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Measurement of free and bound malondialdehyde in plasma by high-performance liquid chromatography as the 2,4-dinitrophenylhydrazine derivative

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Abstract

We established a method for the detection of free and total (free and bound) malondialdehyde (MDA) in human plasma samples after derivatisation with 2,4-dinitrophenylhydrazine (DNPH). Free MDA was prepared by perchloric acid deproteinisation whereas an alkaline hydrolysation step for 30 min at 60°C was introduced prior to protein precipitation for the determination of total MDA. Derivatisation was accomplished in 10 min at room temperature subsequently chromatographed by HPLC on a reversed-phase 3 μm C_{18} column with UV detection (310 nm). The detection limit was 25 pmol/ml for free and 0.3 nmol/ml for total MDA. The recovery of MDA added to different human plasma samples was 93.6% ($n=11$; RSD 7.1%) for the hydrolysation procedure. In samples from 12 healthy volunteers who underwent a hypoxic treatment (13% O_2 for 6 h) we estimated a baseline value of total MDA of 2.16 nmol/ml (SD 0.29) (ambient air) with a significant increase to 2.92 (nmol/ml, SD 0.57; $P=0.01$) after the end of this physiological oxidative stress challenge. Plasma values of free MDA in these samples were close to our detection limit. The presented technique can easily performed with an isocratic HPLC apparatus and provides highly specific results for MDA as do sophisticated GC–MS methods.

Keywords: Derivatisation, LC; Malondialdehyde; 2,4-Dinitrophenylhydrazine

1. Introduction

The non-enzymatic, metabolically uncoupled lipid peroxidation plays a crucial role in the degeneration associated with ageing and in the pathogenesis of a number of diseases [1].

An important step in the degradation of cell membranes is the reaction of reactive oxygen species with the double-bonds of polyunsaturated fatty acids (PUFAs) to yield lipid hydroperoxides. On breakdown of such hydroperoxides a great variety of aldehydes can be formed. These aldehydes are relatively stable and at the same time biologically active, and therefore may be considered as ''cytotoxic second messengers'' [2,3].

Among numerous analytical approaches for the estimation of oxygen radical mediated damage in biological systems (for reviews see Refs. [4–6]), the determination of malondialdehyde, as one of the major aldehyde species, has been employed most

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frequently. In biological matrices malondialdehyde (MDA) exists both free and bound to SH and NH_2 groups of macromolecules, e.g., proteins and nucleic acids [2,7,8]. Only low amounts of free MDA are present in biological samples and its assay is often hampered by the detection limit of the used method [7,9,10]. Therefore, usually the total (free and bound) MDA is evaluated, most frequently by the thiobarbituric acid (TBA) test which has been the favoured assay for more than four decades because of its simple handling and sensitivity [11,12]. This is true despite the fact that the TBA test is intrinsically non-specific for MDA since various non-lipid-related materials are TBA positive, e.g., carbohydrates, several amino acids and deoxyribose, bile pigments, sialic acids as well as other decomposition products like alkanals, alkenals and alkadienals (for reviews see Refs. [2,5,13]). Furthermore, the TBA test shows very variable results when applied to human plasma samples [2,5,11,13–16] and it has been suggested that an additional high-performance liquid chromatography (HPLC) separation step may improve the assay [7,17–21]. But, as mentioned above, identical MDA–TBA adducts were yielded from different substances [5,13] only depending on the reaction conditions. In such a situation an additional HPLC separation step would be useless [5,22].

MDA and aldehydes in general can be determined by a variety of other methods like various fluorometric procedures (for reviews see Refs. [14,15]), by derivatisation with *N*-methyl-2-phenylindole [23,24], or by treatment with *O*-pentafluorobenzyl hydroxylamine for gas chromatographic mass spectrometric (GC–MS) analysis [3,25]. The reaction of the carbonylic compound with hydrazines to form stable derivatives is well known and has been used for GC–MS [8,26] or HPLC analysis, mainly for urine samples [9,15,27–30]. This derivatisation presents clear advantages such as: the hydrazone formation proceeds readily at room temperature under mild acid pH conditions; the hydrazones formed are unique for a given aldehyde and can therefore easily be separated by GC or HPLC to improve selectivity. Despite these benefits, the determination of free and bound MDA in blood with these technique was successfully practised only by the above cited GC–MS methods. With a HPLC method Kawai et al. [9] failed to detect MDA in human plasma.

Compared to the sophisticated GC–MS method we try to establish a more simple HPLC procedure. Therefore, we combined two defined sample preparation protocols and the derivatisation with 2,4-dinitrophenylhydrazine (DNPH) to measure both, free and total MDA in human plasma.

The method was applied to monitor the time course of plasma MDA concentrations in healthy male volunteers during and after a 6-h exposure to hypoxia.

2. Experimental

2.1. Reagents

Acetonitrile, methanol, sulfuric acid, hydrochloric acid, perchloric acid, acetaldehyde, formaldehyde, acetone and ethanol were purchased from Merck (Darmstadt, Germany). All reagents were of analytical grade unless stated otherwise.

Butylated hydroxy toluene (BHT; ICN, Eschwege, Germany) was prepared as a 2% (w/v) solution in ethanol. The DNPH reagent (2,4-dinitrophenylhydrazine containing 25% water, Acros Organics, New Jersey, NJ, USA) was prepared as a 5 m*M* solution in 2 *M* hydrochloric acid. MDA–hydrazone was prepared by reacting 30 ml of DNPH stock solution with 1 mmol of 1,1,3,3-tetraethoxypropane 95% (TEP; Aldrich, Steinheim, Germany), under stirring for 10 min. The hydrolysis of TEP in acidic solution and the following MDA–hydrazone formation proceed rapidly at room temperature. Due to the low solubility of the hydrazones in aqueous solution, MDA–hydrazone precipitates and was filtered and dried. A hydrazone stock solution of 1 mg/ml in methanol was prepared. Hydrazones of other carbonyl compounds were prepared analogously. These derivatives were used to test the selectivity of the chromatographic system.

2.2. Sample collection

Blood was collected by venipuncture into K-EDTA Monovettes (Sarstedt, Germany) from 12 healthy male volunteers (18–30 years, non-smokers). Blood samples were taken 1 h before, at 0, 2, 4, 6 h during exposure to hypoxia and at the time points 8,

12, 24 and 48 h (i.e., after termination of hypoxia at 6 h) [31]. After centrifugation (4000 *g* for 10 min at 4°C), plasma was immediately frozen on dry ice and stored at −80°C.

The hypoxic treatment was carried out by breathing an artificial Medical gas mixture containing 13% oxygen and 87% nitrogen through a air-tight face mask under strictly standardised and controlled study conditions. The primary objective of the study was to quantitate the influence of hypoxic conditions on the endogenous erythropoetin production. The study was conducted according to the principles of the Declaration of Helsinki and was approved by the local Ethics Committee and all volunteers gave their written, informed consent.

2.3. Preparation of standard curves and reference samples

MDA standard was prepared by dissolving 25 μl TEP in 100 ml of water to give a 1 m*M* stock solution. Working standard was prepared by hydrolysis of 1 ml TEP stock solution in 50 ml 1% sulfuric acid and incubation for 2 h at room temperature [32]. The resulting MDA standard of 20 nmol/ml was further diluted with 1% sulfuric acid to yield the final concentrations of 10, 5, 2.5, 1.25 and 0.625 nmol/ml to get the standard curve for the estimation of total MDA and to spike plasma samples for determination of the recovery.

Because TEP is readily hydrolysed to MDA during the protein precipitation step with perchloric acid (described in Section 2.4) the standard preparation for the analysis of free MDA can be performed in a similar manner by dilution of TEP stock solution with water instead of sulfuric acid to final concentrations of 1, 0.5, 0.25, 0.1 and 0.05 nmol/ml.

Preparation of reference samples was done with blood from untreated healthy male volunteers mixed with 15 ml of EDTA (Titriplex III) to yield a final concentration of 1.6 mg EDTA/ml blood and centrifuged for 10 min at 4000 *g* at room temperature. The received plasma was either spiked with 200 pmol/ml for free MDA or 2 nmol/ml for total MDA. Aliquots of native and spiked plasma were kept frozen at −80° until analysis.

Standard and reference samples were processed under the same conditions as described in Section 2.4.

2.4. Sample preparation

2.4.1. Free MDA

To 1 ml of plasma sample in a 2.0-ml Eppendorf cup 0.5 ml 12% (v/v) perchloric acid was added and the mixture vortexed to precipitate protein. After centrifugation at 14 000 *g* for 10 min, 1 ml of clear supernatant was transferred to a 10-ml glass tube with a conical glass stopper. A 0.1-ml volume of DNPH solution was then added, mixed and incubated for 10 min at room temperature. From this step onward samples need be protected from light. The aqueous phase was extracted twice with 4 ml of hexane by shaking the sample gently for 5 min. The organic extracts were combined, evaporated under a stream of nitrogen at 40°C in a heating block and reconstituted in 60 μl of mobile phase. A 40-μl volume was injected onto the HPLC system.

2.4.2. Total MDA

For alkaline hydrolysis of protein bound MDA 50 μl of 6 *M* aqueous sodium hydroxide were added to 0.25 ml of plasma in a 1.5-ml Eppendorf cup and the sample was incubated in a 60°C water bath for 30 min. The hydrolyzed sample was acidified with 0.125 ml of 35% (v/v) perchloric acid. After centrifugation at 14 000 *g*, 0.25 ml of supernatant was transferred to a 2-ml Eppendorf cup, mixed with 25 μl DNPH solution and incubated for 10 min. A 30-μl volume of the reaction mixture was directly injected onto the HPLC system or the samples were extracted twice with 1.2 ml of hexane as described for free MDA and the dried extract was reconstituted with 0.1 ml of mobile phase.

For each analytical run a reagent blank underwent the same procedure as the calibration standards and plasma samples.

2.5. HPLC analysis

Analytical HPLC separations were performed with a Model 655A-12 liquid chromatograph (Merck, Darmstadt, Germany), equipped with an autoinjector (655A-40) and a variable-wavelength UV detector (655A) operated at 310 nm (0.0025 AUFS) on a

125×3 mm Nucleosil C_{18} column of 3 μm particle size with integrated precolumn (SepServ, Berlin, Germany). The column was kept at 30°C in a column oven with a mobile phase flow-rate of 0.6 ml/min.

HPLC mobile phase was prepared by mixing 380 ml acetonitrile with 620 ml of distilled water, acidified with 0.2% (v/v) acetic acid and degassed under reduced pressure. The resulting back-pressure was $1.85 \cdot 10^4$ kPa. Chromatograms were registered and peak areas determined with a Merck D 2000 Chromato-Integrator. The peak purity analysis was performed on a Gynkotek chromatographic system (Gynkotek, Germering, Germany) with a diode array detector (UVD 340 S). The obtained UV spectra from 210 to 360 nm was processed by a specialised software (Chromeleon; V 4.20).

3. Results

3.1. HPLC separation of the DNPH derivatives

Different columns were tested (125×4.6 mm, 5 μm CN or C_{18} phase and 125×3 mm, 3 μm, C_8 and C_{18} phase). The best chromatographic separation was obtained on a 3 μm C_{18} RP column (Nucleosil; 125×3 mm; SepServ). A stable baseline separation of the MDA peak from interfering substances was achieved with a mobile phase of acetonitrile–distilled water (38:62, v/v) containing 0.2% (v/v) acetic acid. Although the separation was not sensitive to changes in temperature it is recommendable to keep the temperature constant with a column oven in order to obtain stable retention times. With a solvent flow-rate of 0.6 ml/min at a temperature of 30°C the resulting back pressure was $1.85 \cdot 10^4$ kPa. The retention time of the MDA–hydrazone averaged to 6.93 min (SD=0.07; RSD=1.01%) for 20 consecutive daily runs. One analytical run was finished within 15 min with a baseline separation from interfering peaks as shown in Fig. 1.

In Fig. 2 the chromatographic separation of the DNPH derivatives of other short-chain water-soluble carbonylic compounds namely formaldehyde, acetaldehyde and acetone is shown. Though the absorption maximum of the formaldehyde hydrazone is at 350 nm, this aldehyde gives a measurable signal at 310 nm in low concentrations even in a water blank (Fig. 2b). Furthermore a number of unknown peaks are detectable in a reagent blank or in water solubilized standards (Fig. 1a, Fig. 2b–d) compared to the synthesised hydrazones (e.g., MDA, Fig. 2a) where these peaks are totally absent. Therefore, it is absolutely necessary to introduce a reagent blank in the analytical procedure.

The standard curve gave a linear response for MDA concentrations and HPLC peak areas in the range from 0.625 nmol/ml to 20 nmol/ml for total MDA ($y=5688.4x+524.02$; $r=0.9995$, for y=peak area, x=MDA concentration) and from 0.05 nmol/ml to 1 nmol/ml for free MDA. The lower limit of detection was 8 pmol injected on column, corresponding to plasma concentrations of 0.3 nmol/ml for total and 0.025 nmol/ml for free MDA.

For the daily control of the HPLC system, we injected 20 μl of the MDA–hydrazone solution. The day-to-day precision of the corresponding peak area was 4.4% (RSD) for 20 consecutive daily runs.

3.2. Sample preparation for free MDA analysis

The derivatisation with DNPH proceeds readily under room temperature and we further optimised the sample preparation conditions in a way that we used all the chemicals in concentrations as low as possible. Strong acidic conditions can lead to a release of bound MDA from its protein binding so we precipitated proteins according to the method described by Carbonneau et al. [7] but with reduced perchloric acid concentration. We used 0.6 *M* perchloric acid as the final concentration of the sample instead of 1.8 *M* by Carbonneau et al. Furthermore, we reduced the concentration of the derivatisation reagent in order to minimise artificial signals from an excess of DNPH reagent. Referred to the used plasma volume we employed 1.1 μ*M* DNPH reagent to derivatise a 1-ml sample whereas Kawai et al. [9] and Bagchi et al. [29] employed 2.5 μ*M* and 6.3 μ*M* DNPH. The chromatographic resolution is shown in Fig. 1, left column. It is necessary to protect derivatised samples from light otherwise the signals are reduced to 20% of their initial magnitude within 1 h (data not shown).

Because of the expected small concentrations of free MDA in plasma samples extraction with hexane and enrichment of MDA was necessary. The re-

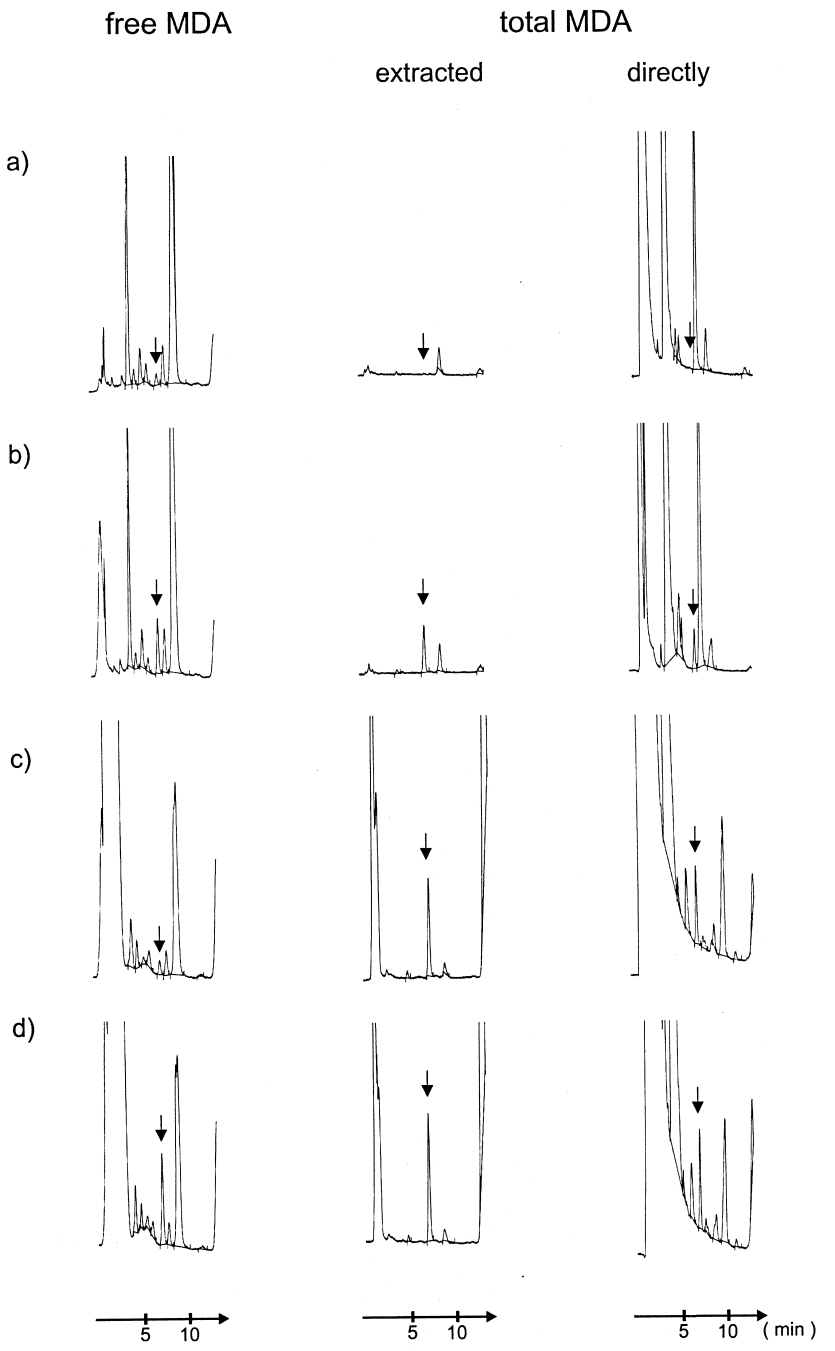

Fig. 1. Chromatographic separation of (from left) free MDA and total MDA (either extracted in hexane or injected directly). Arrow indicates MDA peak. (a) Reagent blank; (b) standard MDA (100 pmol/ml for free MDA, 2 nmol/ml for total MDA); (c) plasma sample (with approximately 25 pmol/ml for free MDA and 3.8 nmol/ml for total MDA) and (d) plasma sample spiked with MDA (200 pmol/ml for free MDA and 2 nmol/ml for total MDA).

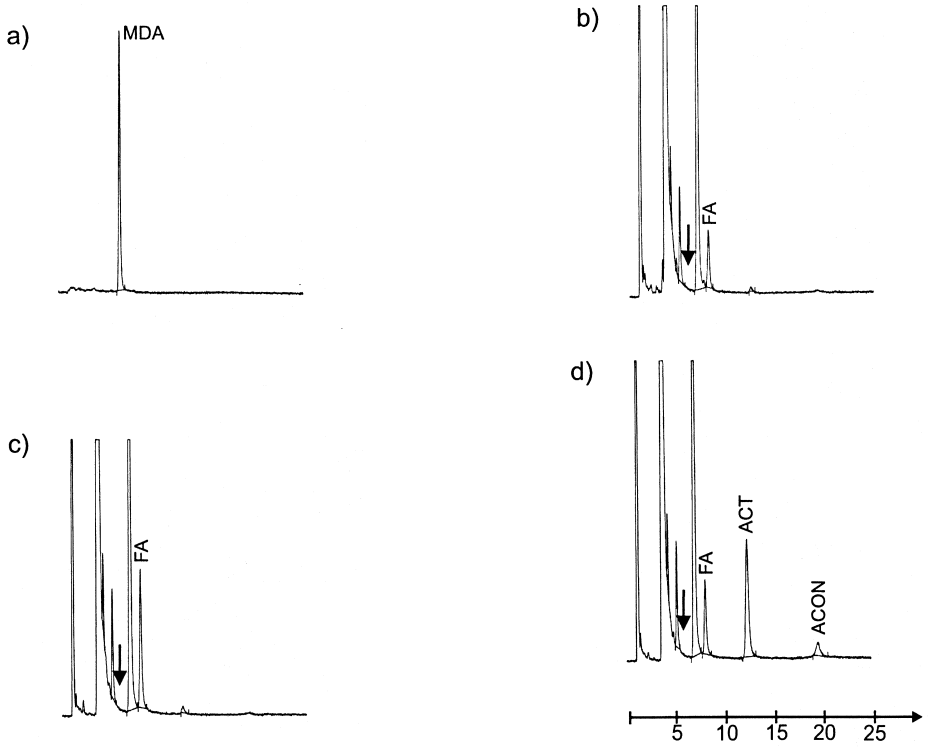

Fig. 2. Chromatographic separation of MDA and the short-chain carbonyls formaldehyde (FA), acetaldehyde (ACT) and acetone (ACON). (a) 170 pmol MDA was injected as the methanolic hydrazone solution. (b) Reagent blank (bidistilled water) or (c) formaldehyde, (d) acetaldehyde and acetone were dissolved in water and 250 μl of each was derivatised with 25 μl DNPH solution. A 30-μl volume was injected corresponding to 80 pmol formaldehyde and 800 pmol acetaldehyde and acetone. In the water blank there are measurable amounts of formaldehyde of approx. 4.5 nmol/ml. Other peaks are not identified. Arrows indicate the position of the MDA peak.

covery of 100 pmol/ml TEP added to plasma samples was determined as 98.2% ($n=6$; RSD 10.7%). Concentrations of free MDA in unspiked reference sample were below the lowest calibrator of the assay (50 pmol/ml). The signals observed approximately correspond to 25 pmol/ml (Fig. 1c). For reference samples spiked with 200 pmol/ml TEP, on average 220 pmol/ml (SD: 17.1; RSD: 7.4%) were measured (Fig. 1d). Similarly, in the samples obtained from the volunteers during the hypoxygenation study, free MDA concentrations were below the quantitation limit and no changes in the free MDA concentration due to oxygenation status could be registered.

3.3. Sample preparation for analysis of total MDA

With an alkaline incubation we hydrolyzed the protein bound MDA from their nucleophilic counterparts. We adopted the conditions used by Carbonneau et al. [7] who incubated the samples with a final concentration of 0.5 *M* NaOH at 60°C for 30 min. Because of variable results we enhanced the NaOH concentration to one molar. This resulted in an approximate 80% increase of the total MDA values and a much better reproducibility. With this NaOH concentration we further tested the influence of the incubation time. Comparing the relative amounts measured for three different plasma samples incubated for 15, 30 and 60 min (in four different runs), the average increase in peak area was 9.8% from 15 to 30 min and only 6.7% after further incubation up to 60 min. This indicates a gradual completion of the hydrolysis procedure. If we compared MDA standards measured before and after 30 min of alkaline incubation (60°C) a slight decrease in peak area (−13%, $n=4$) could be found. As a consequence of these results measurement of total MDA was per-

formed with an incubation of plasma samples with 1 *M* NaOH at 60°C for 30 min. The recovery of MDA added to plasma samples of different untreated healthy male volunteers (5 nmol/ml added 1 h before alkaline incubation) was determined as 93.6% ($n=11$; RSD 7.1%). With our reference samples we estimate the within-assay precision (RSD 7.8%, $n=8$) and the between-assay precision (RSD 9.8%, for 20 different runs).

3.3.1. Effect of the antioxidant BHT

To ensure that no lipid oxidation occurs during the assay, many authors have added the strong antioxidant BHT to the sample [8,15,18,20,33]. Therefore, we tested the effect of the commonly used 2% BHT solution in absolute ethanol by adding 10 μl of this solution to 1 ml of plasma prior to alkaline hydrolyzation. The addition of ethanol alone reduced the MDA peak (Fig. 3). The presence of BHT did not improve this situation. We could therefore see no advantage in the use of BHT as a presumptive antioxidant.

3.3.2. Effect of sample extraction into hexane

After the alkaline hydrolysis the following protein precipitation with perchloric acid yielded a clear supernatant which could be injected directly onto the HPLC system without further purification after the short derivatisation procedure. We compared the results obtained with 30 μl of the derivatised sample injected directly and the values recorded after additional hexane extraction of the remaining samples. With both techniques total MDA concentrations in plasma could be reliably quantitated. The additional extraction step of the derivatised supernatant into hexane before HPLC measurement gave cleaner chromatograms and a further enhanced sensitivity as shown in Fig. 1. The recovery of the hexane extraction procedure estimated from 15 different plasma samples was 92.3% (RSD 6.9%).

The diode array spectrum (210 to 360 nm) gave the same absorption curve for a plasma derivative and a MDA standard compared with the pure MDA–hydrazone. Standard and plasma samples were injected directly without extraction into hexane.

3.3.3. Plasma values under hypoxia and reoxygenation

The concentration–time course of total MDA in the plasma from the volunteers who underwent hypoxia was monitored using the procedure which included the additional extraction step (Fig. 4). The mean plasma value of total MDA at the beginning of hypoxic treatment was 2.16 nmol/ml ($n=12$; SD 0.29) and increased significantly to 2.92 nmol/ml (SD 0.57, $P=0.01$) 2 h after the end of the hypoxic period.

It is noteworthy that some subjects showed no

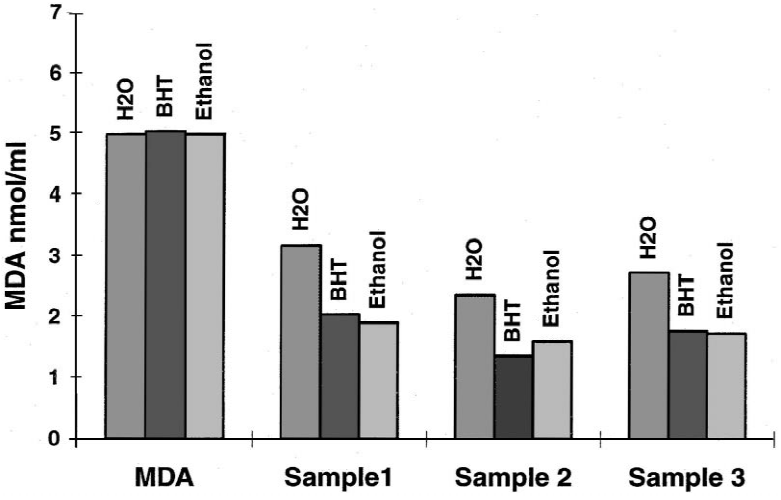

Fig. 3. Amount of MDA measured after adding 10 μl/ml of either water, the antioxidant butylated hydroxytoluene BHT (2% solution in ethanol) or pure ethanol to MDA standard and plasma samples from three different healthy male volunteers prior to incubation with sodium hydroxide for 30 min at 60°C.

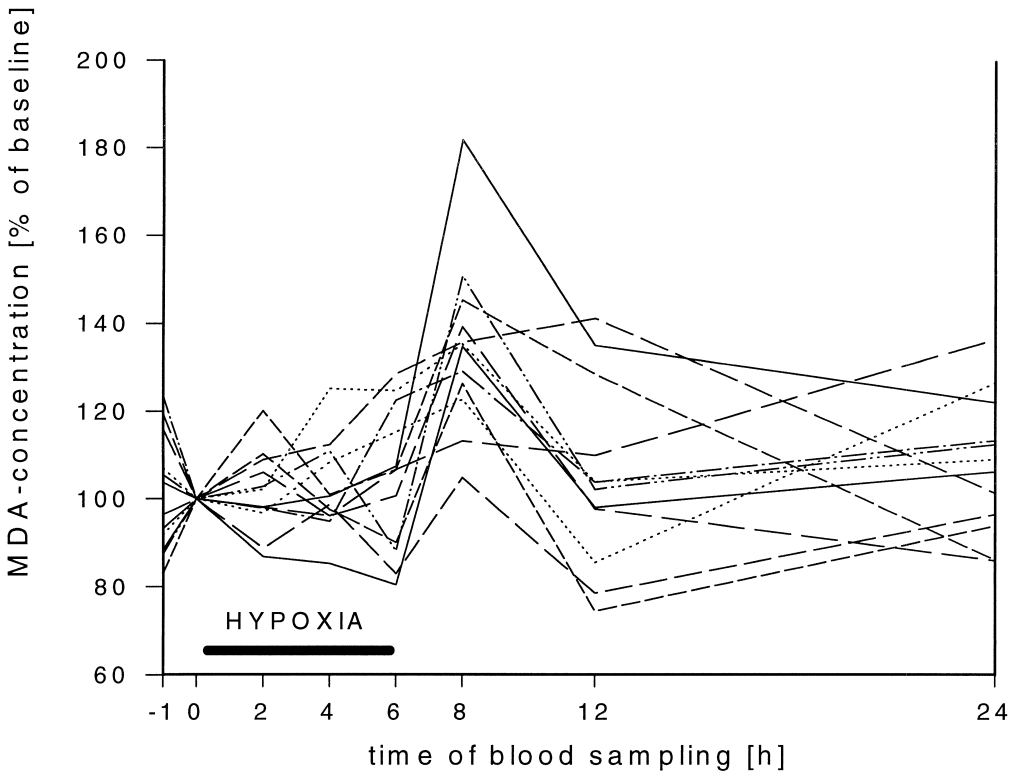

Fig. 4. Time course of plasma MDA values from 12 healthy male volunteers during a 6-h hypoxic treatment (13% oxygen in N_2) and during reoxygenation. Values expressed in % of baseline (value before exposure to hypoxia at 0 h) which was set at 100%.

MDA response during reoxygenation whereas others responded strongly, indicating a high interindividual variability of the physiological reaction to a short exposure to regular air.

The MDA–hydrazones were stable for more than 20 h at room temperature when protected from light and one analytical run was finished within 15 min so that batch analyses with HPLC could be performed. This finding is of importance for the routine use of any method for the determination of MDA.

4. Discussion

The estimation of MDA from samples with complex biological matrix like plasma is difficult. Even the use of sophisticated and complex methods like GC–MS does not guarantee reproducible results. Our objective was therefore to develop a method for MDA estimation with a high selectivity for MDA and being simple enough for routine determination. So we preferred HPLC with robust UV detection. The samples were derivatised with DNPH since this procedure proceeds rapidly under mild acid pH and temperature conditions and the resulting derivatives are unique for a given aldehyde. They are poorly soluble in aqueous solutions allowing a quantitative recovery by extraction with organic solvents. Furthermore, these derivatives are stable in the absence of light and could be separated by HPLC to get a specific signal for MDA. These are important advantages for analysing MDA content in samples with complex biological matrix in relation to the commonly used TBA assay. They enhance the certainty to measure true MDA as a main endproduct of lipid peroxidation.

In Table 1 we list a selection of recent papers where MDA values in plasma from naive human volunteers have been reported. Overall, these values range from 0.03 to 3.42 nmol/ml MDA corre-

Table 1
MDA or TBARS values reported for human plasma (values for free MDA in parentheses if a different sample preparation is used)

MDA (nmol/ml)	Specification of the assay (detection/fraction derivatised/extraction of derivatives)	Ref.
TBA test without HPLC		
3.42	FD/lipids and proteins/butanol extraction	[34]
2.51	FD (assay kit)/whole sample/butanol extraction	[35]
0.34	UV/whole sample under argon/butanol extraction	[33]
0.88	UV/whole sample/butanol extraction and back extraction into 4 *M* NaOH (same values with HPLC)	[22]
TBA test with additional HPLC separation		
0.60	UV/whole sample/methanol–NaOH extraction	[17]
0.55	UV (assay kit)/whole sample/derivatives directly injected	[37]
0.45 (0.04)	UV/clear supernatant/derivatives directly injected	[7]
0.12	UV/clear supernatant/butanol extraction	[18]
1.08	FD/whole sample/derivatives directly injected	[19]
0.68	FD/whole sample/derivatives directly injected	[21]
0.27	FD/whole sample (prior incubated with KJ for reduction of lipid hydroperoxides)/extraction into isobutyl alcohol	[20]
Use of other derivatisation reagents		
1.48	LPO-568 assay (*N*-methyl-2-phenylindole)	[24]
0.03	GC–MS (pentafluorophenylhydrazine)	[26]
0.20	GC–MS (pentafluorobenzylhydroxylamine)	[25]
1.30 (0.14)	GC–MS (phenylhydrazine)	[8]
2.16 (<0.05)	HPLC–UV (dinitrophenylhydrazine)	Present paper

sponding to a 100-fold difference. Higher values reported in earlier publications were excluded (for review see Ref. [2]).

Even among GC–MS methods the reported values differed more than 40-fold which indicates inherent problems in all assays and the profound influence of differences in the used sample preparation conditions [2,8]. For critical comparison of the MDA values it is necessary to realize that plasma MDA may originate from different sources that is (I) free MDA; (II) protein bound MDA and (III) MDA that may have been liberated from endogenous lipoperoxide molecules during the assay, and even minor modifications of a given assay procedure may change the contribution from the sources I, II or III to the MDA signal measured. Furthermore, we must recognise that the measured values of MDA reflect the sum of the enzymatically catalyzed PUFA oxygenation (particularly the arachidonic acid pathway) and the metabolically uncoupled ''autooxidation'' of PUFAs and PUFA-esters if we inferred from the MDA values about oxidative stress [2,13–15].

With our method we could not quantitate free MDA in normal human plasma because the signals observed were below the lowest calibrator of the assay (50 pmol/ml) approximately correspond to 25 pmol/ml. Recently, Cighetti et al. [8] measured 140 pmol/ml free MDA with a phenylhydrazine derivatisation and a GC–MS method without protein precipitation. Others measured 117 pmol/ml [18], 43 pmol/ml [7] or failed to detect free MDA in human plasma [9,10]. Therefore, we conclude that values for free MDA in human plasma must be very low (<0.2 nmol/ml) and all values reported to be higher are most likely the sum of free and some fraction of bound MDA or stem from additional MDA liberated from lipoperoxide precursors even though results were declared to represent free MDA.

Bound MDA in plasma samples can only be measured after acid or alkaline hydrolysis of the protein binding. We used alkaline hydrolysis with 1 *M* NaOH for 30 min under heating to 60°C which was found to be optimal. This is in good agreement with the findings of Chighetti et al. [8] who demonstrated that MDA hydrolysis was complete only with the use of 1 *M* NaOH for 60 min or 2.6 *M* acetic

acid for 20 min. Additionally, we could confirm their observation that after incubation with 0.5 *M* NaOH, as carried out by Carbonneau et al. [7], only approximately 50% of the MDA signal could be detected. As shown in Table 1 with our method we found 2.16 nmol/ml of total MDA in human plasma from untreated volunteers. This value is approximately 60% higher than those reported by Cighetti et al. [8]. Despite the fact that the sample preparation is quite similar, they incubated their samples in the presence of the antioxidant BHT while we did not use this reagent. With another GC–MS method, Yeo et al. [26] found only 0.03 nmol/ml which was lower than the reported values for free MDA. They performed hydrolysis of protein bound MDA with 0.5 *M* H_2SO_4 at room temperature for 10 min conditions under which hydrolysis has been shown to be uncomplete [8].

The variations in hydrolysis conditions may be one reason for the variable results of the estimation of total MDA. Additionally, the production of ''artifacts'' if plasma samples were boiled under strong acidic conditions (as necessary for the TBA test) may be another source for variability [2,13,15]. Beside the frequently mentioned artifacts it is most important to recognise which compound an assay was designed to measure. The so-called TBA test was introduced in 1976 by Yagi [12] for the measurement of lipid oxygenation in serum, and plasma samples. This method was never intended for the estimation of endogenous MDA alone. The sample preparation was consequently optimised for the analysis of oxygenated lipids by removing other TBA reactive substances such as water soluble aldehydes and glucose from the reaction mixture [34]. Some authors have nevertheless tried to employ the TBA test for the selective measure of endogenous MDA, e.g., the estimation of free MDA after modification of the protein precipitating step and derivatisation of the clear supernatant [7,18].

Another popular modification consists of the addition of the strong antioxidant BHT dissolved in ethanol or methanol to the sample with the idea that this would prevent further lipid peroxidation during the assay procedure [15]. For our assay conditions we found that not BHT but the ethanolic solution alone is able to reduce the signal to about 65% of the initial values. Therefore, the antioxidant BHT has no protective effect in our assay. However, the modification of the sample by spiking with ethanol (1%, v/v) can profoundly alter the results. It has frequently been reported that the addition of BHT to the sample before incubation was considered essential to prevent lipid oxidation [8,15,18,20,33] whereas others found no effect [17,21,35]. We conclude that additional modifications of the sample preparation must be carefully proven for the given assay so that a reduction of the MDA signal may not be misinterpreted for example as an inhibition of artificial lipid peroxidation.

Reoxygenation after ischemia or extreme hypotension is a situation where superoxide radicals are generated [1]. We were able to demonstrate a significant increase of total MDA concentration in the first plasma sample of the normoxic period following 6 h of hypoxic treatment but we could not detect any free MDA. This may be due to the sampling schedule with the first sample during reoxygenation taken 2 h after the end of hypoxia. It has been assumed that oxygen radical generation and lipid peroxidation occurs only in a narrow time window related to the stressful event [11]. For the detection of changes in the free MDA content the sampling intervals probably were to wide, while changes in total MDA may persist for a longer time. The total MDA vs. time plot (Fig. 4) reveals pronounced interindividual differences as far as the response to physiological stress is concerned. These differences are thought to reflect different individual capacity in counteracting the generation of free radicals. Both, differences in baseline MDA concentrations [36] and more pronounced in MDA increase after a challenge can be explained by this way. A simple and selective assay for the determination of MDA could be a valuable tool in the investigation of an individual's capacity to scavenge free radicals as well as his ability to generate and accumulate this toxic aldehyde.

Studying the role of lipid peroxidation in the generation and progression of various diseases some of the simple established assays together with new enzyme-linked immunosorbent assay (ELISA) methods may be useful to provide preliminary data [6]. For more definitive studies specific GC–MS or HPLC methods especially with the hydrazine derivatisation are recommendable.

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