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_2α and nitric oxide (**NO)

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A B S T R A C T

Malondialdehyde (MDA, CH_3(CH=O)_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_3}]MDA (d_3-MDA, CH_3(CH=O)D) are derivatized in aqueous acetone (400 μL) with 
pentafluorobenzyl (PFB) bromide (10 μL). The reaction products were identified as C(PFB)_2(CH)=O (molecular weight, 432) and C(PFB)_2(CD)=O (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_3-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_3-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_2α and nitric oxide (**NO).

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1. Introduction

Malondialdehyde (MDA, OHC−CH=O; see Scheme 1) is an 
end-product of enzyme- and free radical-catalyzed lipid peroxida-
tion of polyunsaturated fatty acids including arachidonic acid 
[1,2]. Circulating MDA is one of the commonly and widely used
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-dinitrophenhydrazine (DNPH) [11–13] and 2,4-diaminonaphthalene (DAN) [14]. In GC–MS methods, methylhydrazine [15], phenylhydrazine [16], 2,4-dinitrophenhydrazine [17], 2-hydradino-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-cromo-2,3,4,5,6-pentafluorotoluene is a versatile derivatization reagent both for organic and inorganic anions including nitrate 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–CH2–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 acetonitrile–water solutions of neutral pH to form the strongly electron-capturing MDA–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 didemutero-MDA (ODC–CH2–CHO, d0–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. [1,3–2H2]-1,1,3,3-Tetraethoxypropane (declared as minimum 95% atom %2H) was from Cambridge Isotope Laboratories (Andover, MA, USA). Acetone (supra pure) 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 (d0-MDA) and [1,3–2H2]–MDA (d2-MDA) stock solutions were prepared from 1,1,3,3-tetraethoxypropane and [1,3–2H2]–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 Spector 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 d0–MDA and d2–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 d0–MDA and d2–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; PFBBr 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 0.5 to 2 μM d5-MDA (11 concentration points) using d3-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]:

\[ \text{Rec} = \frac{[C_{\text{M}} - C_{0}]}{C_{+}} \times 100 \]  

(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 GC–MS/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 Macherrey-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 kPa) were used as the carrier and the reagent gases for ECNCl, 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/m2) 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 Hanover 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 F2-isoprostane 15(S)-8-iso-prostaglandin F2α [15(S)-8-iso-PGF2α] 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-PGF2α excretion which is reported in μg 15(S)-8-iso-PGF2α 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 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 we included male and female patients aged 43–76 years who had undergone angioplasty. In the PAOD study we included Caucasian male and female patients older than 40 years with a peripheral arterial occlusive disease at Fontaine stage IIb and who had undergone cardiological 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 Hanover Medical School. Written informed consent was obtained from each patient.

CAD and PAOD patients received two NaHCO3-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 d₀-MDA (A) and d₂-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-d₀-MDA-PFB (A; molecular mass, 432) and PFB-d₂-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-PGF₂α in 1-mL aliquots of urine and free plus esterified 15(S)-8-iso-PGF₂α 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-PGF₂α excretion which is reported in nmol 15(S)-8-iso-PGF₂α 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 d2-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 d0-MDA and m/z 253 for d2-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-NO2, which emerged from the same GC column at 3.5 min under the same GC conditions, suggest that the reaction products of d0-MDA and d2-MDA with PFB-Br carry two PFB moieties, i.e., they are PFB-d0-MDA-PFB and PFB-d2-MDA-PFB, respectively. The lack of the molecular radical anions [M]+ at m/z 432 and m/z 434 in the ECNICI mass spectra of PFB-d0-MDA-PFB and PFB-d2-MDA-PFB, respectively, suggests that their ECNICI is accompanied by loss each of a PFB radical (PFB•, 181 Da) from PFB-d0-MDA-PFB and PFB-d2-MDA-PFB. Thus, the intense ions m/z 251 and m/z 253 are due to [PFB-d0-MDA]- and [PFB-d2-MDA]−, respectively (see Supplement). The difference of 2 Th indicates that the aldehydic H/D atoms are not lost during ECNICI of PFB-d0-MDA-PFB and PFB-d2-MDA-PFB.

The ECNICI production ion mass spectra generated by CID of m/z 251 for d0-MDA and m/z 253 of d2-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 d0-MDA m/z 253 of d2-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 [C6F5]−.

3.2. Modes of quantification

The multiple product ions described above indicate that the CID of m/z 251 for d0-MDA and m/z 253 for d2-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 d0-MDA and m/z 253 d2-MDA. In GC–MS/MS, quantification was carried out by SRM of the mass transition m/z 251 → m/z 175 for d0-MDA and of the mass transition m/z 253 → m/z 177 for d2-MDA. The peak area was calculated and considered in quantitative analyses.

Separate derivatization of three 100-μL aliquots of a 1-μM d2-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 d0-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 d0-MDA and d2-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., [d0-MDA]) in biological fluids was performed by multiplying the known concentration of the internal standard (i.e., [d2-MDA]) added to the sample by the respective PAR measured either in the SIM or SRM mode, i.e., [d0-MDA] = [d2-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 acetonitrile 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 a 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 d₀-MDA (A) and m/z 253 for d₂-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 d₀-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.
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 d2-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 d2-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 d2-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

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**Scheme 2.** Proposed chemical structures of and CID mechanisms for the product ions observed in product ion mass spectra of d0-MDA (A) and d2-MDA (B) upon CID of the precursor ions at m/z 251 and m/z 253, respectively (Fig. 2), generated by ECNICI of PFB-d0-MDA-PFB and PFB-d2-MDA-PFB (Fig. 1).
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 d₂-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.

<table>
<thead>
<tr>
<th>Day</th>
<th>Added (nM)</th>
<th>Measured (mean ± SD, nM)</th>
<th>Recovery (%)</th>
<th>Imprecision (RSD, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GC–MS</td>
<td>GC–MS/MS</td>
<td>GC–MS</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>260 ± 37</td>
<td>272 ± 31</td>
<td>N.A.</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>187 ± 4</td>
<td>200 ± 9</td>
<td>N.A.</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>250 ± 9</td>
<td>223 ± 20</td>
<td>N.A.</td>
</tr>
<tr>
<td>4</td>
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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 (rLLOQ) [25]. The value of rLLOQ is calculated by Formula (F2), i.e., by dividing the value of the lower limit of quantification GLOQ, usually the lowest added analyte concentration to the matrix, by the experimentally measured basal analyte concentration value of C0 and by multiplying the observed molar ratio by 100:

$$rLLOQ = \left( \frac{C_{LOQ}}{C_0} \right) \times 100$$ (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 rLLOQ expresses the percentage fraction of the analyte which, upon addition to the biological sample that contains this analyte at the basal concentration C0, 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 rLLOQ value of the GC–MS/MS method for MDA in plasma is calculated to be 80 nM.

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 d2-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 1.00, indicating complete recovery of MDA added to the urine sample examined. Imprecision (RSD) ranged between 0.7% and 1.1% in GC–MS and between 0.1% and 1.7% 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 d2-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 d2-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

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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 d21-MDA as the 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 d21-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. 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 d21-MDA as internal standard. The results are shown as mean ± SD.
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$_{2\alpha}$
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-isopGF2α as well as urinary 15(S)-8-isopGF2α are summarized in Table 3. In both groups, significantly lower concentrations of circulating MDA and 15(S)-8-isopGF2α, but not of urinary 15(S)-8-isopGF2α, were measured after 3 months (PAOD study) and 6 months (CAD study). We found considerable correlations for MDA and 15(S)-8-isopGF2α 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-isopGF2α correlated with MDA as well (Fig. 9D). In contrast, circulating and urinary 15(S)-8-isopGF2α did not correlate each with other (data not shown).

4. Discussion

4.1. Analytical considerations

Malondialdehyde (MDA, OHC—CH₂—CHO) is a CH-acidic dicarboxylic compound, a protonic acid in aqueous solution, even more acid (pKₐ, 4.46) [34, 35] than aliphatic carboxylic acids including acetic acid (pKₐ, 4.7). Compared to the inorganic nitrous acid (O=–N=O–H; pKₐ, 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 CH₂ group of MDA are substituted by two PFB moieties via nucleophilic substitution reactions (presumably S₂/C of the MDA carbanion to form the 2,2′-dipentafluorobenzyl-1,3-propanedial, i.e., C(C₆F₅CH₂)$_{2}$–(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 nitrate 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
case of MDA we observed a single derivatization product. We have no evidence for the formation of a PFB ether derivative such as C\textsubscript{6}F\textsubscript{5}CH\textsubscript{2}O\textsubscript{2}CH\textsubscript{2}OCH\textsubscript{2}CH\textsubscript{2}CHO (C\textsubscript{6}F\textsubscript{5}CH\textsubscript{2}OCH\textsubscript{2}CH\textsubscript{2}C\textsubscript{2}H\textsubscript{4}CHO) which could be formed by the nucleophilic attack of the enolate anion of MDA (i.e., OH\textsubscript{2}C\textsubscript{6}F\textsubscript{5}CH\textsubscript{2}OCH\textsubscript{2}CH\textsubscript{2}CHO) on PFB-Br. Being a CH\textsubscript{2}-acidic compound, the nucleophilic attack of the carbanion of MDA, i.e., OH\textsubscript{2}C\textsubscript{6}F\textsubscript{5}CH\textsubscript{2}OCH\textsubscript{2}CHO and even of OH\textsubscript{2}C\textsubscript{6}F\textsubscript{5}CH\textsubscript{2}OCH\textsubscript{2}CHO is less hindered than 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\textsubscript{6}F\textsubscript{5}CH\textsubscript{2})\textsubscript{2}C(CHO)\textsubscript{2} (MW, 432) with methane as the reagent/buffer gas is likely to easily produce the anion (C\textsubscript{6}F\textsubscript{5}CH\textsubscript{2})\textsubscript{2}C(CHO)\textsubscript{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\textsubscript{6}F\textsubscript{5}CH\textsubscript{2})\textsubscript{2}C(CHO)\textsubscript{2}. CID of

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**Table 3**

Effects of chronic oral L-arginine administration on MDA in plasma and 15(S)-8-iso-PGF\textsubscript{2a} 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).

<table>
<thead>
<tr>
<th>Study</th>
<th>Group</th>
<th>MDA T0</th>
<th>T6</th>
<th>P</th>
<th>15(S)-8-iso-PGF\textsubscript{2a} T0</th>
<th>T6</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAD study</td>
<td>Plasma</td>
<td>Arginine</td>
<td>1284 [1192–1515]</td>
<td>1114 [990–1261]</td>
<td>0.001</td>
<td>2130 [1870–2620]</td>
<td>1350 [1030–1520]</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>Arginine</td>
<td>n.d.</td>
<td>n.d.</td>
<td>49.4 [43.6–68.1]</td>
<td>44.8 [41.9–60.4]</td>
<td>0.819</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>n.d.</td>
<td>n.d.</td>
<td>39.8 [38.5–55.0]</td>
<td>35.2 [32.0–67.2]</td>
<td>0.452</td>
<td></td>
</tr>
</tbody>
</table>

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

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**Fig. 9** Spearman correlation between MDA (in plasma) and 15(S)-8-iso-PGF\textsubscript{2a} (in plasma and urine) in the PAOD study.
C6F5CH2–C(CDO)2 at m/z 253 generates several intense product ions which contain both D atoms in each carbonyl functional group of d2-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 C6F5CH2-O-CH(C6F5CH2)=CHO.

d2-MDA of high isotopic purity at 2H can be easily and quantitatively prepared by HCl-catalyzed hydrolysis of the commercially available synthetic standard [1,3-2H2]-1,1,3,3-tetraethoxypropene. Despite the small difference of 2 Da between the internal standard d3-MDA and the endogenous MDA, i.e., d0-MDA, the isotopic contribution of the two O atoms of d0-MDA with the low natural abundance of the 18O 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 added to various amino acids, such as N-epsilon-(2-propenyl) lysine [36], N-alpha-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-isopGF2α, 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-isopGF2α, 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-isopGF2α 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-isopGF2α 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-isopGF2α as biomarkers of free radical-induced oxidative stress seems to be limited.

L-Ariginine 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-isopGF2α in both groups, i.e., in the Arginine and Placebo groups, yet without appreciable changes in the urinary excretion of 15(S)-8-isopGF2α. These observations suggest that circulating MDA and 15(S)-8-isopGF2α 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-isopGF2α and other F2-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-isopGF2α in stored plasma samples is the most likely explanation for the lower MDA and 15(S)-8-isopGF2α concentrations measured in the younger plasma samples of both studies. In contrast, artefactual formation of 15(S)-8-isopGF2α 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-isopGF2α 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 sulphhydryl (SH) group may enhance enzymatic formation of MDA and 15(S)-8-isopGF2α [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-isopGF(2α) and other 8-isopGF(2α) 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-isopGF(2α) and other F₂-isoprostanes [48]. It seems that there is no circadian rhythm for circulating MDA and urinary F₂-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 CH₂ group of MDA and derivatized free MDA in buffer, plasma and urine by PFBr-Br in acetonitrile solution (1:4, v/v) and quantified MDA by GC–MS and GC–MS/MS using d₂-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-isopGF(2α) 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-isopGF(2α) and of oxidative stress.

References


