healthy humans, DMA concentration was measured by Teerlink et al. [5] to range 1.0–5.5 μM, whereas in dialysis patients DMA concentration were much higher, i.e. in the range 14.4–49.8 μM [5]. DMA serum concentrations of this order of magnitude in healthy subjects and in haemodialysis patients were also reported by Baba et al. [17]. Because of the potentially relatively wide range for circulating DMA concentrations in healthy and ill subjects, we investigated the usefulness of various concentrations (1–10 μM) of the internal standard d_6-DMA to be added to human plasma for quantitative analyses under optimum conditions. There was no statistically significant difference (not shown) for the tested d_6-DMA concentrations, and endogenous DMA concentration in the CPD plasma was determined to be (mean ± SD) 2.06 ± 0.18 μM (RSD, 8.9%). In order to cover the potentially whole range for circulating DMA concentration in health and disease, we decided to use the internal standard d_6-DMA at an added final concentration of 10 μM in plasma and serum in quantitative measurements in the present study.

3.3. Validity of the method

The results from intra- and inter-day validation of the method are summarized in Tables 1–3. Intra-day recovery and imprecision ranged between 81 and 110%, and between 0.02 and 8.8%, respectively (Tables 1 and 2). Table 2 shows that DMA added to plasma at 100 nM, i.e. of about 5% of the basal DMA concentration, can be accurately (81%) and precisely (0.3%) measured by the method. Thus, 100 nM could be defined as the LOQ of the method. Inter-day recovery and imprecision ranged between 91.5 and 111.5%, and between 0.01 and 3.7%, respectively (Table 3). The slopes and correlation coefficients of all regression equations from validation experiments are each close

Table 1
Intra-assay accuracy (recovery) and imprecision (RSD) of the GC–MS method for DMA in human CPD plasma for an extended range

Added DMA (μM)	Measured DMA (μM) (mean ± SD, $n = 10$)[a]	Recovery (%)	Imprecision (%)
0	1.84 ± 0.16	N.A.	8.8
2	3.74 ± 0.22	95.0	5.9
4	5.64 ± 0.14	95.0	2.6
6	7.52 ± 0.18	94.7	2.4
8	9.88 ± 0.35	100.5	3.5
10	12.0 ± 0.32	101.6	2.7

[a] The experiment was repeated five times within the same day and analyses were performed in duplicate for each concentration in all analyses. N.A., not applicable.

Table 2
Intra-assay accuracy (recovery) and imprecision (RSD) of the GC–MS method for DMA in human CPD plasma for a narrow range

Added DMA (nM)	Measured DMA (nM) (mean ± SD, $n = 2$)	Recovery (%)	Imprecision (%)
0	1822 ± 86	N.A.	4.7
100	1903 ± 6	81.0	0.3
300	2132 ± 39	103.3	1.8
600	2481 ± 95	109.8	3.8
1200	2923 ± 0.5	91.8	0.02
1600	3347 ± 7	95.3	0.2
2400	4284 ± 129	102.6	3.0

N.A., not applicable.

to 1.0 (not shown) indicating complete recovery and excellent linearity for DMA added to plasma samples.

3.4. Concentration of circulating DMA in healthy young women

The method was applied to measure DMA in plasma and serum of healthy young volunteers without history of hepatic, renal, cardiovascular or other diseases. The study and the characteristics of the study participants have been reported in detail recently [18]. In the present study, EDTA plasma, lithium heparin plasma and serum from 18 volunteers of the placebo group at baseline of the previous study [18] were analyzed for DMA in duplicate within one run alongside with each of three quality control (QC1, QC2, QC3) samples using the CPD plasma. QC1 was analyzed without external addition of DMA, QC2 and QC3 samples were spiked with 2 and 10 μM of DMA, respectively. The basal DMA concentrations measured in plasma and serum of the study volunteers are summarized in Table 4. QC samples were analyzed with an imprecision (RSD) of 3.3, 3.4 and 2.6%,

Table 3
Inter-assay accuracy (recovery) and imprecision (RSD) of the GC–MS method for DMA in human CPD plasma

Day	DMA (μM)								
	0			2			4		
	Mean ± SD[a] (μM)	Recovery (%)	Imprecision (%)	Mean ± SD (μM)	Recovery (%)	Imprecision (%)	Mean ± SD (μM)	Recovery (%)	Imprecision (%)
A	2.05 ± 0.06	N.A.	2.8	4.11 ± 0.11	103	2.7	5.81 ± 0.00	94	0.01
B	1.96 ± 0.02	N.A.	1.0	3.93 ± 0.07	98.5	1.7	5.71 ± 0.09	93.8	1.6
C	1.93 ± 0.02	N.A.	1.2	3.84 ± 0.07	95.5	1.8	5.59 ± 0.12	91.5	2.1
D	1.75 ± 0.01	N.A.	0.5	3.67 ± 0.07	96.0	1.8	5.81 ± 0.04	101.5	0.7
E	1.80 ± 0.06	N.A.	3.7	3.59 ± 0.06	89.5	1.7	5.49 ± 0.04	92.3	0.8
Day	DMA (μM)								
	6			8			10		
	Mean ± SD[a] (μM)	Recovery (%)	Imprecision (%)	Mean ± SD (μM)	Recovery (%)	Imprecision (%)	Mean ± SD (μM)	Recovery (%)	Imprecision (%)
A	8.27 ± 0.19	103.7	2.3	10.4 ± 0.18	104.4	1.7	12.2 ± 0.09	101.5	0.8
B	7.65 ± 0.14	94.8	1.8	9.49 ± 0.21	94.1	2.2	12.1 ± 0.00	101.1	0.1
C	7.70 ± 0.13	96.2	1.7	9.88 ± 0.14	99.4	1.4	12.8 ± 0.00	108.7	0.01
D	7.45 ± 0.15	95.0	2.0	9.58 ± 0.02	97.9	0.2	12.9 ± 0.05	111.5	0.4
E	7.50 ± 0.01	95.0	0.2	9.53 ± 0.09	96.6	0.9	12.1 ± 0.32	103.0	2.6

[a] Measured concentration of DMA. N.A., not applicable.

Table 4
Concentration of DMA measured by GC–MS in EDTA plasma, heparinized plasma and serum samples of healthy young women

Subject no.	Age (years)	Weight (kg)	DMA (μM) [mean ± SD (RSD, %)][a]		
			EDTA plasma	Lithium heparin plasma	Serum
1	20	72	9.19 ± 0.1 (1.1)	2.11 ± 0.01 (0.3)	1.66 ± 0.04 (2.1)
2	24	49	8.52 ± 0.33 (3.9)	1.83 ± 0.04 (1.9)	1.31 ± 0.04 (2.7)
3	21	63	8.13 ± 0.01 (0.1)	1.73 ± 0.06 (3.2)	1.68 ± 0.03 (1.7)
4	23	64	8.89 ± 0.52 (5.8)	1.71 ± 0.13 (7.5)	1.18 ± 0.01 (0.6)
5	22	47	10.3 ± 0.5 (4.9)	1.69 ± 0.1 (5.9)	1.33 ± 0.02 (1.6)
6	29	79	11.8 ± 0.07 (0.6)	1.79 ± 0.0 (0)	1.83 ± 0.01 (0.8)
7	24	59	7.85 ± 0.0 (0)	1.54 ± 0.01 (0.5)	1.42 ± 0.16 (10.9)
8	20	60	9.67 ± 0.38 (3.9)	1.67 ± 0.06 (3.4)	1.46 ± 0.04 (2.4)
9	24	82	9.38 ± 0.36 (3.8)	1.57 ± 0.06 (4.1)	1.19 ± 0.03 (2.4)
10	24	60	9.03 ± 0.21 (2.3)	1.39 ± 0.08 (5.6)	1.07 ± 0.04 (3.9)
11	22	65	8.59 ± 0.36 (4.2)	1.81 ± 0.04 (1.9)	1.16 ± 0.06 (4.9)
12	23	75	9.00 ± 0.04 (0.4)	1.57 ± 0.01 (0.4)	1.33 ± 0.07 (5.3)
13	24	62	9.36 ± 0.18 (1.9)	1.53 ± 0.03 (1.8)	1.32 ± 0.01 (0.5)
14	24	73	11.3 ± 0.07 (0.6)	1.89 ± 0.21 (11.2)	1.70 ± 0.21 (12.5)
15	23	63	10.3 ± 0.83 (8.1)	1.87 ± 0.02 (1.1)	1.46 ± 0.04 (2.4)
16	25	56	11.2 ± 0.28 (2.5)	1.80 ± 0.06 (3.2)	1.40 ± 0.06 (4.0)
17	23	63	12.9 ± 0.14 (1.1)	1.73 ± 0.01 (0.4)	1.45 ± 0.00 (0)
18	23	66	11.8 ± 0.42 (3.6)	1.88 ± 0.09 (4.9)	1.81 ± 0.07 (3.9)
Mean ± SD	23.2 ± 2.1	63.8 ± 9.3	9.84 ± 1.43 (15)	1.73 ± 0.17 (9.8)	1.43 ± 0.23 (16.1)

[a] All samples were analyzed in duplicate within a day in the order listed.

respectively. QC2 and QC3 were analyzed with an accuracy (recovery) of 103 and 107%, respectively. Fig. 6 shows a partial chromatogram from the GC–MS (A) and GC–tandem MS (B) analysis of DMA in EDTA plasma of a healthy volunteer.

Table 4 indicates that mean values and ranges for DMA in EDTA plasma, lithium heparin plasma and serum samples differ greatly, suggesting a potential effect of blood anticoagulation on circulating DMA concentration. The highest DMA concentrations were measured in EDTA plasma samples, i.e. 9.84 ± 1.43 μM (range 7.85–12.9 μM). Considerably lower DMA concentrations were measured in lithium heparin plasma samples, i.e. 1.73 ± 0.17 μM (range 1.39–2.1 μM), and in serum samples, i.e. 1.43 ± 0.23 μM (range 1.07–1.83 μM). No significant correlation was observed between DMA concentrations measured in heparin and EDTA plasma, and only a very weak correlation was obtained between DMA concentrations measured in serum and EDTA plasma ($R = 0.47$, $P = 0.047$). By contrast, serum and heparin DMA concentrations correlated fairly ($R = 0.60$, $P = 0.009$).

The DMA concentrations measured in EDTA plasma by GC–MS were confirmed by GC–tandem MS. The values obtained by GC–tandem MS were constantly higher than those obtained by GC–MS. Linear regression analysis between the DMA concentrations measured by GC–MS (y) and those measured by GC–tandem MS (x) revealed a straight line with the regression equation $y = -0.07 + 0.81x$ ($R = 0.99$, $P < 0.0001$, $n = 18$). Therefore, interferences by co-eluting substances can be excluded as an explanation for differences in circulating DMA concentration found in EDTA plasma, especially in comparison with those measured in serum and lithium heparin plasma (Table 4).

Two investigator groups have reported DMA concentrations in serum of normal subjects using different HPLC methods [5,17]. Teerlink et al. [5] measured DMA serum concentrations of 3.3 ± 1.5 μM (range 1.0–5.5 μM) in eight healthy controls (42 ± 14 years of age). Baba et al. [17] measured DMA serum concentrations of 3.3 ± 0.9 μM (range of 2.4–6.6 μM) in 20 healthy controls (21–49 years of age). The DMA serum concentrations measured in the present study overlap with those measured by Teerlink et al. [5], but they are lower than those reported by Baba et al. [17]. In the present study, younger subjects (only women) participated as compared to the older subjects (men and women) examined by Teerlink et al. [5] and Baba et al. [17]. However, whether age and/or gender may influence circulating DMA concentrations is not yet known.

3.5. DMA as a contaminant in vacutainer tubes and in the chemical EDTA

The most likely explanation for the clearly higher DMA concentrations measured in EDTA plasma as compared to lithium heparin plasma and serum in the present study seems to be presence of DMA as a contaminant in EDTA-containing vacutainer tubes. Indeed, determination of the DMA content in monovettes containing different anticoagulants revealed that from the vacutainer tubes tested the 9-ml EDTA monovettes contained by far the highest amount of DMA, i.e. 9.3 ± 1.9 nmol/monovette (Fig. 7). The lowest DMA content was found in the 3-ml citrate monovettes. Also, the 7.5-ml serum monovettes were measured to contain relatively low amounts of DMA. The 4.5-ml ammonium heparin monovettes contained double as much DMA as the 9-ml lithium heparin monovettes. It is noteworthy that the 7.5-ml serum monovettes were found to contain considerably less DMA as a contaminant in comparison with the 9-ml lithium heparin monovettes. This could be, at least in part, the rea-

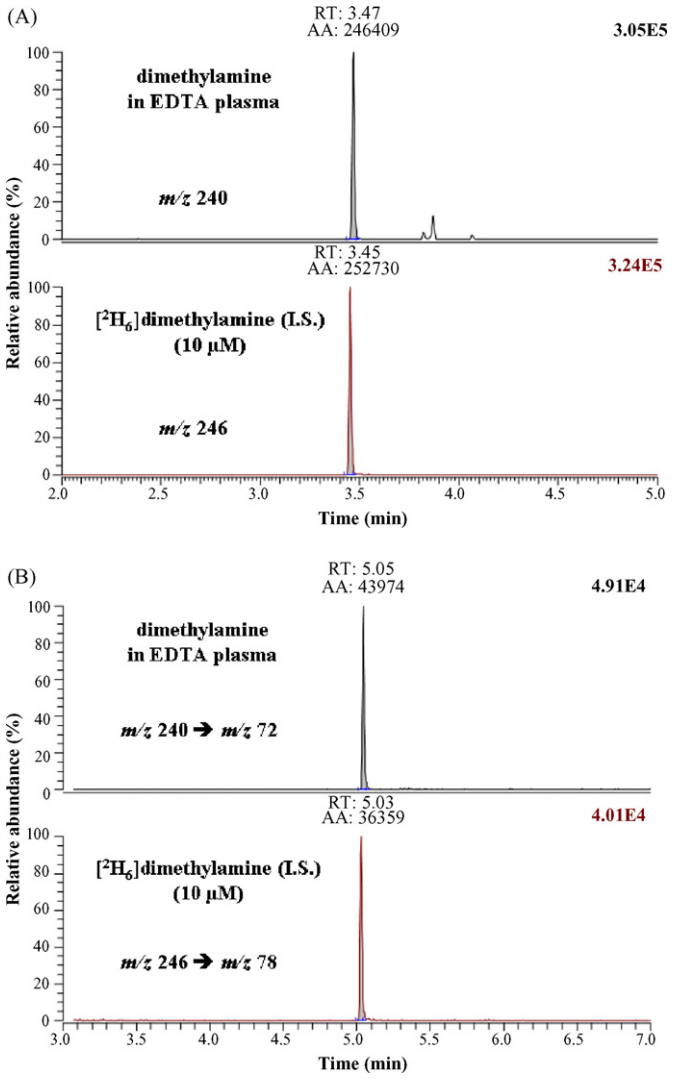

Fig. 6. Partial chromatograms from the quantitative determination of DMA in EDTA plasma of a healthy young female (volunteer No. 5, see Table 4) by (A) GC–MS (SIM mode) and (B) GC–tandem MS (SRM mode) for DMA (upper tracings) and the internal standard (d_6-DMA, lower tracings) which was added to the plasma sample at a final concentration of 10 μM.

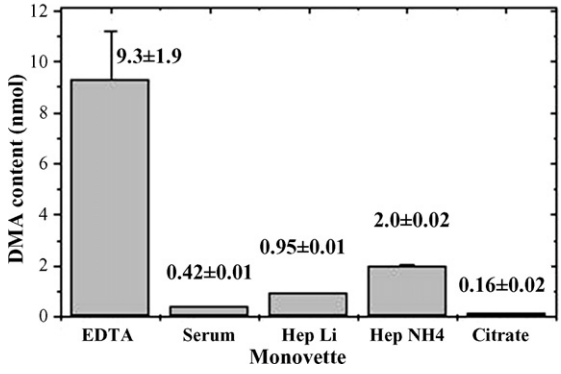

Fig. 7. DMA content of tested vacutainer tubes as measured by GC–MS. Each six monovettes were used. See the text for more details. HepLi: lithium heparin; HepNH4: ammonium heparin.

son for the slightly lower DMA concentrations measured in serum samples compared to those determined in heparin plasma samples (Table 4), as well as for the merely moderate correlation ($R = 0.60$, $P = 0.009$) found between DMA concentrations measured in heparin plasma and serum samples.

EDTA added to PBS at final concentrations of up to 10 mM was found not to contribute to DMA when measured by the present GC–MS method (data not shown). This finding suggests that the chemical EDTA does not contain DMA. The failure to detect DMA in EDTA-containing PBS suggests that EDTA itself does not contribute to DMA by thermal decomposition or by other ways during GC–MS analysis, most likely because EDTA is not derivatized with PFBoylCl and EDTA itself is not extracted by toluene. All findings together strongly suggest that DMA measured in EDTA monovettes is produced during sterilization by γ irradiation.

The present study does not allow reliable evaluation of the contribution of the DMA contaminant in the vacutainer tubes to the total DMA concentration measured in the clinical study samples (Table 4) for the following reasons. The monovettes used to measure DMA contamination in the present study are not from the same charge that was previously used in the clinical study [18]; the blood volumes taken and the plasma/serum volumes generated are unknown; the distribution of DMA between plasma or serum and erythrocytes is unknown; and the DMA contamination may vary considerably, notably in EDTA monovettes (Fig. 7). Nevertheless, our present study cannot exclude alternative, EDTA-involving mechanisms by which endogenous substances, such as lecithin, sarcosine and dimethylglycine [9,10], could have contributed to circulating DMA measured in EDTA plasma. Because ADMA concentrations in plasma and serum are very similar and furthermore lower, i.e. about 0.4 μM [19,20], than plasma/serum DMA concentrations measured by our and other groups [5,17], it is unlikely that the anticoagulant EDTA could have enhanced DDAH-catalyzed hydrolysis of ADMA to DMA and L-citrulline during blood sampling to produce several μM-units of DMA. It is noteworthy that blood anticoagulation may be a major determinant of circulating L-arginine concentration, with the underlying mechanism being still unknown; obviously, L-arginine concentrations are constantly higher (by 58%) in serum as compared with plasma and not related to contaminating L-arginine [19].

4. Conclusions

The present GC–MS method involving simultaneous derivatization and extraction of DMA from human plasma or serum is accurate, precise, simple and rapid. Routinely, 100 plasma/serum samples can be worked up by a person per day and can be analyzed daily by this method using our GC–MS and GC–tandem MS instruments. These characteristics allow for automated and high-throughput quantitative determination of DMA in human plasma/serum in the frame of clinical studies.

Contamination of vacutainer tubes and other laboratory equipment with nitrite and nitrate as well as dietary intake of these anions are well recognized and the most prominent

example for the importance of pre-analytical factors in the area of the L-arginine/NO pathway from the analytical standpoint [21]. Our previous study on urinary DMA [15] and the present study on circulating DMA show that the quantitative determination of DMA in urine and plasma/serum may also be hampered by pre-analytical factors. The present study identifies DMA as a considerable contaminant in EDTA-containing vacutainer tubes, but not in the chemical EDTA itself. EDTA monovettes may not be suited for the measurement of circulating DMA, at least when measured by the present method and when EDTA-containing monovettes are sterilized by γ irradiation. In addition to the contribution of EDTA to DMA upon γ irradiation, there may be additional mechanisms by which EDTA may cause enhanced formation of DMA from alternative endogenous sources in human blood. This interesting issue remains, however, to be investigated. From the contamination point of view, serum or citrated plasma seems to be the most appropriate matrices for measuring DMA in human blood. In serum of healthy young women, we measured DMA concentrations in the range 1.07–1.83 μM.

The GC–MS method reported here may be useful for the determination of DDAH activity, either by quantifying endogenous DMA or by measuring stable-isotope labelled DMA produced from the DDAH-catalyzed hydrolysis of an ADMA analogue labelled with stable isotopes on the N^G-dimethylamino group as has been recently suggested [22].

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