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Development, validation and biomedical applications of stable-isotope dilution GC–MS and GC–MS/MS techniques for circulating malondialdehyde (MDA) after pentafluorobenzyl bromide derivatization: MDA as a biomarker of oxidative stress and its relation to 15(*S*)-8-*iso*-prostaglandin $F_{2\alpha}$ and nitric oxide (•NO)

Dimitrios Tsikas[a,*], Sabine Rothmann[a], Jessica Y. Schneider[a], Maria-Theresia Suchy[a], Arne Trettin[a], Darko Modun[b], Nadine Stuke[c], Norbert Maassen[c], Jürgen C. Frölich[a]

[a] *Bioanalytical Research Laboratory for NO, Oxidative Stress and Eicosanoids (BIOFORNOX20), Centre of Pharmacology and Toxicology, Hannover Medical School, Hannover, Germany*
[b] *Department of Pharmacology, University of Split School of Medicine Split, Croatia*
[c] *Institute of Sport Medicine, Hannover Medical School, Hannover, Germany*

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ABSTRACT

Malondialdehyde (MDA, CH_2—$(CHO)_2$) is one of the best investigated and most frequently measured biomarkers of lipid peroxidation in biological fluids, a constituent of the so called thiobarbituric acid reactive substances (TBARS). The reaction of thiobarbituric acid with MDA and other carbonyl compounds is the basis for the batch TBARS assay, one of the most commonly and widely used assays of oxidative stress. Yet, the TBARS assay lacks specificity even if combined with HPLC separation prior to visible absorbance or fluorescence detection. In this article, we report highly specific and sensitive stable-isotope dilution GC–MS and GC–MS/MS methods for the quantitative determination of MDA in human plasma (0.1 mL). These methods utilize the acidity (pK_a, 4.46) of the two methylene H protons of MDA in aqueous solution, which are as acidic as acetic acid. Endogenous MDA in native plasma and the externally added internal standard [1,3-$^2H_2$]-MDA (d_2-MDA, CH_2—$(CDO)_2$) are derivatized in aqueous acetone (400 μL) with pentafluorobenzyl (PFB) bromide (10 μL). The reaction products were identified as $C(PFB)_2$—$(CHO)_2$ (molecular weight, 432) and $C(PFB)_2$—$(CDO)_2$) (molecular weight, 434), respectively. After solvent extraction with toluene (1 mL) quantification is performed by selected-ion monitoring (SIM) in GC–MS and by selected-reaction monitoring (SRM) in GC–MS/MS in the electron-capture negative-ion chemical ionization (ECNICI) mode. In the SIM mode, the anions $[M - PFB]^-$ at *m/z* 251 for MDA and *m/z* 253 for d_2-MDA are detected. In the SRM mode, the mass transitions *m/z* 251 to *m/z* 175 for MDA and *m/z* 253 to *m/z* 177 for d_2-MDA are monitored. The method was thoroughly validated in human plasma. Potential interfering substances including anticoagulants and commercially available monovettes commonly used for blood sampling were tested. The lowest MDA concentrations were measured in serum followed by heparinized and EDTA plasma. The GC–MS and GC–MS/MS methods were found to be specific, precise, accurate and sensitive. Thus, the LOD of the GC–MS/MS method was determined to be 2 amol (2×10^{-18} mol) MDA. The GC–MS/MS method is exceedingly useful in clinical settings. We report several biomedical applications and discuss the utility of circulating MDA as a biomarker of lipid peroxidation, especially in long-term clinical studies, and its relation to the F_2-isoprostane 15(*S*)-8-*iso*-prostaglandin $F_{2\alpha}$ and nitric oxide (•NO).

1. Introduction

Malondialdehyde (MDA, OHC—CH_2—CHO; see Scheme 1) is an end-product of enzyme- and free radical-catalyzed lipid peroxidation of polyunsaturated fatty acids including arachidonic acid [1,2]. Circulating MDA is one of the commonly and widely used

* Corresponding author at: Bioanalytical Research Laboratory for NO, Oxidative Stress and Eicosanoids (BIOFORNOX20), Centre of Pharmacology and Toxicology, Hannover Medical School, Carl-Neuberg-Strasse 1, D-30625 Hannover, Germany. Fax: +49 511 532 2750.
E-mail address: tsikas.dimitros@mh-hannover.de (D. Tsikas).

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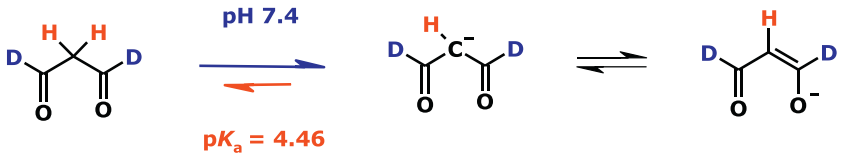


Scheme 1. Chemical structure of dideutero-malondialdehyde [1,3-$^2H_2$]-MDA. MDA is weak acid (pK_a, 4.46) and ionizes at the physiological plasma pH 7.4 to produce the carbanion which is in equilibrium with its isomeric enolate anion. In theory, both anions can undergo nucleophilic reactions with electrophiles such as pentafluorobenzyl (PFB) bromide to form *C*- and *O*-PFB derivatives.

biomarkers of oxidative stress [1,2]. Oxidative stress is generally considered a major contributor to diseases such as cancer, diabetes, asthma, and atherosclerosis [2]. MDA exists primarily in two forms, i.e., free or covalently bound to proteins, nucleic acids, lipoproteins and particular amino acids. MDA-amino acid adducts are excreted in the urine and serve as biomarkers of oxidative stress as well.

Various analytical methods have been applied to measure MDA in biological samples so far. Analysis of MDA started with the measurement as a species among the so-called thiobarbituric acid-reactive substances (TBARS) [3]. In the batch TBARS assay, derivatives are formed that can be measured by spectrophotometry [4] or fluorimetry [5]. The batch thiobarbituric acid (TBA) method lacks specificity, as many chemically reactive carbonyl groups-containing compounds from different classes of substances including oxidized polyunsaturated fatty acids and carbohydrates from endogenous sources and foods present in body fluids can react with TBA, too. As an example, HPLC analysis of TBA-treated extracts of oxidized methyl esters of linoleic acid and arachidonic acid revealed formation not only of the TBA-MDA derivative, but also several others not identified TBA derivatives [6]. Furthermore, MDA and MDA-like substances are formed from various precursors during the derivatization step that requires high temperatures [7]. The specificity of the TBARS assay is considerably improved by separating the MDA-TBA adduct from TBA adducts of other TBARS substances by means of HPLC [8–10].

In addition to the advanced TBARS assays, other HPLC and later GC–MS methods were introduced for the measurement of MDA. These methods use derivatization reagents that react exclusively with the carbonyl functional groups of TBARS. In HPLC methods, the derivatization reagents include 2,4-dinitrophenylhydrazine (DNPH) [11–13] and 2,4-diaminonaphthalene (DAN) [14]. In GC–MS methods, methylhydrazine [15], phenylhydrazine [16], 2,4-dinitrophenylhydrazine [17], 2-hydrazino-benzthiazol [18] and pentafluorophenylhydrazine [19] have been used, thus far. HPLC and GC–MS methods are by far more specific and sensitive than batch TBARS assays and can be used to determine the concentration of free and adducted MDA [14,16].

2,3,4,5,6-Pentafluorobenzyl (PFB) bromide (PFB-Br) or α-bromo-2,3,4,5,6-pentafluorotoluene is a versatile derivatization reagent both for organic and inorganic anions including nitrite and nitrate in aqueous media such as plasma and urine [20]. Given the acidity of the H atoms of the methylene group of MDA (OHC—CH_2—CHO; pK_a, 4.46; Scheme 1), we assumed that MDA anions would also react with PFB-Br to form PFB derivatives. Indeed, we found that MDA reacts with PFB-Br in acetonic aqueous solutions of neutral pH to form the strongly electron-capturing OHC—C(PFB)$_2$—CHO derivative, suggesting that both methylene H atoms of MDA are substituted by PFB residues (see Supplementary material). In the present article, we describe in detail this unique derivatization method for MDA, the development and validation of GC–MS and GC–MS/MS methods and their application in the quantification of MDA in human plasma in several clinical settings. The method uses dideutero-MDA (ODC—CH_2—CDO, d_2-MDA) as the internal standard and electron-capture negative-ion chemical ionization (ECNICI). Previously, a few applications of the GC–MS/MS method have been reported yet without its detailed description [21–23].

2. Experimental

2.1. Reagents and materials

1,1,3,3-Tetraethoxypropane and 2,3,4,5,6-pentafluorobenzyl bromide (PFB-Br) were obtained from Aldrich (Steinheim, Germany). PFB-Br is corrosive and an eye irritant. Inhalation and contact with skin and eyes should be avoided. All work should be performed in a well-ventilated fume hood. [1,3-$^2H_2$]-1,1,3,3-Tetraethoxypropane (declared as minimum 95% atom% at ^2H) was from Cambridge Isotope Laboratories (Andover, MA, USA). Acetone (supra solve) was from Merck (Darmstadt, Germany) and toluene from Mallinckrodt Baker (Deventer, The Netherlands). All monovettes used to blood sampling were obtained from Sarstedt (Nümbrecht, Germany).

2.2. Preparation of MDA stock solutions

MDA (d_0-MDA) and [1,3-$^2H_2$]-MDA (d_2-MDA) stock solutions were prepared from 1,1,3,3-tetraethoxypropane and [1,3-$^2H_2$]-1,1,3,3-tetraethoxypropane, respectively, as described [24]. Briefly, the chemicals (12.5 mg each) were dissolved in 50 mL 0.1 M HCl and incubated for 60 min at 40 °C. Then the solutions were diluted 1:12 (v/v) with 0.1 M HCl and analysed by UV/vis spectrophotometry on a Specord 50 spectrometer from Analytik Jena (Jena, Germany). Their absorbance was measured at 246 nm and the molar absorptivity of 13.7 mM^{-1} cm^{-1} was used to calculate the concentration of d_0-MDA and d_2-MDA in their solutions (see Supplementary material). The stock solutions were diluted with 0.1 M HCl to obtain 10-μM standard solutions for further use. Stock solutions and dilutions of d_0-MDA and d_2-MDA were stored in a refrigerator at 8 °C.

2.3. Derivatization procedure

The derivatization procedure used for MDA is a modification of the procedure originally reported for nitrite and nitrate [20]. Several experimental conditions were investigated and optimized and were found to be very similar to those required for nitrate derivatization with PFB-Br. Thus, in quantitative analyses, derivatization with PFB-Br was performed by adding acetone (400 μL) and PFB-Br (10 μL) to plasma aliquots (100 μL) and by incubating the whole sample for 60 min at 50 °C. The final concentration of PFB-Br in the sample is about 130 mM, i.e., in high molar excess over MDA and other inorganic and organic substances that can react with PFB-Br. After derivatization, samples were cooled to room temperature and evaporated to complete dryness under a nitrogen stream for 25 min. Note that in the GC–MS method for nitrite and nitrate only acetone is evaporated [20]. Reaction products were extracted by vortex-mixing with toluene (1 mL) for 1 min, and an 800-μL aliquot of the organic phase was transferred into a 1.8-mL autosampler glass vial.

2.4. Method validation in human plasma

The following experimental parameters were used in method validation. Plasma volume, 100 μL; acetone volume, 400 μL; PFB-Br volume, 10 μL; derivatization temperature, 50 °C; derivatization time, 60 min; evaporation time, 25 min. We used a CPD (citric acid, phosphate, dextrose) pooled plasma sample from the local blood bank. The method was validated in the relevant concentration range of 0 to 2 μM d_0-MDA (11 concentration points) using d_2-MDA at the fixed concentration of 1 μM. All analyses were performed in duplicate on 5 different days (i.e., day A–E).

The precision (more correctly the imprecision) of the method was expressed as relative standard deviation (RSD, %). The accuracy of the method was expressed as the recovery (%) which was calculated by using the Formula (F1) [25]:

$$\text{Rec} = [(C_M - C_0) : C_+] \times 100 \quad \text{(F1)}$$

where Rec is the recovery value (in %), C_M is the MDA concentration measured in the spiked biological sample, C_0 is the basal MDA concentration measured in the unspiked biological sample, and C_+ is the known nominal concentration of MDA added to the sample.

2.5. GC–MS and GC–MS/MS conditions for MDA analysis

GC–MS and GCkMS/MS analyses were carried out on a Thermoquest TSQ 7000 apparatus (San Jose, CA, USA) connected directly to a Thermoquest Carlo Erba Instruments gas chromatograph Trace 2000 equipped with an autosampler model AS 2000. The GC column Optima 5-MS (30 m × 0.25 mm i.d., 0.25-μm film thickness) from Macherey-Nagel (Düren, Germany) was used.

Aliquots (1 μL, splitless) were injected in the programmable temperature vaporization (PTV) mode using the following temperature program: the injector was held at 70 °C for 1 min and then increased to 320 °C at a rate of 10 °C/s. The oven temperature was held at 80 °C for 2 min and then increased to 340 °C at a rate of 20 °C/min. Helium (124 kPa, constant flow of 1 mL/min) and methane (530 Pa) were used as the carrier and the reagent gases for ECNICI, respectively. Electron energy was set to 200 eV and emission current to 300 μA. For collision-induced dissociation (CID) argon (0.27 Pa) was used. The optimum collision energy was 15 eV. Constant temperatures of 180 °C and 280 °C were kept at the ion source and interface, respectively. In GC–MS, the dwell-time was 100 ms for each ion in the selected-ion monitoring (SIM). In GC–MS/MS, the dwell-time was 100 ms for each mass transition in the selected-reaction monitoring (SRM) mode. For more details see the Section 3.

2.6. Biomedical applications

2.6.1. Effects of paracetamol and acetyl salicylic acid on plasma MDA in healthy humans

Ten healthy male volunteers (age, 20–35 years; body mass index (BMI), 24.2 ± 1.8 kg/m^2) who were normolipidemic, nonsmoking with no history of alcohol abuse and who did not take any medications were involved in the study. Subjects were asked to abstain from exercise, fruits, vegetables, dietary supplements, tea, alcoholic beverages, and caffeine- or theobromine-containing foods for 24 h before each visit. After an overnight fast, the subjects randomly consumed placebo, paracetamol (500 mg) or aspirin (600 mg) in a cross-over design over the period of 3 weeks. Each drug was taken once only by every subject and two weeks elapsed (wash out time) before the next drug was ingested. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University of Split School of Medicine (Croatia). All subjects gave written consent prior to their participation in the study.

All experiments were carried out in a quiet, temperature-controlled room maintained around 24 °C and were started at 8 a.m. Subjects rested for 15 min in a supine position before baseline measurement. Through the 4-h study, the subjects abstained from any food or beverages, except water ad libitum. A venous cannula (22 G) was inserted into a large subcutaneous vein in the left antecubital fossa to allow blood sampling. Blood samples were collected into heparin vacutainers (Becton Dickinson, Plymouth, UK), immediately before (baseline) and 30, 60, 90, 120, 180 and 240 min after ingestion of placebo, paracetamol or aspirin. At the same seven time points, urine samples were collected. Blood samples were immediately centrifuged at 4 °C. Plasma and urine samples were refrigerated at −80 °C until shipping on dry ice to the Hannover Medical School (Germany) where the samples were stored at −80 °C until analysis. Upon receipt, all samples were analyzed within a week. After thawing on ice, plasma samples were analyzed for MDA by the present GC–MS/MS method. The F_2-isoprostane 15(*S*)-8-*iso*-prostaglandin $F_{2\alpha}$ [15(*S*)-8-*iso*-$PGF_{2\alpha}$] was determined in 1-mL urine samples by GC–MS/MS as described elsewhere [26]. Urinary creatinine was measured by GC–MS [27] and used for correction of urinary 15(*S*)-8-*iso*-$PGF_{2\alpha}$ excretion which is reported in μg 15(*S*)-8-*iso*-$PGF_{2\alpha}$ per g creatinine.

Statistical analyses were performed using Statistica 6.0 (StatSoft Inc., Tulsa, USA). One-way ANOVA for repeated measures was used to evaluate drug dependent changes in biochemical parameters over time. When statistical significance was reached by ANOVA ($P < 0.05$), Bonferroni test was used for the post hoc analysis. Area-under-curve (AUC) analyses were performed using GraphPad Prism 4.03 for Windows (GraphPad Software, San Diego, USA).

2.6.2. Effects of chronic oral administration of L-arginine in patients with coronary artery disease or peripheral arterial occlusive disease

We performed double-blind, placebo-controlled studies on patients suffering from coronary artery disease (CAD) or peripheral arterial occlusive disease (PAOD). These studies have been described in part elsewhere [28–30]. In the CAD study were included male and female patients aged 43–76 years who had undergone angioplasty. In the PAOD study were included Caucasian male and female patients older than 40 years with a peripheral arterial occlusive disease at Fontaine stage IIb and who had undergone cardiologic and angioplastic examination, with PAOD being the main limiting factor of the absolute walking distance. The PAOD and CAD studies were approved by the Ethics Committee of the Hannover Medical School. Written informed consent was obtained from each patient.

CAD and PAOD patients received two $NaHCO_3$-based effervescent tablets (C. Hedenkamp GmbH, Hövelhof, Germany) that contained 2 g Arg hydrochloride (1.66 g Arg; Arginine group) or 2 g mannitol (Placebo group), thrice a day for 3 months in the PAOD study and for 6 months in the CAD study, resulting in a total daily amount of 9.96 g Arg in the Arginine groups in both studies. The patients of the PAOD and CAD studies were not on special diets during the whole study periods.

Venous blood (9 mL EDTA monovettes) and urine samples from spontaneous micturition (40 mL) were collected in the morning after an overnight fasting at the start, after 3 months in the PAOD and CAD studies, and after 6 months in the CAD study. Blood was taken immediately before and immediately after the treadmill exercise, and urine was collected before the treadmill exercise in the PAOD study. Urine and plasma samples were stored immediately after collection at −20 °C and −80 °C, respectively, until analysis. The analysis of the plasma and urine samples was started after completion of the studies, i.e., not earlier than 3 months in the PAOD study and 6 months in the CAD studies. MDA was determined in 100-μL plasma aliquots by the present GC–MS/MS method. Free

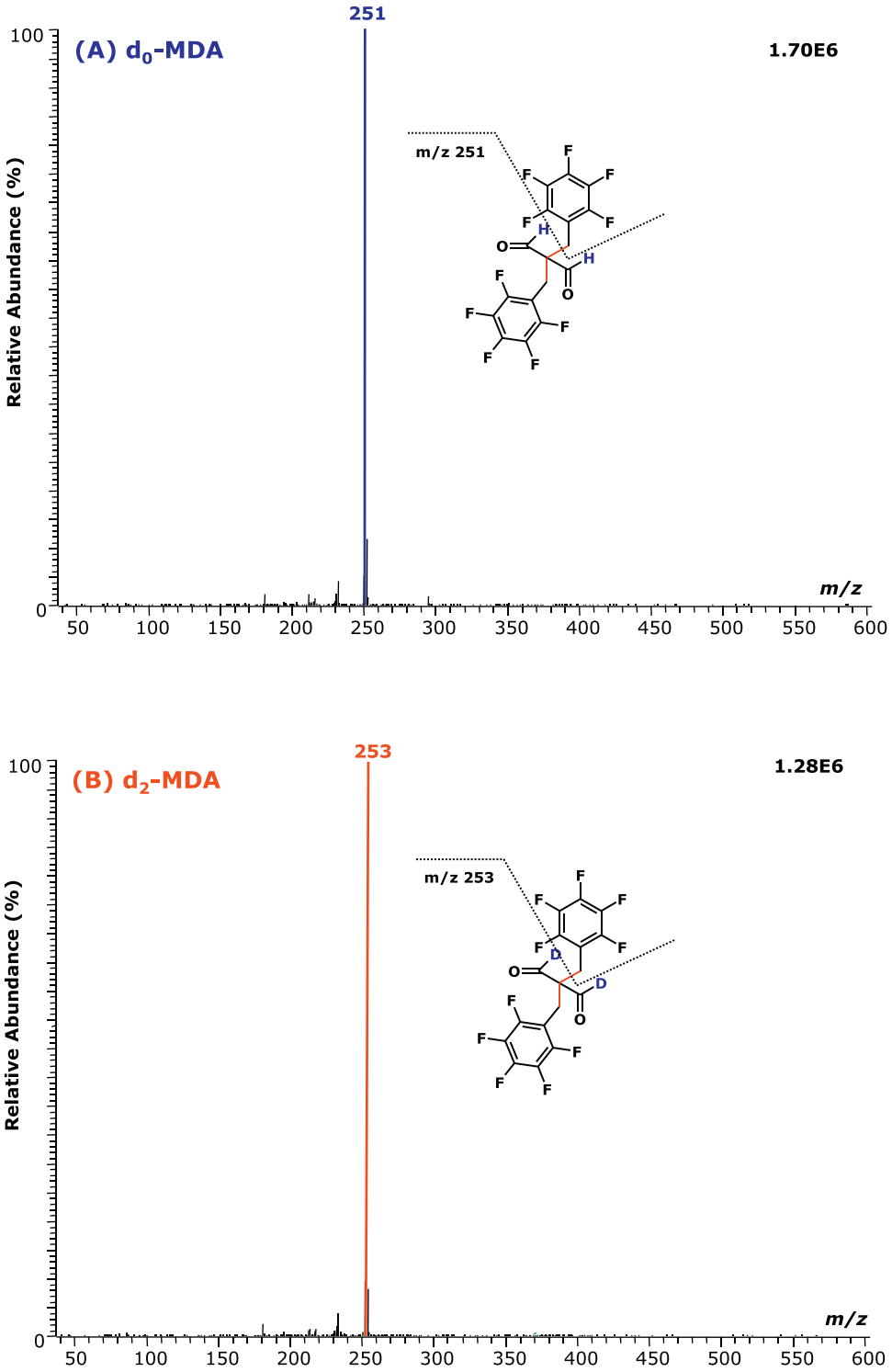


Fig. 1. Electron capture negative-ion chemical ionization (ECNICI) mass spectra of the reaction product of d_0-MDA (A) and d_2-MDA (B) with PFB-Br in aqueous acetone (50 °C, 60 min). The mass spectra were derived from the GC–MS peaks emerging from the column each at 10.58 min. Insets indicate the proposed structures for the derivatives PFB-d_0-MDA-PFB (A; molecular mass, 432) and PFB-d_2-MDA-PFB (B; molecular mass, 434) and the anions at *m/z* 251 and *m/z* 253 which are produced each by neutral loss of a PFB radical.

15(*S*)-8-*iso*-$PGF_{2\alpha}$ in 1-mL aliquots of urine and free plus esterified 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in 1-mL aliquots of plasma were determined by GC–MS/MS [26]. Urinary creatinine was measured by GC–MS [27] and used for correction of urinary 15(*S*)-8-*iso*-$PGF_{2\alpha}$ excretion which is reported in nmol 15(*S*)-8-*iso*-$PGF_{2\alpha}$ per mol creatinine (1 nmol/mol corresponds to 3.23 ng/g).

Statistical analyses were performed by means of the Software SPSS 14.0 for Windows. Graphs were constructed by GraphPad Prism 5.04 (GraphPad Prism Software Inc., San Diego, California, USA). Distribution of variables was tested by Kolmogorov–Smirnov or D'Agostino and Pearson omnibus K2 test. Normally distributed parameters were compared by parametric tests (Student's *t* test).

Non-normally distributed parameters were analyzed by non-parametric tests (Mann–Whitney test) and are presented as median and interquartile range (25–75th percentile). Correlations between variables were assessed by Pearson (parametric) or Spearman (non-parametric) correlation. P values < 0.05 were considered as statistically significant.

2.7. Data presentation

In method validation, quantitative analyses were performed at least in duplicate as specified in the respective sections. Values are reported as mean ± standard deviation (SD). Data from clinical studies are expressed as mean ± standard error of the mean (SEM) or median [25–75th percentile].

3. Results

3.1. Structural identification of the derivatization products

GC–MS analysis of toluene extracts obtained from derivatization of d_2-MDA in phosphate buffer (pH 7.4) showed formation of several GC peaks (data not shown). The ECNICI mass spectra of the compounds eluting at about 10.57 min contain each a single intense ion at m/z 251 for d_0-MDA and m/z 253 for d_2-MDA (Fig. 1). These data together with the relatively long retention time of 10.57 min when compared to that of the PFB derivative of nitrite, i.e., PFB-NO_2, which emerged from the same GC column at 3.5 min under the same GC conditions, suggest that the reaction products of d_0-MDA and d_2-MDA with PFB-Br carry two PFB moieties, i.e., they are PFB-d_0-MDA-PFB and PFB-d_2-MDA-PFB, respectively. The lack of the molecular radical anions $[M]^{\bullet -}$ at m/z 432 and m/z 434 in the ECNICI mass spectra of PFB-d_0-MDA-PFB and PFB-d_2-MDA-PFB, respectively, suggests that their ECNICI is accompanied by loss each of a PFB radical (PFB$^\bullet$, 181 Da) from PFB-d_0-MDA-PFB and PFB-d_2-MDA-PFB. Thus, the intense ions m/z 251 and m/z 253 are due to $[\text{PFB-}d_0\text{-MDA}]^-$ and $[\text{PFB-}d_2\text{-MDA}]^-$, respectively (see Supplement). The difference of 2 Th indicates that the aldehydic H/D atoms are not lost during ECNICI of PFB-d_0-MDA-PFB and PFB-d_2-MDA-PFB.

The ECNICI product ion mass spectra generated by CID of m/z 251 for d_0-MDA and m/z 253 of d_2-MDA are shown in Fig. 2. The most intense product ions are m/z 175 and m/z 177, respectively, also suggesting that the aldehydic H/D atoms are present in these CID product ions. The proposed structures for these and other product ions obtained from CID of m/z 251 for d_0-MDA m/z 253 of d_2-MDA and of the mechanisms by which the products ions may be formed are shown in Scheme 2. Other paired product ions with a 2-Th difference due to the presence of 2 D atoms are m/z 231/m/z 233, m/z 203/m/z 205, m/z 187/m/z 189. Other paired product ions with a 1-Th difference due to the presence of 1 D atom are m/z 211/m/z 212, m/z 183/m/z 184, m/z 155/m/z 156, and m/z 149/m/z 150. The common anion at m/z 167 is due to $[C_6F_5]^-$.

3.2. Modes of quantification

The multiple product ions described above indicate that the CID of m/z 251 for d_0-MDA and m/z 253 for d_2-MDA is complex (Scheme 2) and suggest that quantification of MDA in biological samples should be highly specific by GC–MS/MS.

In GC–MS, quantification was performed by SIM of m/z 251 for d_0-MDA and m/z 253 d_2-MDA. In GC–MS/MS, quantification was carried out by SRM of the mass transition m/z 251 → m/z 175 for d_0-MDA and of the mass transition m/z 253 → m/z 177 for d_2-MDA. The peak area was calculated and considered in quantitative analyses.

Separate derivatization of three 100-μL aliquots of a 1-μM d_2-MDA solution in phosphate buffer (pH 7.4) and GC–MS analysis in the SIM mode resulted in a peak area ratio (PAR) of 0.0216 ± 0.0037 for m/z 251 to m/z 253. GC–MS/MS analysis of the same samples in the SRM mode resulted in a PAR of 0.0023 ± 0.0001 for m/z 175 to m/z 177. Separate derivatization of three 100-μL aliquots of a 1-μM standard solution of d_0-MDA in phosphate buffer (pH 7.4) and analysis resulted in a PAR of 0.0126 ± 0.0007 for m/z 253 to m/z 251 (SIM mode) and in a PAR of 0.0037 ± 0.0002 for m/z 177 to m/z 175 (SRM mode). These data indicate no appreciable cross-contribution between d_0-MDA and d_2-MDA. Furthermore, derivatization and analysis of unspiked phosphate buffer by SIM and SRM did not result in peaks eluting at about 10.58 min, indicating that MDA is not present as a contamination in the buffer, the chemicals and the glass vials used. Thus, contaminating MDA should not represent an appreciable concern in the quantitative analysis of MDA in biological samples, unlike in the nitrite and nitrate analysis [20].

In consideration of the above described results, calculation of MDA concentrations (i.e., [d_0-MDA]) in biological fluids was performed by multiplying the known concentration of the internal standard (i.e., [d_2-MDA]) added to the sample by the respective PAR measured either in the SIM or SRM mode, i.e., [d_0-MDA] = [d_2-MDA] × PAR.

3.3. Derivatization procedure

Derivatization of inorganic and organic ions in biological samples with PFB-Br demands use of a water-miscible organic solvent such as acetone, acetonitrile, methanol or ethanol as solubilizer for PFB-Br which is not miscible with water. Because PFB derivatives need to be extracted prior to GC–MS analysis, quite volatile solvents such as acetone (boil point 56 °C) are preferentially used [20]. We used acetone for the derivatization of MDA with PFB-Br in aqueous phases in a volume ratio of 4:1 to enable formation of a single homogenous sample. In case of protein-rich biological samples, proteins are precipitated upon acetone addition. Yet, PFB-Br derivatization is performed in the presence of precipitated proteins.

Formation of PFB-MDA-PFB from PFB-Br and MDA in acetonic phosphate buffer (pH 7.4) was found to depend upon reaction temperature and time. We investigated by GC-MS in the SIM mode the effect of two reaction temperatures, i.e., 50 °C and 80 °C, and various derivatization times, i.e., between 5 min to 120 min, on the formation of PFB-MDA-PFB from MDA (1 μM) in the presence of a great molar excess of PFB-Br (10 μL). Maximum formation of PFB-MDA-PFB was observed after heating the samples for 60 min at 50 °C (data not shown). These conditions are comparable to those required for the derivatization of nitrate [20], suggesting that MDA is quite inert towards PFB-Br. We observed that the accuracy of the method depended upon the evaporation time of the derivatization mixture. Unlike in nitrite and nitrate analysis, where acetone but not the aqueous phase is evaporated [20], we found (data not shown) that analytically acceptable accuracy and precision were obtained after complete evaporation of the whole derivatization mixture including the aqueous phase under a gentle stream of nitrogen. By using a nitrogen pressure of ≤35 kPa, the evaporation process regularly requires about 25 min on the nitrogen evaporator model TurboVap LV from Zymark (Frankfurt am Main, Germany).

3.4. Method validation in human plasma

The results from the validation of the method are summarized in Table 1. Typical chromatograms from the GC–MS and GC–MS/MS analysis of MDA in human plasma are shown in Fig. 3. In the concentration range investigated, imprecision ranged between 0.1% and 25.3% in GC–MS and between 0.1% and 28.4% in GC–MS/MS; recovery ranged between 51% and 139% in GC–MS and between 73% and 144% in GC–MS/MS (Table 1). Expectedly, precision and accuracy

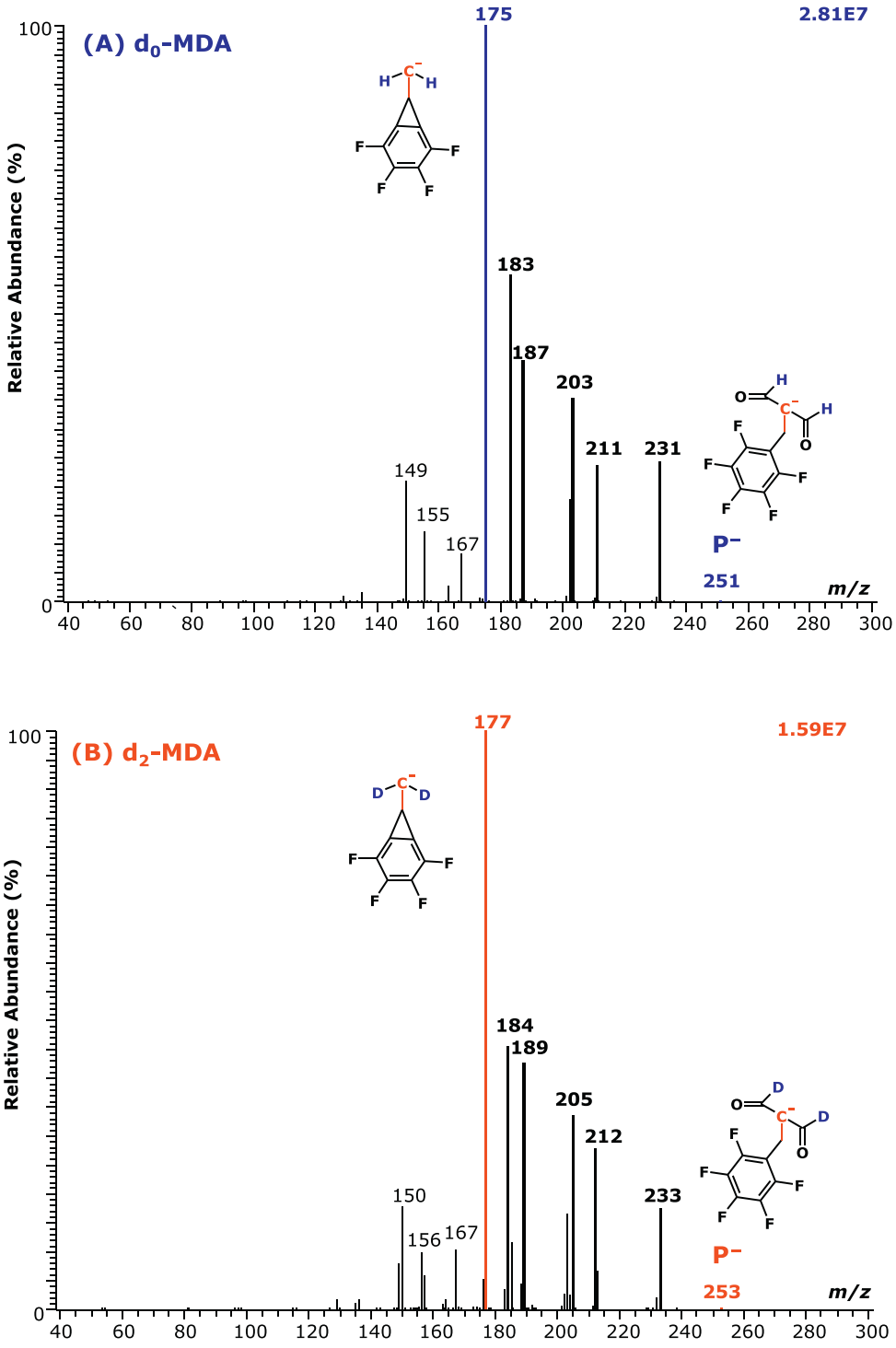
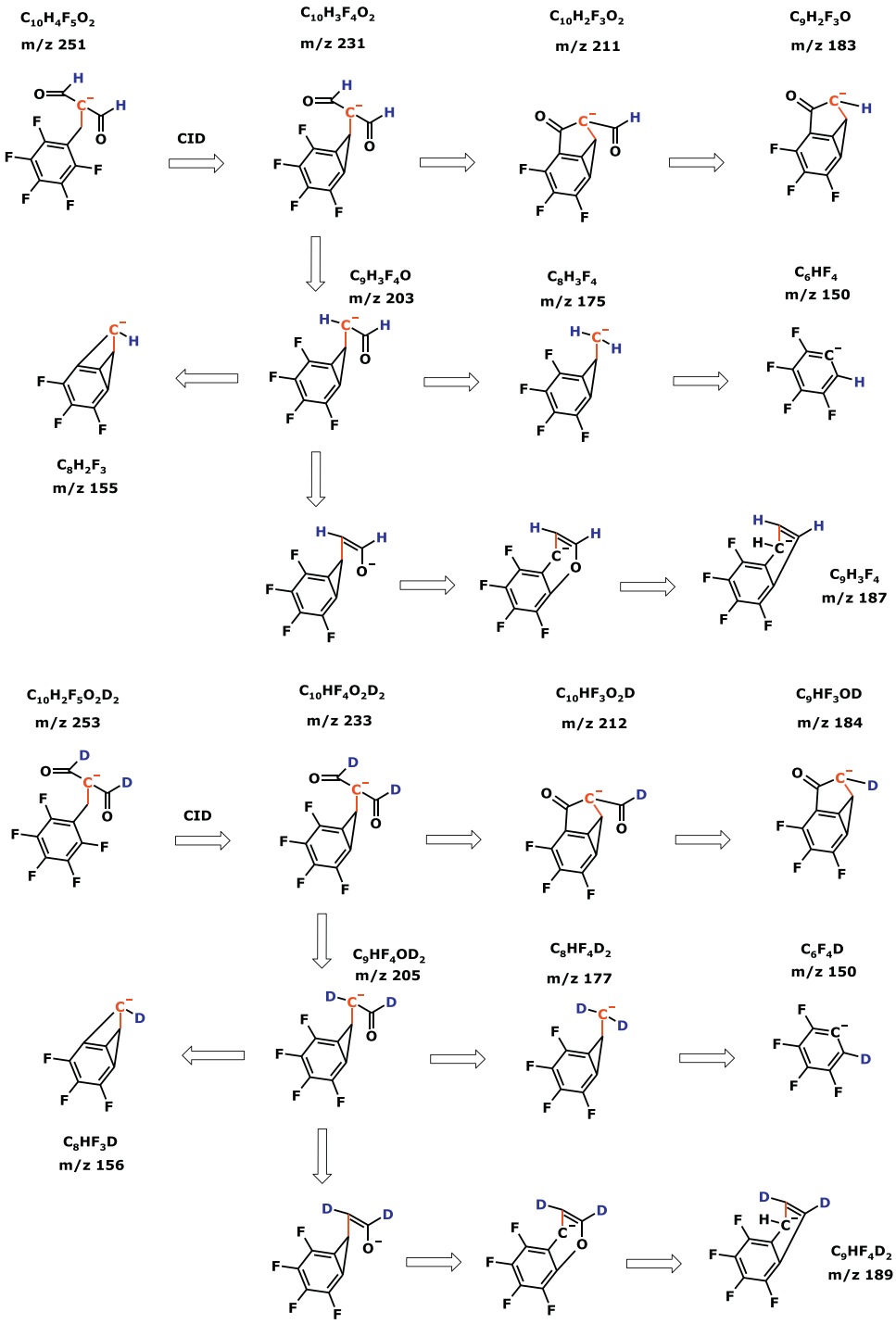


Fig. 2. Product ion mass spectra obtained by collision-induced dissociation (CID) of the parent ions (P^-) at m/z 251 for d_0-MDA (A) and m/z 253 for d_2-MDA (B) which were generated by electron capture negative-ion chemical ionization (ECNICI) of the pentafluorobenzyl derivatives emerging from the column each at 10.58 min. Insets indicate the proposed structures for the parent ions m/z 251 and m/z 253 and the respective most intense product ions m/z 175 and m/z 177. The proposed mechanisms and the structures of these and other product ions are shown in Scheme 2. See also Fig. 1.

decreased with decreasing concentrations of MDA added to the CPD plasma.

Linear regression analysis was performed between the PAR of m/z 251 to m/z 253 (GC-MS) or m/z 175 to m/z 177 (GC–MS/MS) measured and the concentration of d_0-MDA added to plasma. The y-axis intercept value corresponded to MDA concentrations of 257 ± 49 nM in GC–MS and 249 ± 53 nM in GC-MS/MS, indicating a basal MDA concentration of about 250 nM in the CPD plasma used. The slope values of the regression equations obtained by plotting the measured PAR values versus the added MDA concentrations ranged between 1.061 and 1.225 in GC–MS and between 1.102 and 1.249 in GC–MS/MS. The values of the coefficient of correlation R ranged between 0.9895 and 0.9988 in GC–MS and between 0.9965 and 0.9992 in GC–MS/MS.

These data indicate that MDA can be precisely and accurately measured in human plasma both by GC–MS and GC–MS/MS



Scheme 2. Proposed chemical structures of and CID mechanisms for the product ions observed in product ion mass spectra of d_0-MDA (A) and d_2-MDA (B) upon CID of the precursor ions at *m/z* 251 and *m/z* 253, respectively (Fig. 2), generated by ECNICI of PFB-d_0-MDA-PFB and PFB-d_2-MDA-PFB (Fig. 1).

in pathophysiologically relevant MDA concentrations (see also below).

3.4.1. Limits of detection and quantitation

The limits of detection (LOD) of the methods were determined as follows. Each three aliquots (100 μL) of 1-μM solutions of d_2-MDA in phosphate buffer (pH 7.4) were derivatized, reaction products were extracted, and 1-μL aliquots of the toluene phases (1000 μL) were injected in the splitless mode and analyzed by GC–MS in the SIM mode (*m/z* 251 and *m/z* 253). On the assumption of quantitative derivatization and extraction, 1-μL toluene phase volumes injected would contain 100 fmol of d_2-MDA. They produced peak areas with a mean signal-to-noise ratio (S/N) of 144:1 (RSD, 17%), suggesting an approximate LOD value of 2 fmol in GC–MS. Due to higher sensitivity in GC–MS/MS, dilutions of the above mentioned toluene phase were prepared, and 100 amol of d_2-MDA were injected and analyzed by GC–MS/MS in the SRM mode (*m/z* 175 and *m/z* 177). This amount produced peak areas with a mean S/N ratio of 183:1 (RSD, 5.6%), suggesting an approximate LOD value of 2 amol in GC-MS/MS.

The limits of quantitation (LOQ) of the methods were determined as follows. The lowest added concentration of MDA to the

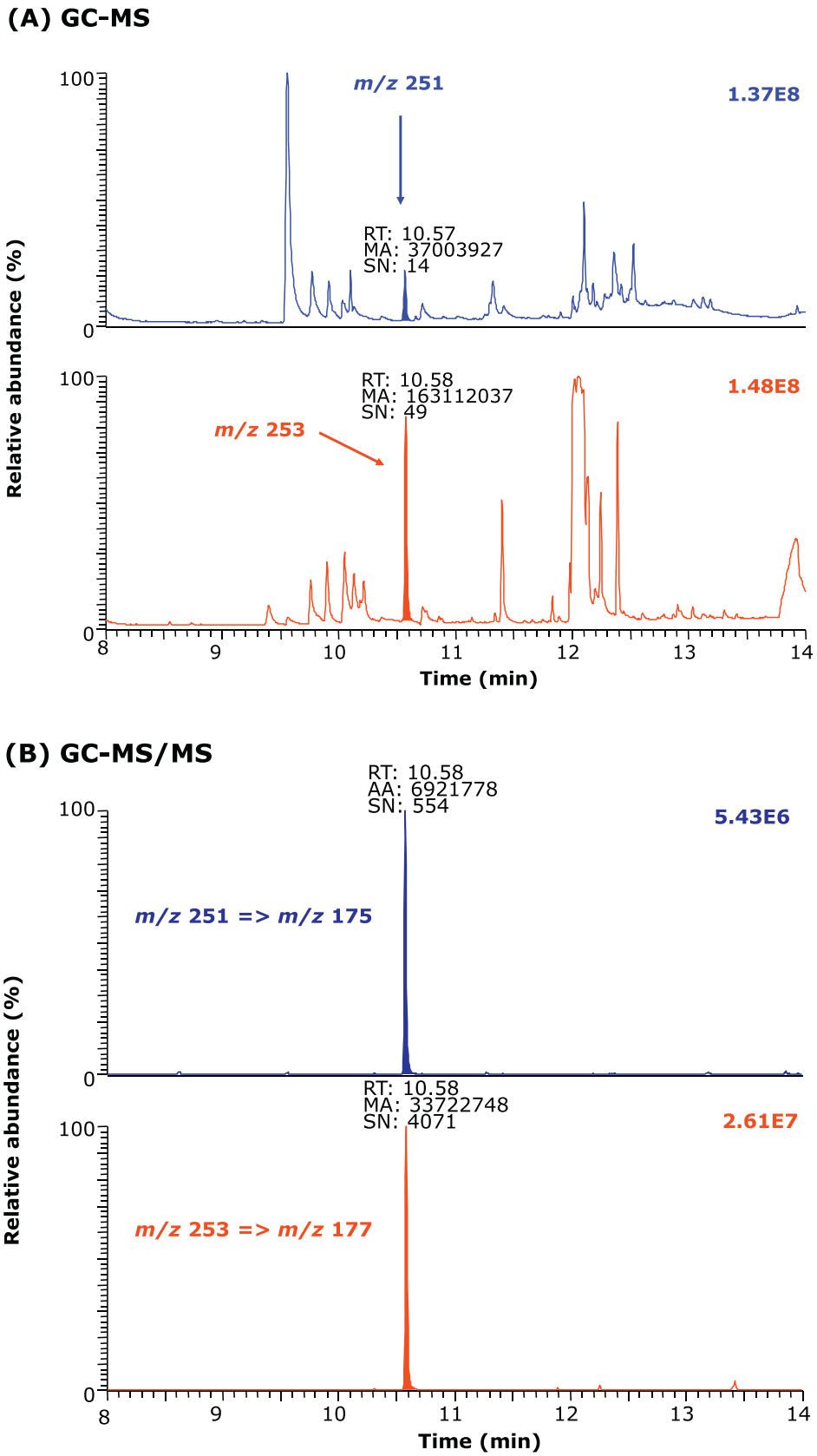


Fig. 3. Typical chromatograms from the analysis of MDA in plasma of a healthy subject after derivatization with PFB-Br and subsequent analysis by GC–MS in the SIM mode (A) and by GC–MS/MS in the SRM mode (B). The concentration of the internal standard d_2-MDA was 1000 nM. The concentration of endogenous MDA in this plasma sample is determined to be 227 nM by GC–MS and 205 nM by GC–MS/MS.

Table 1
GC–MS and GC–MS/MS intra- and inter-assay accuracy (recovery, %) and precision (RSD, %) for MDA in CPD human plasma.

Day	Added (nM)	Measured (mean ± SD, nM)		Recovery (%)		Imprecision (RSD, %)	
		GC–MS	GC–MS/MS	GC–MS	GC–MS/MS	GC–MS	GC–MS/MS
1	0	260 ± 37	272 ± 31	N.A.	N.A.	14.2	11.4
2	0	187 ± 4	200 ± 9	N.A.	N.A.	2.0	4.4
3	0	260 ± 9	223 ± 20	N.A.	N.A.	3.4	8.9
4	0	323 ± 13	331 ± 3	N.A.	N.A.	4.1	0.8
5	0	252 ± 14	219 ± 13	N.A.	N.A.	5.7	5.9
1	200	384 ± 4	431 ± 25	62.0	79.5	1.1	5.8
2	200	288 ± 67	431 ± 25	51.0	73.0	23.4	23.0
3	200	454 ± 58	429 ± 41	97.0	103	12.7	9.5
4	200	554 ± 1	429 ± 41	115	93.0	0.1	0.5
5	200	488 ± 123	470 ± 133	118	126	25.3	28.4
1	400	672 ± 38	709 ± 8	103	109	5.7	1.1
2	400	571 ± 1	637 ± 8	96.0	109	0.2	1.3
3	400	656 ± 73	634 ± 28	99.0	103	11.1	4.3
4	400	734 ± 21	747 ± 1	103	104	2.8	0.1
5	400	656 ± 90	661 ± 74	101	111	13.7	11.2
1	600	927 ± 215	1137 ± 259	111	144	23.2	22.8
2	600	843 ± 16	894 ± 25	109	116	1.9	2.8
3	600	920 ± 40	937 ± 1	110	119	4.4	0.1
4	600	1095 ± 4	1009 ± 15	97.0	113	2.0	1.5
5	600	1131 ± 57	904 ± 1		114	0.5	0.1
1	800	1040 ± 42	1204 ± 48	97.0	117	4.0	4.0
2	800	952 ± 8	1096 ± 8	96.0	112	0.8	0.8
3	800	1376 ± 203	1306 ± 127	139	135	14.7	9.4
4	800	1095 ± 4	1191 ± 10	96.0	104	0.4	1.7
5	800	1131 ± 57		110	122	5.0	0.9
1	1000	1365 ± 80	1403 ± 31	110	113	5.9	2.0
2	1000	1315 ± 25	1378 ± 11	113	118	1.9	0.8
3	1000	1425 ± 29	1476 ± 45	117	125	2.0	3.1
4	1000	1428 ± 53	1416 ± 22	110	109	3.7	1.6
5	1000	1349 ± 90	1454 ± 22	110	124	6.7	1.5
1	1200	1511 ± 179	1673 ± 67	104	117	11.8	4.0
2	1200	1514 ± 40	1686 ± 10	111	124	2.7	0.6
3	1200	1630 ± 24	1732 ± 19	114	126	1.4	1.1
4	1200	1525 ± 14	1574 ± 28	100	104	0.9	1.8
5	1200	1627 ± 130	1773 ± 157	115	130	8.0	8.9
1	1400	1641 ± 9	1911 ± 11	99.0	117	0.5	0.6
2	1400	1646 ± 6	1861 ± 101	104	119	0.4	5.4
3	1400	1799 ± 145	1932 ± 35	110	122	8.0	1.8
4	1400	1777 ± 56	1844 ± 12	104	108	3.1	0.7
5	1400	1831 ± 4	1967 ± 78	113	125	0.2	4.0
1	1600	1893 ± 3	2112 ± 4	102	115	0.1	0.2
2	1600	1975 ± 5	2184 ± 2	112	124	2.3	0.1
3	1600	2106 ± 28	2156 ± 52	115	121	1.3	2.4
4	1600	1972 ± 71	2114 ± 11	103	111	3.6	0.5
5	1600	1991 ± 25	2114 ± 15	109	118	1.2	0.7
1	1800	2121 ± 19	2347 ± 4	103	115	0.9	0.2
2	1800	2162 ± 93	2391 ± 18	110	122	4.3	0.8
3	1800	2181 ± 57	2379 ± 7	107	119	2.6	0.3
4	1800	2292 ± 43	2292 ± 8	109	109	1.9	0.3
5	1800	2280 ± 25	2343 ± 8	113	118	1.1	0.3
1	2000	2435 ± 25	2670 ± 95	109	121	1.0	3.5
2	2000	2800 ± 597	2627 ± 47	131	121	21.3	1.8
3	2000	2561 ± 233	2659 ± 1	115	122	9.1	0.1
4	2000	2442 ± 54	2520 ± 1	106	109	2.2	0.1
5	2000	2452 ± 83	2654 ± 1	110	121	3.4	0.1

N.A., not applicable

CPD plasma samples used in method validation was 200 nM. The recovery and imprecision data observed for 200 nM MDA suggest that this concentration may serve as the lower limit of quantitation (LLOQ) of the GC–MS/MS method for MDA both in plasma. For endogenous analytes such as MDA, the lowest concentration used in method validation is rather arbitrary and may depend on the basal concentration of the analyte in the biological sample being investigated. To circumvent this difficulty we proposed the use of the relative lower limit of quantitation (*r*LLOQ) [25]. The value of *r*LLOQ is calculated by Formula (F2), i.e., by dividing the value of the lower limit of quantification C_{LLOQ}, usually the lowest added analyte concentration to the matrix, by the experimentally measured basal analyte concentration value of C_0 and by multiplying the observed molar ratio by 100:

$$r\mathrm{LLOQ} = \left(C_{\mathrm{LLOQ}} : C_0\right) \times 100 \qquad \text{(F2)}$$

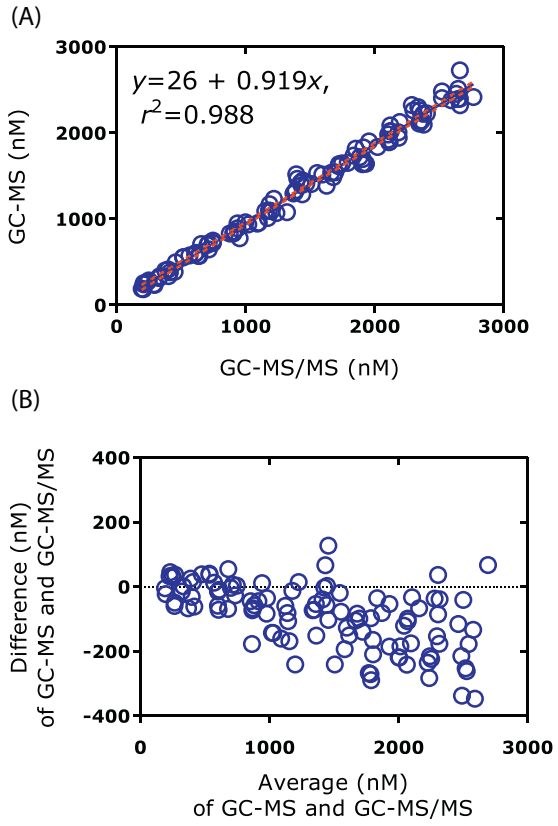


Fig. 4. (A) Linear regression analysis and (B) Bland–Altman analysis of the MDA concentrations measured by GC–MS and GC–MS/MS in method validation (see Table 1). MDA was added to the plasma sample at concentrations in the range 0–2000 nM. The concentration of the internal standard was 1000 nM.

By definition, the *r*LLOQ expresses the percentage fraction of the analyte which, upon addition to the biological sample that contains this analyte at the basal concentration C_0, can be measured therein with acceptable accuracy (e.g., recovery of 100 ± 20%) and imprecision (e.g., RSD ≤ 20%) or with an acceptable total error (recovery + precision) of ≤30% [25]. On the basis of these criteria and the validation data for plasma (Table 1), the *r*LLOQ value of the GC–MS/MS method for MDA in plasma is calculated to be 80%.

3.4.2. Comparison between GC–MS and GC–MS/MS for plasma MDA

The GC–MS and GC–MS/MS chromatograms for human plasma MDA analysis shown in Fig. 3 indicate a single peak in GC–MS/MS but several peaks in GC–MS including those for endogenous MDA and the internal standard d_2-MDA, suggesting that GC–MS and GC–MS/MS may provide different results. The GC–MS and GC–MS/MS methods were therefore compared both by linear regression analysis and the method of Bland and Altman [31] (Fig. 4). Linear regression analysis between the concentrations of MDA measured in plasma by GC–MS and those measured by GC–MS/MS for all 5 standard curves (see Table 1), resulted in an *y*-axis intercept of 26 ± 16 nM, a slope value of 0.919 ± 0.01, correlation coefficient *R* ranging between 0.9783 and 0.9989, and an overall precision (RSD) of 7.2% (Fig. 4A). The plot of the differences of the GC–MS and GC–MS/MS methods against the average of the two methods by the method of Bland and Altman is shown in Fig. 4B. Analysis by GC–MS gave plasma MDA concentrations being constantly slightly lower than those measured by GC–MS/MS. The difference (bias) between MDA concentrations measured by GC–MS and GC–MS/MS was only −89 ± 99 nM. Best agreement between GC–MS and GC–MS/MS was observed for MDA concentrations <1 μM. The GC–MS-to-GC–MS/MS ratio was 0.95 ± 0.08.

3.5. Method validation in human urine

Under physiological conditions, the pH value of human urine ranges between 5.5 and 7.8. In order to adjust and buffer the pH of urine samples around a value of 7, 50-μL aliquots of urine samples were mixed with 50-μL aliquots of a 67-mM phosphate buffer (pH 7.4). The derivatization of the diluted urine samples (100 μL) with PFB-Br was performed as described for plasma (acetone, 400 μL; PFB-Br, 10 μL; 50 °C, 60 min, evaporation time, 25 min).

To test the validity of the method for urine samples two standard curves (range, 0-2 μM; 10 and 11 concentration points; duplicate analyses) were performed (Table 2). Linear regression analysis between the PAR measured and the MDA concentration added yielded *y*-axis intercept values of 222 ± 37 nM in GC–MS and 33.3 ± 4 nM in GC–MS/MS, slope values (range in GC–MS, 0.0922–0.0971; range in GC–MS/MS, 0.1015–0.1022) close to the theoretical value of 0.100, and coefficient of correlation *R* values (range in GC–MS, 0.9895–0.9988; range in GC–MS/MS, 0.9965–0.9992) close to 1.000, indicating complete recovery of MDA added to the urine sample examined. Imprecision (RSD) ranged between 0.7% and 11.1% in GC–MS and between 0.1% and 11.7% in GC–MS/MS; recovery ranged between 74% and 102% in GC–MS and between 84% and 107% in GC–MS/MS (Table 2). The clearly different baseline values of MDA measured in the urine samples of 222 nM in GC–MS and 33 nM in GC–MS/MS indicate that there is a lack of specificity in one of the methods used. The higher values for MDA in urine determined by GC–MS could be due to the measurement of co-eluting isobaric substances with an *m*/*z* value of 251. These potential interferences are likely to derive from isobaric ions (with an *m*/*z* value of 251) produced by substances different from MDA, which are eliminated by the second mass separation step that follows CID in GC–MS/MS.

3.6. Biomedical applications of the stable-isotope dilution GC–MS/MS method

In the sections that follow we describe the effects of coagulation/anticoagulation on the measurement of MDA in human blood and the application of the GC–MS/MS method in clinical studies.

3.6.1. Contaminating MDA in blood sampling monovettes and effects of coagulation and anticoagulation on circulating MDA concentration

Each six commercially available monovettes for blood sampling (volume/anticoagulants: 3 mL/citrate, 9 mL/EDTA, 9 mL/lithium heparin, and 4.5 mL/ammonium heparin for plasma generation, and 5.5 mL/no anticoagulants for serum generation) were filled with 67 mM phosphate buffer (pH 7.4) and gently shaken. In 100-μL aliquots taken from these monovettes and from the same buffer the concentration of MDA was determined by GC–MS/MS using 1 μM d_2-MDA as internal standard. With the exception of the citrate monovettes all other monovettes were found not to be contaminated by MDA (Fig. 5). In the buffer used the MDA concentration was measured to be about 5 nM. This concentration is likely to be caused by the amount of unlabeled MDA in d_2-MDA.

By means of the present GC–MS/MS method we determined the concentration of MDA in serum, heparin- and EDTA-plasma samples collected in a previously reported randomized, double-blind, double-dummy, three-period crossover design study [32]. Inclusion criteria of the study were female, age 18–35 years, healthy, intake of oral contraceptives, willingness to observe sexual abstinence during the study to avoid contamination of urine samples by seminal fluid. Exclusion criteria included acute or chronic dis-

Table 2
GC–MS and GC–MS/MS intra- and inter-assay method accuracy (recovery, %) and precision (RSD, %) for MDA in human urine.

Day	Added (nM)	Measured (mean ± SD, nM)		Recovery (%)		Imprecision (RSD, %)	
		GC–MS	GC–MS/MS	GC–MS	GC–MS/MS	GC–MS	GC–MS/MS
1	0	249 ± 15	36.4 ± 4.3	N.A.	N.A.	6.0	11.7
	200	412 ± 46	223 ± 6	82.0	93.0	11.1	2.9
	400	543 ± 13	373 ± 4	74.0	84.0	2.3	1.0
	600	798 ± 15	644 ± 5	92.0	101	1.9	0.8
	800	983 ± 12	852 ± 3	92.0	102	1.2	0.4
	1000	1146 ± 27	1058 ± 2	90.0	102	2.4	0.2
	1200	1363 ± 30	1249 ± 1	93.0	101	2.2	0.1
	1400	1459 ± 97	1392 ± 97	86.0	97.0	6.7	7.0
	1600	1666 ± 70	1639 ± 31	89.0	100	4.2	1.9
	1800	1916 ± 14	1846 ± 28	93.0	101	0.7	1.5
	2000	2086 ± 115	2064 ± 14	92.0	101	5.5	0.7
2	0	196 ± 14	30.2 ± 0.01	N.A.	N.A.	7.4	0.4
	200	385 ± 9	199 ± 10	94.0	84.0	2.4	5.2
	400	598 ± 37	427 ± 9	101	102	6.2	2.1
	600	759 ± 56	657 ± 50	94.0	104	7.4	7.6
	800	1000 ± 47	833 ± 3	100	100	4.7	0.3
	1000	1132 ± 56	1043 ± 8	94.0	101	5.0	0.7
	1200	Samples not available					
	1400	1630 ± 48	1502 ± 68	102	105	3.0	4.5
	1600	1819 ± 164	1739 ± 157	101	107	9.0	9.0
	1800	1842 ± 23	1839 ± 32	91.0	100	1.2	1.8
	2000	2148 ± 76	2011 ± 21	98.0	99.0	3.5	1.0

N.A., not applicable.

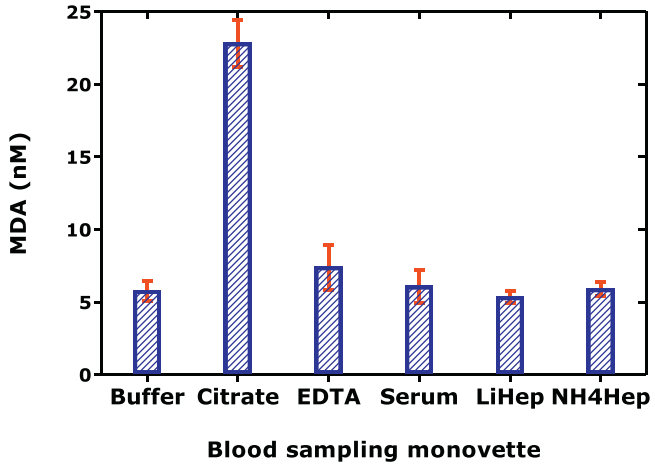


Fig. 5. Measurement of MDA contamination in commercially available monovettes used clinically for blood sampling. Each six monovettes (3-mL citrate, 9-mL EDTA, 5.5-mL serum, 9-mL lithium heparin (LiHep) and 4.5-mL ammonium heparin (NH4Hep)) were filled with 67 mM phosphate buffer (pH 7.4). In 100-μL aliquots taken from these monovettes and from the same buffer the concentration of MDA was determined by GC–MS/MS using 1000 nM d_2-MDA as the internal standard. The results are shown as mean ± SD.

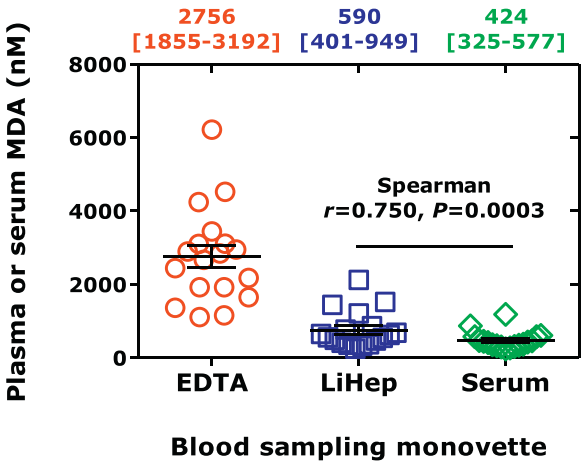


Fig. 6. MDA concentration measured in serum, heparin- and EDTA-plasma samples from venous blood taken consecutively using 5-mL monovettes from 18 healthy subjects. Serum and plasma samples had been stored frozen at -80 °C for three years. MDA was measured in 100-μL aliquots by GC–MS/MS method using 1 μM d_2-MDA as internal standard. The results are shown as mean ± SD.

eases, medical history of ulcer disease and pregnancy [32]. Venous blood was taken from 18 non-medicated healthy young women (age range, 20–31 years) consecutively using 5-mL monovettes and centrifuged immediately for generation of serum and plasma. Serum and plasma samples were separated immediately and frozen aliquoted at −80 °C until analysis for various biochemical parameters. About three years after sample collection, MDA was measured in 100-μL aliquots of the serum and plasma samples by the present GC-MS/MS method using 1 μM d_2-MDA as internal standard. The results of these analyses are illustrated in Fig. 6. The MDA concentration in EDTA-plasma (median [25–75th]) 2756 [1855–3192] nM was much higher compared to heparin-plasma 590 [401–949] nM and serum 424 [325–577] nM (Fig. 6). Interestingly, there was a considerable correlation ($r = 0.750$, $P = 0.0003$) between the MDA concentration in serum and heparin plasma (Fig. 6), whereas no correlation was found between the MDA concentration in EDTA-plasma and heparin-plasma or between the MDA concentration in EDTA-plasma and serum. These observations together with the finding shown in Fig. 5 suggest that blood sampling using EDTA-containing monovettes is associated with considerable ex vivo formation of MDA during plasma storage, although EDTA acts as an antioxidant by complexing transition metal ions. It is worth mentioning that in the same samples similar differences were observed for dimethyl amine (DMA) between serum (1430 ± 230 nM) or heparin-plasma (1730 ± 170 nM) and EDTA-plasma (9840 ± 1430 nM) [33]. The reasons for these effects of EDTA on MDA (see Section 4) and DMA, the major urinary metabolite of the nitric oxide (•NO) synthase inhibitor asymmetric dimethylarginine (ADMA), are unknown and remain to be elucidated. For reliable comparison of analytical methods and of experimental and clinical studies, reporting the type of anticoagulation is essential for circulating MDA as well.

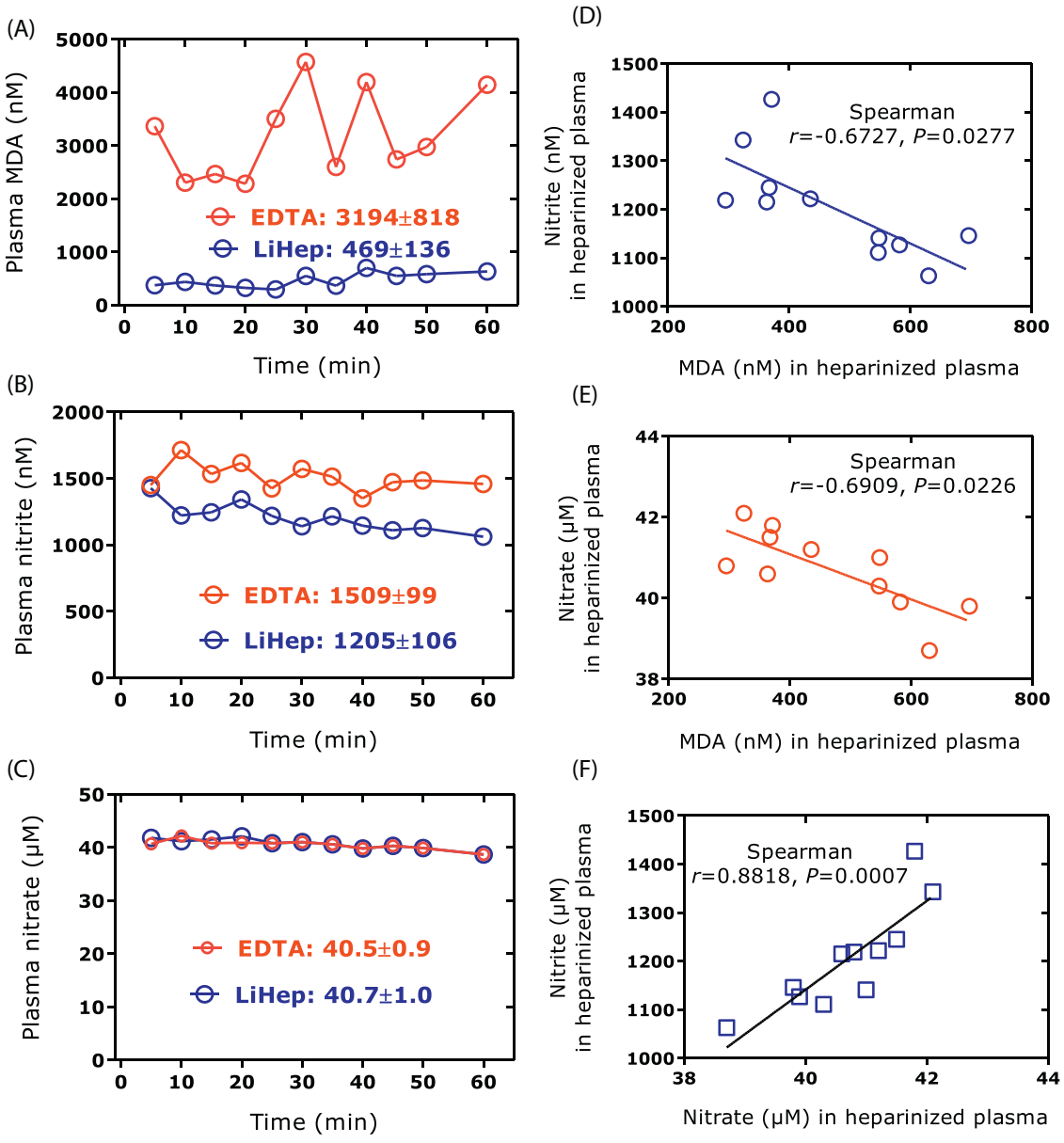


Fig. 7. Measured MDA, nitrite and nitrate concentrations in plasma freshly prepared from venous blood donated by a healthy volunteer, which was collected using EDTA and heparin monovettes. Plasma MDA concentration is associated with considerable intra-individual variation. In heparinized plasma, MDA is inversely correlated with nitrite and nitrate, whereas nitrite is positively correlated with nitrate.

3.6.2. Relationship between MDA and nitrite or nitrate in plasma

We observed a similar finding for MDA but not for nitrite and nitrate on freshly generated EDTA-plasma and heparin-plasma from antecubital venous blood donated consecutively by a healthy non-medicated male volunteer (age, 60 years) who underwent intermittent ischemia/reperfusion of the forearm for 60 min (Fig. 7). Fig. 7A indicates that measurement of MDA is associated with considerable intra-individual variation both in EDTA-plasma (RSD, 26%) and in heparinized plasma (RSD, 29%), yet with the average MDA concentration being about 7 times lower in the heparinized compared to EDTA-containing plasma samples. In this pilot study we observed considerable inverse correlations between nitrite (Fig. 7D) or nitrate (Fig. 7E) and MDA in heparinized plasma, and a tight positive correlation between nitrite and nitrate in the heparinized plasma samples (Fig. 7F), indicating a close equilibrium between nitrite and nitrate in the circulation. These observations may suggest that repeated intermittent ischemia/reperfusion of the forearm in healthy humans may not only decrease •NO bioavailability (reduction of plasma nitrite) due to elevated oxidative stress but also systemic •NO synthesis in the circulation.

3.6.3. Effects of paracetamol and aspirin on plasma MDA in healthy humans

We performed a cross-over, interventional study on ten healthy male volunteers who did not take any medication two weeks before. Study subjects randomly consumed either placebo, aspirin or paracetamol under fasting conditions. This design should eliminate possible confounders like gender and seasonal or circadian variations. Although there were significant oscillations between subjects within all three experimental groups for values of both plasma MDA (Fig. 8A) and 15(*S*)-8-*iso*-$PGF_{2\alpha}$ (Fig. 8B), after 60 min there was a significant and concomitant decrease of both biomarkers in the aspirin group in comparison to the placebo group. Furthermore, the baseline-normalized AUC values of the creatinine-corrected excretion rate of 15(*S*)-8-*iso*-$PGF_{2\alpha}$

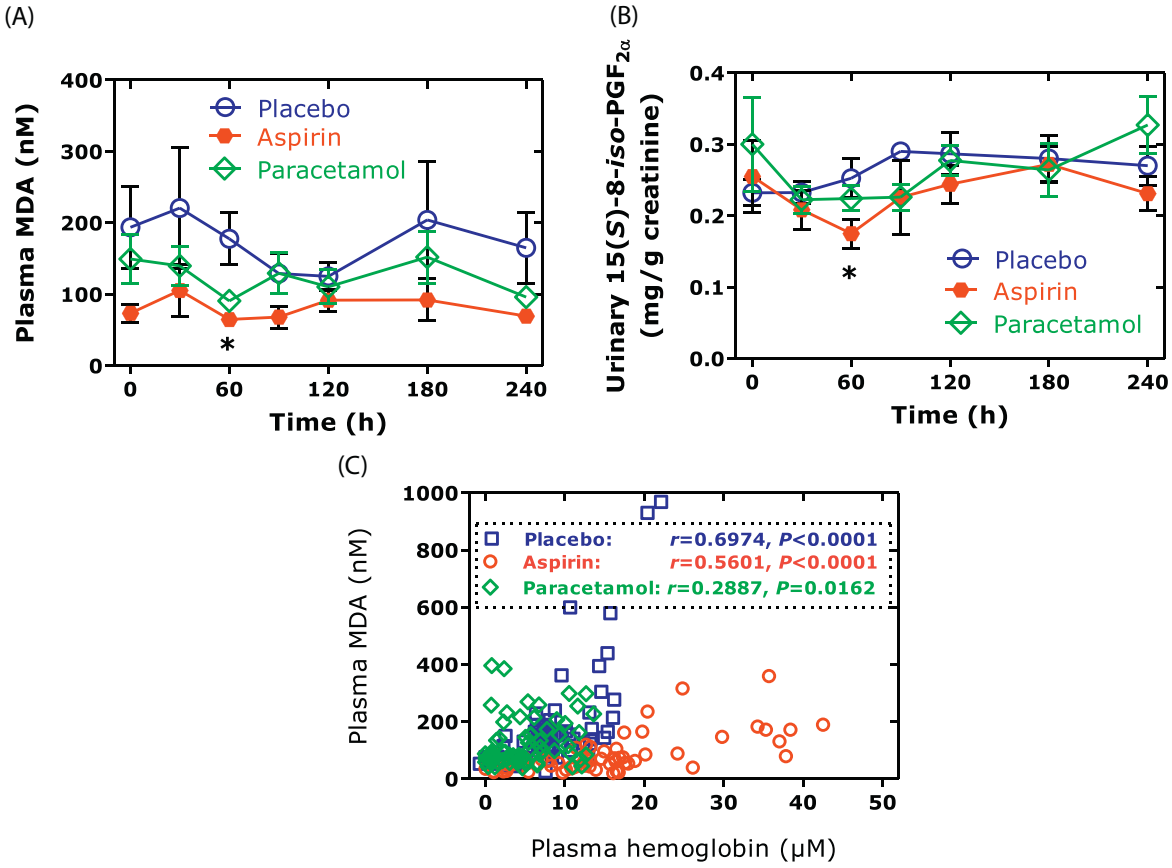


Fig. 8. MDA plasma concentration (A) and creatinine-corrected excretion of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ (B) in ten healthy, non-medicated male subjects after ingestion of placebo, aspirin (600 mg) or paracetamol (500 mg). Data are mean ± SEM. An asterisk indicates $P < 0.05$ Aspirin vs. Placebo (one-way ANOVA for repeated measures). (C) Spearman correlation between MDA and hemoglobin concentration in plasma of the volunteers. Hemoglobin was measured spectrophotometrically and the absorbance values at wavelengths of 562, 577 and 602 nm were used in calculations of hemoglobin concentrations.

(Fig. 8B) were by 62% lower in the aspirin group than in the placebo group. Some plasma samples of this study indicated clinical-chemistry relevant hemolysis. The plasma MDA concentration correlated with the plasma hemoglobin concentration in the placebo group ($r = 0.697$) more strongly than in the aspirin group ($r = 0.560$) and paracetamol group ($r = 0.289$) (Fig. 8C). The hemolysis-related finding in the placebo group of the present study is in agreement with the observation in healthy untreated young men [21]. Yet, it is worth mentioning that even in the placebo group the correlation of plasma MDA with plasma hemoglobin was evident only in three out of ten volunteers of this study.

3.6.4. *Effects of L-arginine on plasma MDA in CAD and PAOD patients*

The results of these studies investigating effects of chronic oral L-arginine administration to the CAD and PAOD patients of the present study on plasma MDA and 15(*S*)-8-*iso*-$PGF_{2\alpha}$ as well as urinary 15(*S*)-8-*iso*-$PGF_{2\alpha}$ are summarized in Table 3. In both groups, significantly lower concentrations of circulating MDA and 15(*S*)-8-*iso*-$PGF_{2\alpha}$, but not of urinary 15(*S*)-8-*iso*-$PGF_{2\alpha}$, were measured after 3 months (PAOD study) and 6 months (CAD study). We found considerable correlations for MDA and 15(*S*)-8-*iso*-$PGF_{2\alpha}$ concentrations measured at baseline and those after 3 months of L-arginine administration in the CAD study (Fig. 9A–C). Interestingly, circulating 15(*S*)-8-*iso*-$PGF_{2\alpha}$ correlated with MDA as well (Fig. 9D). In contrast, circulating and urinary 15(*S*)-8-*iso*-$PGF_{2\alpha}$ did not correlate each with other (data not shown).

4. Discussion

4.1. Analytical considerations

Malondialdehyde (MDA, OHC—CH_2—CHO) is a CH-acidic dicarbonyl compound, a protonic acid in aqueous solution, even more acidic (pK_a, 4.46) [34,35] than aliphatic carboxylic acids including acetic acid (pK_a, 4.7). Compared to the inorganic nitrous acid (O=N—O—H; pK_a, 3.4), MDA is about one order of magnitude less acidic. Analogous to nitrous acid and carboxylic acids, we utilized the remarkable acidity of the H atoms of the methylene group of MDA and derivatized it with pentafluorobenzyl bromide (PFB-Br) in aqueous acetone. Our results suggest that both H atoms of the CH_2 group of MDA are substituted by two PFB moieties via nucleophilic substitution reactions (presumably S_N2) of the MDA carbanion to form the 2,2′-dipentafluorobenzyl-1,3-propanedial, i.e., $C(C_6F_5CH_2)_2$—$(CHO)_2$. On the basis of this novel derivatization procedure we developed, validated and used stable-isotope dilution GC–MS and GC–MS/MS methods for the quantification of MDA in 100-μL aliquots of human plasma or serum without changing the plasma pH value, and in human urine after dilution (1:1, v/v) with 67 mM phosphate buffer of pH 7.4 to ensure complete dissociation of MDA.

The PFB-Br derivatization method used for MDA closely resembles the PFB-Br derivatization method we previously used for nitrite (O=N—O^-) [20], the conjugate base of nitrous acid. Although the O atom of nitrite is negatively charged, the reaction product of nitrite and PFB-Br in aqueous acetone is not the nitrous acid ester PFB-ONO, but is the nitro derivative PFB-NO_2 [20]. In the

Table 3
Effects of chronic oral L-arginine administration on MDA in plasma and 15(*S*)-8-*iso*-PG $F_{2\alpha}$ in plasma and urine of the CAD and PAOD patients of the present study at baseline (T0) and after 3 months (T3) or after 6 months (T6).

	MDA T0	T6	*P*	15(*S*)-8-*iso*-$PGF_{2\alpha}$ T0	T6	*P*
CAD study						
Plasma						
Arginine group	1284 [1192–1515]	1114 [990–1261]	0.001	2130 [1870–2620]	1350 [1030–1520]	<0.001
Placebo group	1316 [1240–1620]	1092 [987–1427]	0.005	2320 [1980–3060]	1100 [1020–1640]	<0.001
Urine						
Arginine group	n.d.	n.d.		49.4 [43.6–68.1]	44.8 [41.9–60.4]	0.819
Placebo group	n.d.	n.d.		39.8 [38.5–55.0]	35.2 [32.0–67.2]	0.452
PAOD study						
Plasma						
Arginine group	1640 [1206–1888]	762 [629–1218]	<0.001	753 [556–937]	428 [281–605]	<0.001
Placebo group	1419 [1037–2074]	910 [732–1344]	0.004	684 [561–763]	382 [222–648]	<0.001
Urine						
Arginine group	n.d.	n.d.		194 [136–299]	195 [125–332]	0.989
Placebo group	n.d.	n.d.		179 [120–288]	184 [149–380]	0.325

Data are presented as median [25–75th percentile]; n.d., not detected.

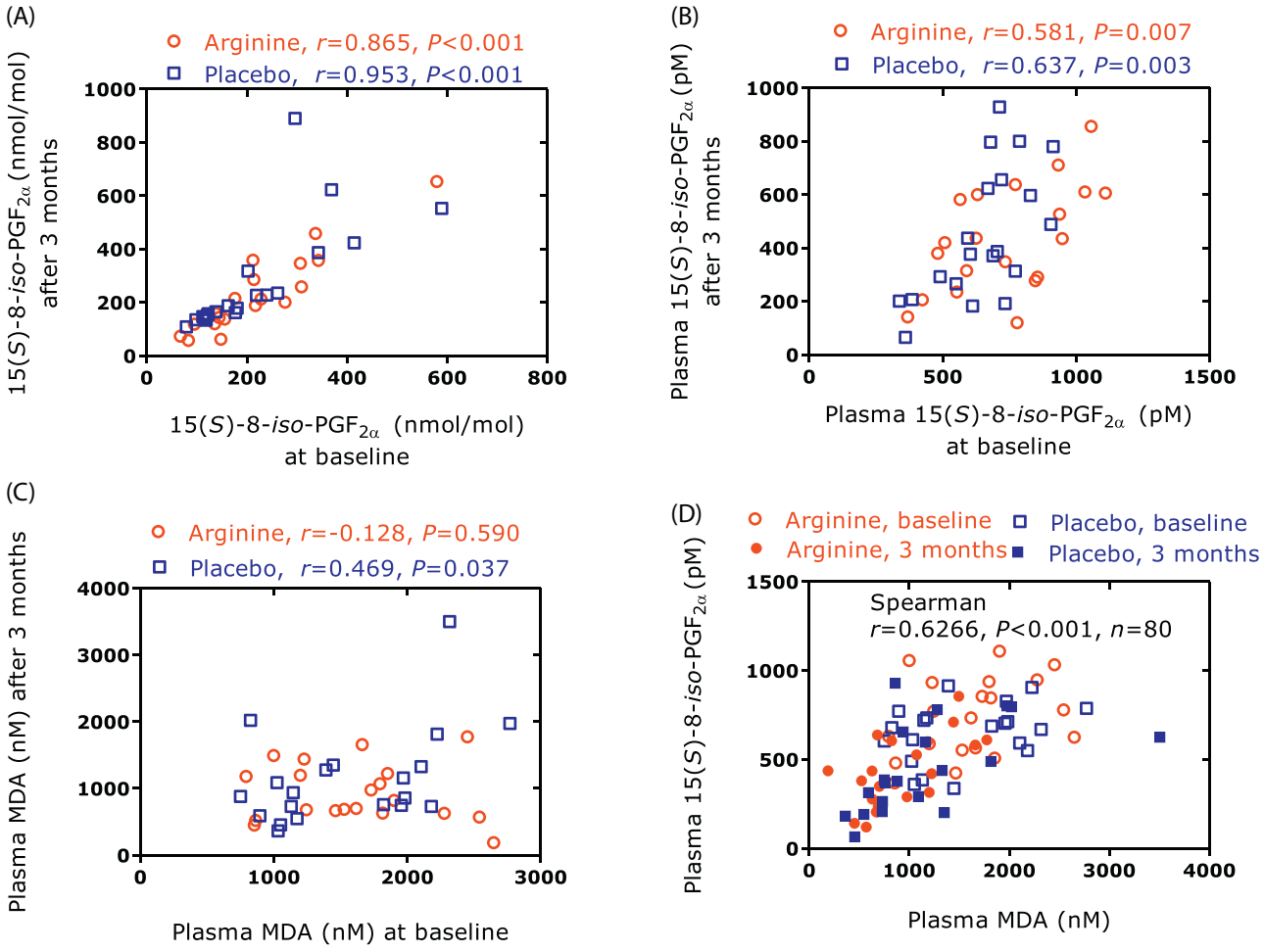


Fig. 9. Spearman correlation between MDA (in plasma) and 15(*S*)-8-*iso*-$PGF_{2\alpha}$ (in plasma and urine) in the PAOD study.

case of MDA we observed a single derivatization product. We have no evidence for the formation of a PFB ether derivative such as $C_6F_5CH_2$—O—CH=CH—CHO or $C_6F_5CH_2$—O—CH=C($C_6F_5CH_2$)—CHO which could be formed by the nucleophilic attack of the enolate anion of MDA (i.e., OHC—CH=CH—O^-) on PFB-Br. Being a CH-acidic compound, the nucleophilic attack of the carbanion of MDA, i.e., OHC—HC^-—CHO and even of OHC—($C_6F_5CH_2$)C^-—CHO rather than of the enolate anion of MDA seems to be favored despite the considerable steric hindrance. The examples of nitrite and MDA indicate that formation of stable C—N- and C—C-bonds seems to be the decisive factor in reactions of anions with PFB-Br in aqueous acetone.

ECNICI of ($C_6F_5CH_2$)$_2$C(CHO)$_2$ (MW, 432) with methane as the reagent/buffer gas is likely to easily produce the anion $C_6F_5CH_2$—C^-(CHO)$_2$ with *m*/*z* 251 in high abundance. This is caused by the large number of strongly electron-capturing F atoms, the neutral loss of the very stable PFB radical, an excellent leaving group, and is due to the acidity of the remaining second H atom of the central carbon atom in $C_6F_5CH_2$—CH(CHO)$_2$. CID of

$C_6F_5CH_2$—$C^-(CDO)_2$ at *m/z* 253 generates several intense product ions which contain both D atoms in each carbonyl functional group of d_2-MDA. This finding and the double neutral loss of CO (28 Da) argue against the formation of an ether PFB derivative of MDA such as $C_6F_5CH_2$—O—CH=C($C_6F_5CH_2$)—CHO.

d_2-MDA of high isotopic purity at ^2H can be easily and quantitatively prepared by HCl-catalyzed hydrolysis of the commercially available synthetic standard [1,3-$^2H_2$]-1,1,3,3-tetraethoxypropane. Despite the small difference of 2 Da between the internal standard d_2-MDA and the endogenous MDA, i.e., d_0-MDA, the isotopic contribution of the two O atoms of d_0-MDA with the low natural abundance of the ^{18}O isotope of oxygen of 0.2% results in an extended linearity over several orders of magnitude and a simple calculation mode for MDA concentrations in biological samples. MDA as PFB derivative can be quantified in human plasma and urine samples by GC–MS/MS. GC–MS is feasible for reliable MDA measurement only in human plasma samples.

In plasma ultrafiltrate (e.g., cut off 10 kDa), we were not able to measure MDA above the blank level of about 3–5 nM, suggesting that MDA has a very high binding to plasma proteins. For this reason, PFB-Br derivatization of MDA was performed in native plasma samples without preceding protein removal. Because of the relatively mild conditions of MDA derivatization with PFB-Br used in the present method (60 min and 50 °C), measurement of MDA from human plasma or serum as PFB derivative is likely to be more specific and much less prone to artefactual generation/release of MDA from different MDA-containing species as it is likely to be the case in most TBARS assays which require high temperatures and strong acids such as sulfuric acid. Also, because PFB-Br derivatization of MDA is based on the acidity of the CH-bond of MDA, it is unlikely that PFB-Br reacts with other MDA-containing species adducted via the carbonyl groups, in contrast to other derivatization reagents which react with the aldehyde functionalities of MDA and other aldehydes.

Many pre-analytical factors such as type of the anticoagulant, hemolysis due to the blood sampling technique or for other reasons may compromise measurement of numerous compounds in blood, plasma or serum. In our method, monovettes that contain citrate for anticoagulation were found to comprise the highest amount of contaminating MDA, which is close to the lowest MDA concentrations we measured in the present study.

MDA is mainly excreted in the urine adducted to various amino acids, such as *N*-epsilon-(2-propenal) lysine [36], *N*-*a*-acetyl-(epsilon)-(2-propenal) lysine [37], *N*-(2-propenal) [38] and *N*-(2-propenal) ethanolamine [39]. Higher MDA urine levels (e.g., 200–800 nM) similar to those measured in the present studies were found by another group [40]. These contradictory findings demand further research to determine urinary levels of free MDA in a large cohort of healthy humans. It needs to be evaluated whether measurement of urinary MDA in its free form is indeed of minor importance, as MDA is extensively metabolized and only very low concentrations (<50 nM) of free MDA seem to be excreted in the urine.

4.2. Biomedical considerations regarding oxidative stress

MDA and other aldehydes such as 2-hydroxy-hexanal are considered to be formed exclusively by free radical-catalyzed oxidation of unsaturated fatty acids including arachidonic acid. Yet, MDA and 15(*S*)-8-*iso*-$PGF_{2\alpha}$, currently two of the most frequently measured biomarkers of oxidative stress [2], can also be formed enzymatically from non-esterified arachidonic acid. Thus, in vitro recombinant cyclooxygenase (COX) catalyzes concomitantly the formation of MDA and 15(*S*)-8-*iso*-$PGF_{2\alpha}$, which, moreover, is promoted by the antioxidant tripeptide glutathione (GSH) [23]. In vivo in humans, daily administration of 600 mg aspirin (acetylic salicylic acid), an inhibitor of COX activity, has been shown to inhibit concomitantly thromboxane and MDA formation in platelets [41]. By measuring MDA in plasma of ten healthy male subjects who received a single 600-mg aspirin dose we found that both plasma MDA concentration and urinary 15(*S*)-8-*iso*-$PGF_{2\alpha}$ decreased slightly one hour after drug ingestion as compared to placebo. This may suggest that MDA is formed in considerable amounts in other tissues. As discussed recently [23], 15(*S*)-8-*iso*-$PGF_{2\alpha}$ has been shown by many groups to be formed in vivo in humans by the COX activity. For these reasons, the utility of MDA and 15(*S*)-8-*iso*-$PGF_{2\alpha}$ as biomarkers of free radical-induced oxidative stress seems to be limited.

L-Arginine is considered to have antioxidant properties which are attributed to its alpha-amino group [42]. In the PAOD and CAD studies we observed decreases in the plasma concentrations of MDA and 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in both groups, i.e., in the Arginine and Placebo groups, yet without appreciable changes in the urinary excretion of 15(*S*)-8-*iso*-$PGF_{2\alpha}$. These observations suggest that circulating MDA and 15(*S*)-8-*iso*-$PGF_{2\alpha}$ may not be suitable biomarkers of lipid peroxidation under certain conditions. It is worth mentioning that the age of the samples of the Arginine and Placebo groups in the PAOD and CAD studies was quite different at the time of their analysis, because analysis was started after collection of the plasma and urine samples of the last pending patient(s). Thus, in the PAOD study the samples collected at the end of the study were 3 months younger than the samples collected at the start of the study. In the CAD study the difference in the sample age was even higher, i.e., on average 6 months. Given the well-known autoxidation of unsaturated fatty acids in plasma [43] to form 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and other F_2-isoprostanes in human plasma [44] and the different samples age at the time point of analysis, artefactual in vitro formation of MDA and 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in stored plasma samples is the most likely explanation for the lower MDA and 15(*S*)-8-*iso*-$PGF_{2\alpha}$ concentrations measured in the younger plasma samples of both studies. In contrast, artefactual formation of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in urine is much less likely due to its low content in arachidonic acid and other unsaturated fatty acids and lipids. These observations challenge the utility of plasma as a matrix to measure MDA and 15(*S*)-8-*iso*-$PGF_{2\alpha}$ as biomarkers of oxidative stress, with the storage time being an important factor contributing to the extent of their artefactual formation. The effect of this pre-analytical parameter could be minimized by analyzing study samples immediately after their collection, especially in long-term studies such as the CAD and PAOD studies reported in this article.

The effect of anticoagulants on the concentration of MDA in serum and plasma has been investigated by many groups by using different assays including HPLC with visible absorbance detection, yet the reported effects are contradictory [45,46]. Being antioxidants, EDTA and butylated hydroxytoluene (BHT) are in general considered essential to inhibit lipid oxidation. However, our results indicate that EDTA may even increase ex vivo synthesis of MDA. This is paradoxical, yet antioxidants such as GSH which comprises a highly reactive sulfhydryl (SH) group may enhance enzymatic formation of MDA and 15(*S*)-8-*iso*-$PGF_{2\alpha}$ [23]. In our GC–MS and GC–MS/MS methods, BHT did not interfere with the measurement of MDA in plasma, yet BHT (up to 200 μM) added to EDTA blood did not reduce the MDA concentration (data not shown). As BHT possesses low solubility in water, organic solvents such as ethanol and dimethyl sulfoxide (DMSO) may enhance artefactual MDA formation for instance by inducing haemolysis [21].

Reported data on a circadian rhythm of MDA and other commonly used biomarkers of oxidative stress in healthy and diseased humans are very scarce and non-convincing, especially when compared to typical circadian endogenous substances such as melatonin and cortisol [47]. In three healthy male subjects we did not find a circadian rhythm for plasma MDA. The concentration of

MDA in the plasma samples varied over the day and night periods by about 25% (data not shown). The diurnal variation of the excretion of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and other 8-*iso*-$PGF_{2\alpha}$ isomers was also of the same order in urine samples collected by another three healthy subjects (data not shown), confirming results reported by others for urinary 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and other F_2-isoprostanes [48]. It seems that there is no circadian rhythm for circulating MDA and urinary F_2-isoprostanes in humans, so that the timing of sample collection is not an appreciable concern.

4.3. Relationship between plasma MDA, nitrite and nitrate

In a previous study on healthy humans, we found that plasma nitrite (as measured by GC–MS [20]), a surrogate of endothelium-derived •NO, decreased after hyperoxia-induced oxidative stress as revealed by elevated plasma MDA concentrations measured by HPLC [49], suggesting an inverse relationship between MDA and nitrite in the human circulation. Although very preliminary, our present observations from the intermittent ischemia/reperfusion forearm study on a single healthy subject support our previous finding of an inverse relationship between MDA and nitrite in human circulation [49]. While enhancement of oxidative stress is generally assumed to be associated with decreased •NO bioavailability, our pilot study suggests that oxidative stress may even reduce •NO synthesis. However, because a considerable portion of MDA may also derive from enzyme-catalyzed reactions, for instance by cyclooxygenase in platelets and other tissues, the apparent decreases in •NO synthesis and bioavailability seen in our volunteer may also have resulted from an interaction between the cyclooxygenase and •NO synthase pathways.

5. Conclusions

Utilization of specific physicochemical properties of analytes from endogenous or exogenous origin in chemical analysis such as in chemical derivatization may greatly enhance the analytical performance in terms of specificity and sensitivity. We have utilized the remarkable acidity of the H atoms of the CH_2 group of MDA and derivatized free MDA in buffer, plasma and urine by PFB-Br in acetonic solution (1:4, v/v) and quantified MDA by GC–MS and GC–MS/MS using d_2-MDA as the internal standard. The method is rapid, accurate, precise, sensitive, free of interferences and, as demonstrated in the present work, useful in experimental and clinical studies in the area of oxidative stress. On the basis of the analytical performance of the GC–MS/MS method, which is by nature considerably more specific and less susceptible to interferences, this method seems to be the most powerful analytical approach currently available for MDA quantification in the area of oxidative stress research, specifically of lipid peroxidation. Our study shows that the concentration of circulating MDA in humans depends on blood sampling and time of storage of the plasma or serum samples. The lowest MDA concentrations (30–200 nM) were measured in freshly generated serum and heparinized plasma samples of healthy humans. Our studies suggest that circulating MDA should be measured in serum or heparinized plasma samples as soon as possible after their generation. This issue represents a major concern in long-lasting clinical studies in which samples are obtained within a considerable time interval. •NO bioavailability is decisively determined by the extent of oxidative stress, with elevated oxidative stress decreasing •NO bioavailability due to oxidation of •NO by free radicals such as superoxide. The inverse correlation found between circulating nitrite or nitrate and MDA may be due to effects exerted both by free radicals and by enzymatic pathways that generate •NO and MDA, but demands evaluation on a larger population. MDA and 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in the circulation derive from non-enzymatic and enzymatic sources. Taking proper pre-analytical measures and use of unequivocal analytical techniques are absolute requirements to understand the dual nature of MDA and 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and of oxidative stress.

References

[1] H. Draper, M. Hadley, Malondialdehyde determination as index of lipid peroxidation, Methods Enzymol. 186 (1990) 421–431.
[2] D. Giustarini, I. Dalle-Donne, D. Tsikas, R. Rossi, Oxidative stress and human diseases: origin, link, measurement, mechanisms, and biomarkers, Crit. Rev. Clin. Lab. Sci. 46 (2009) 241–281.
[3] J.M.C. Gutteridge, B. Halliwell, The measurement and mechanism of lipid peroxidation in biological systems, Trends Biochem. Sci. 15 (1990) 129–135.
[4] R.O. Sinnhuber, T.C. Yu, T.C. Yu, Characterization of the red pigment formed in the 2-thiobarbituric acid determination of oxidative rancidity, Food Res. 23 (1958) 626–633.
[5] K. Yagi, A simple fluorometric assay for lipoperoxide in blood plasma, Biochem. Med. 15 (1976) 212–216.
[6] K. Yoden, T. Iio, Determination of thiobarbituric acid-reactive substances in oxidized lipids by high-performance liquid chromatography with a postcolumn reaction system, Anal. Biochem. 182 (1989) 116–120.
[7] H. Esterbauer, F.J. Schaur, H. Zollner, Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes, Free Radic. Biol. Med. 11 (1991) 81–128.
[8] R.P. Bird, S.S. Hung, M. Hadley, H.H. Draper, Determination of malonaldehyde in biological materials by high-pressure liquid chromatography, Anal. Biochem. 128 (1983) 240–244.
[9] C.R. Wade, P.G. Jackson, A.M. Van Rij, Quantitation of malondialdehyde (MDA) in plasma, by ion-pairing reverse phase high performance liquid chromatography, Biochem. Med. 33 (1985) 291–296.
[10] L.W. Yu, L. Latriano, S. Duncan, R.A. Hartwick, G. Witz, High-performance liquid chromatography analysis of the thiobarbituric acid adducts of malonaldehyde and *trans*,*trans*-muconaldehyde, Anal. Biochem. 156 (1986) 326–333.
[11] H. Esterbauer, K.H. Cheeseman, M.U. Dianzani, G. Poli, T.F. Slater, Separation and characterization of the aldehydic products of lipid peroxidation stimulated by ADP-Fe^{2+} in rat liver microsomes, Biochem. J. 208 (1982) 129–140.
[12] G.A. Cordis, D.K. Das, W. Riedel, High-performance liquid chromatographic peak identification of 2,4-dinitrophenylhydrazine derivatives of lipid peroxidation aldehydes by photodiode array detection, J. Chromatogr. A 798 (1998) 117–123.
[13] Y. Deng, F. Boomsma, P.H. Yu, Deamination of methylamine and aminoacetone increases aldehydes and oxidative stress in rats, Life Sci. 63 (1998) 2049–2058.
[14] J.-P. Steghens, A.L. van Kappel, I. Denis, C. Collombel, Diaminonaphtalene, a new highly specific reagent for HPLC-UV measurement of total and free malondialdehyde in human plasma or serum, Free Radic. Biol. Med. 31 (2001) 242–249.
[15] K. Fujioka, T. Shibamoto, Improved malonaldehyde assay using headspace solid-phase microextraction and its application to the measurement of the antioxidant activity of phytochemicals, J. Agric. Food Chem. 53 (2005) 4708–4713.
[16] G. Cighetti, S. Debiasi, R. Paroni, P. Allevi, Free and total malondialdehyde assessment in biological matrices by gas chromatography–mass spectrometry: what is needed for an accurate detection, Anal. Biochem. 266 (1999) 222–229.
[17] P. Maboudou, D. Mathieu, H. Bachelet, J.F. Wiart, M. Lhermitte, Detection of oxidative stress. Interest of GC-MS for malondialdehyde and formaldehyde monitoring, Biomed. Chromatogr. 16 (2002) 199–202.
[18] M. Beljean-Leymarie, E. Bruna, Subpicogram determination of malondialdehyde by gas–liquid chromatography with nitrogen phosphorus detector. I. Standardization, Anal. Biochem. 173 (1988) 174–184.
[19] H.C. Yeo, H.J. Helbock, D.W. Chyu, B.N. Ames, Assay of malondialdehyde in biological fluids by gas chromatography–mass spectrometry, Anal. Biochem. 220 (1994) 391–396.
[20] D. Tsikas, Simultaneous derivatization and quantification of the nitric oxide metabolites nitrite and nitrate in biological fluids by gas chromatography/mass spectrometry, Anal. Chem. 72 (2000) 4064–4072.
[21] U. Dreissigacker, M.T. Suchy, N. Maassen, D. Tsikas, Human plasma concentrations of malondialdehyde (MDA) and the F_2-isoprostane 15(*S*)-8-*iso*-$PGF_{2\alpha}$ may be markedly compromised by hemolysis: evidence by GC–MS/MS and potential analytical and biological ramifications, Clin. Biochem. 43 (2010) 159–167.
[22] D. Tsikas, J.Y. Schneider, J.C. Frölich, Antihypertensive inorganic nitrate and nitrite: what is the underlying mechanism? Hypertension 62 (2013) e6.
[23] D. Tsikas, M.T. Suchy, J. Niemann, P. Tossios, Y. Schneider, S. Rothmann, F.M. Gutzki, J.C. Frölich, D.O. Stichtenoth, Glutathione promotes prostaglandin H synthase (cyclooxygenase)-dependent formation of malondialdehyde and 15(*S*)-8-*iso*-prostaglandin $F_{2\alpha}$, FEBS Lett. 586 (2012) 3723–3730.
[24] P. Filipe, J. Haigle, J. Freitas, A. Fernandes, J.C. Mazière, C. Mazière, R. Santus, P. Morlière, Anti- and pro-oxidant effects of urate in copper-induced low-density lipoprotein oxidation, Eur. J. Biochem. 269 (2002) 5474–5483.

[25] D. Tsikas, A proposal for comparing methods of quantitative analysis of endogenous compounds in biological systems by using the relative lower limit of quantification (rLLOQ), J. Chromatogr. B 877 (2009) 2241–2251.
[26] D. Tsikas, E. Schwedhelm, M.T. Suchy, J. Niemann, F.M. Gutzki, V.J. Erpenbeck, J.M. Hohlfeld, A. Surdacki, J.C. Frölich, Divergence in urinary 8-*iso*-PGF(2alpha) (IPF(2alpha)-III 15-F(2*t*)-IsoP) levels from gas chromatography–tandem mass spectrometry quantification after thin-layer chromatography and immunoaffinity column chromatography reveals heterogeneity of 8-*iso*-PGF(2alpha). Possible methodological, mechanistic and clinical implications, J. Chromatogr. B 794 (2003) 237–255.
[27] D. Tsikas, A. Wolf, A. Mitschke, F.M. Gutzki, W. Will, M. Bader, GC–MS determination of creatinine in human biological fluids as pentafluorobenzyl derivative in clinical studies and biomonitoring: inter-laboratory comparison in urine with Jaffe, HPLC and enzymatic assays, J. Chromatogr. B 878 (2010) 2582–2592.
[28] J.Y. Schneider, S. Rothmann, F. Schröder, J. Langen, T. Lücke, F. Mariotti, J.F. Huneau, J.C. Frölich, D. Tsikas, Effects of chronic oral L-arginine administration on the L-arginine/NO pathway in patients with peripheral arterial occlusive disease or coronary artery disease: L-arginine prevents renal loss of nitrite, the major NO reservoir, Amino Acids 47 (2015) 1961–1974.
[29] J.Y. Lachmuth, The APAV Pilot Study. L-Arginine in Patients with Peripheral Arterial Occlusive Disease. PhD Thesis, Hannover Medical School, Hannover, 2007 http://d-nb.info/991618793/34.
[30] S. Rothmann, Pilot Study on the Effects of L-Arginine in Patients with Coronary Artery Disease after Angioplasty and Method Development for the Determination of DMA in Plasma as PFB Derivative. PhD Thesis, Hannover Medical School, Hannover, 2009 http://d-nb.info/1007632712/34.
[31] J.M. Bland, D.G. Altman, Statistical methods for assessing agreement between two methods of clinical measurement, Lancet 1 (1986) 307–310.
[32] D.O. Stichtenoth, V. Marhauer, D. Tsikas, F.M. Gutzki, J.C. Frölich, Effects of specific COX-2-inhibition on renin release and renal and systemic prostanoid synthesis in healthy volunteers, Kidney Int. 68 (2005) 2197–2207.
[33] K. Chobanyan, A. Mitschke, F.M. Gutzki, D.O. Stichtenoth, D. Tsikas, 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, J. Chromatogr. B 851 (2007) 240–249.
[34] R. Hüttel, Über malondialdehyd. 1. Mitteilung, Ber. Detsch. Chem. Ges. 74 (1941) 1825–1829.
[35] M.M. Osman, The acidity of malondialdehyde and the stability of its complexes with nickel(II) and copper(II), Helv. Chim. Acta 55 (1972) 239–244.
[36] H.H. Draper, M. Hadley, L. Lissemore, N.M. Liang, P.D. Cole, Identification of *N*-epsilon-(2-propenal) lysine as a major urinary metabolite of malondialdehyde, Lipids 23 (1988) 626–628.
[37] L.G. McGirr, M. Hadley, H.H. Draper, Identification of *N* alpha-acetyl-epsilon-(2-propenal) lysine as a urinary metabolite of malondialdehyde, J. Biol. Chem. 260 (1985) 15427–15431.
[38] M. Hadley, H.H. Draper, Identification of *N*-(2-propenal) serine as a urinary metabolite of malondialdehyde, FASEB J. 2 (1988) 138–140.
[39] M. Hadley, H.H. Draper, Identification of *N*-(2-propenal) ethanolamine as a urinary metabolite of malondialdehyde, Free Radic. Biol. Med. 6 (1989) 49–52.
[40] M. Tomita, T. Okuyama, Y. Hatta, S. Kawaj, Determination of free malonaldehyde by gas chromatography with an electron-capture detector, J. Chromatogr. 526 (1990) 174–179.
[41] A. Szczeklik, R.J. Gryglewski, L. Grodzińska, J. Musiał, M. Serwońska, E. Marcinkiewicz, Platelet aggregability, thromboxane A_2 and malondialdehyde formation following administration of aspirin to man, Thrombosis Res. 15 (1979) 405–413.
[42] S. Wallner, A. Hermetter, B. Mayer, T.C. Wascher, The alpha-amino group of L-arginine mediates its antioxidant effect, Eur. J. Clin. Invest. 31 (2001) 98–102.
[43] J.D. Evans, N.L. Oleksyshyn, J.M. Waldron, Autoxidation of polyunsaturated fatty acids in human plasma, J. Appl. Physiol. 9 (1956) 301–303.
[44] E. Schwedhelm, R.A. Benndorf, R.H. Böger, D. Tsikas, Mass spectrometric analysis of F2-isoprostanes: markers and mediators in human disease, Curr. Pharmac. Anal. 3 (2007) 39–51.
[45] W.G. Siems, H. Zollner, T. Grune, H. Esterbauer, Metabolic fate of 4-hydroxynonenal in hepatocytes: 1,4-dihydroxynonene is not the main product, J. Lipid Res. 38 (1997) 612–622.
[46] J. Suttnar, L. Masova, J.E. Dyr, Influence of citrate and EDTA anticoagulants on plasma malondialdehyde concentrations estimated by high-performance liquid chromatography, J. Chromatogr. B 751 (2001) 193–197.
[47] E.L. Kanabrocki, D. Murray, R.C. Hermida, G.S. Scott, W.F. Bremner, M.D. Ryan, D.E. Ayala, J.L. Third, P. Shirazi, B.A. Nemchausky, D.C. Hooper, Circadian variation in oxidative stress markers in healthy and type II diabetic men, Chronobiol. Int. 19 (2002) 423–439.
[48] J. Helmersson, S. Basu, F2-isoprostane excretion rate and diurnal variation in human urine, Prostaglandins Leukotrienes Essent. Fatty Acids 61 (1999) 203–205.
[49] D. Modun, M. Krnic, J. Vukovic, V. Kokic, L. Kukoc-Modun, D. Tsikas, Z. Dujic, Plasma nitrite concentration decreases after hyperoxia-induced oxidative stress in healthy humans, Clin. Physiol. Funct. Imaging 32 (2012) 404–408.