Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Chromatography B, 785 (2003) 337–344
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Improved method for plasma malondialdehyde measurement by high-performance liquid chromatography using methyl malondialdehyde as an internal standard

Ah Siew Sim[a], Chris Salonikas[b], Daya Naidoo[b], David Emil Leon Wilcken[a,*]

[a]*Cardiovascular Genetics Laboratory, Department of Medicine, Prince of Wales Hospital, High Street, Randwick, Sydney, NSW, Australia 2031*

[b]*Clinical Chemistry, Prince of Wales Hospital, High Street, Randwick, Sydney, NSW Australia 2031*

Received 5 September 2002; received in revised form 30 October 2002; accepted 18 November 2002

Abstract

Measurement of malondialdehyde (MDA) is an important contribution to the assessment of oxidative stress. We report a sensitive and reproducible high-performance liquid chromatography (HPLC) method for measurement of plasma MDA in the assessment of lipid peroxidation. Using methyl malondialdehyde (Me-MDA) as an internal standard with reversed-phase HPLC and UV detection and derivatisation with 2,4 dinitrophenylhydrazine (DNPH), we obtained maximum MDA values with 60-min incubation of 10% plasma with 1 *M* NaOH at 60 °C. The dilution of the plasma and a longer incubation time in the alkaline hydrolysis step greatly improved recovery of MDA from its bound form. Ratios of peak height of MDA/Me-MDA were linear over a range of 0–100 μ*M* with correlation coefficients >0.99. The recovery was 88.5%. Within and between run variations were <4 and <7%, respectively. The mean MDA value measured in 20 healthy volunteers was 13.8 μ*M* (±1.32).
© 2002 Elsevier Science B.V. All rights reserved.

Keywords: Malondialdehyde

1. Introduction

Tissue damage induced by reactive oxygen species has been implicated in the pathogenesis of cardiovascular and renal disease, and diabetes [1–3]. The break down of lipid peroxides in the biological system produces a number of aldehydes, including 4-hydroxylnonenal (4HNE) and malondialdehyde (MDA) [4]. These aldehydes are relatively stable and have been shown to be cytotoxic and genotoxic by reacting with proteins and nucleic acids [5].

MDA is in many instances the most abundant individual aldehyde resulting from lipid peroxidation. The assay of MDA using thiobarbituric acid (TBA) is commonly employed in lipid peroxidation studies despite the fact that the TBA assay is not specific for MDA [2,5,6]. It has been suggested that an additional high-performance liquid chromatography separation step might improve the assay [2,5,7–9]. However, the assay is still hindered by the harsh conditions used in sample preparation: it involves heating at 96 °C for 1 h at low pH and this

*Corresponding author. Fax: +61-2-9382-4921.
E-mail address: d.wilcken@unsw.edu.au (D.E.L. Wilcken).

1570-0232/02/$ – see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S1570-0232(02)00956-X

could create artefactual intermediates which may form identical MDA-TBA adducts [2,10,11]. In addition, the results of the TBA assay often vary widely in different experimental conditions [12,13].

To overcome the shortcomings of the TBA assay, other means of MDA derivatisation, which could be carried out in milder conditions, have been used. These include diaminonaphthalene (DAN) in acidic medium at 37 °C [14], 1-methyl-2-phenylindole at 45 °C [15] and 2,4-dinitrophenylhydrazine (DNPH) at room temperature [10,13]. More recently, methods using gas chromatography–mass spectrometry (GC–MS) [11,16,17] have also been proposed. These techniques are considered the most reliable. However, they require extensive sample preparation and GC–MS is not always readily available in clinical laboratories.

The use of a stable isotope-labeled internal standard has proved to be successful in the determination of MDA using MS methods. There is little information on a suitable internal standard for other methods. However, Claeson et al. [18] recently reported the potential use of methyl malondialdehyde (Me-MDA) as an internal standard in the determination of MDA in rat brain homogenates using different techniques, including HPLC. In the present study, we have established the use of methyl malondialdehyde (Me-MDA) as an internal standard for the determination of MDA in human plasma [18]. We used a modified HPLC method based on the derivatisation of MDA with 2,4-dinitrophenylhydrazine (DNPH) as reported by Pilz et al. [13]. The hydrazones formed readily at room temperature in mild acid conditions, are unique for a given aldehyde, and can be separated easily by HPLC.

2. Experimental

2.1. Chemicals

1,1,3,3-Tetraethoxypropane (TEP, 97%), 2,4-dinitrophenylhydrazine (DNPH), 3-dimethylamino-2-methyl-2-propenal (DMP) and 2-thiobarbituric acid were from Sigma–Aldrich (Sigma–Aldrich Pty Ltd, Australia). The 2,4-dinitrophenylhydrazine was prepared as a 5 m*M* solution in 2 *M* hydrochloric acid. Hydrochloric acid, 36% was from Ajax (Ajax Chemicals, Australia). Lipid peroxidation (LPO) assay kit, Cat. No. 437634 was from Calbiochem (Calbiochem-Novabiochem Pty Ltd, Australia).

2.2. Sample collection

Peripheral blood samples were collected from fasting subjects into 4-ml vacuettes containing EDTA as anticoagulant. Plasma was separated within 30 min in a refrigerated centrifuge at 4 °C and stored at −70 °C until used.

2.3. Preparation of standard curve and quality control materials

The MDA standard stock solution (10 m*M*) was prepared by acid hydrolysis of 239 μl 1,1,3,3-tetraethoxypropane (TEP) in 100 ml of 1% sulphuric acid for 2 h at room temperature. This solution was further diluted with water to 100 μ*M* to be used as a working standard solution. Calibrations were done in 50 μl of plasma spiked with 0.0, 5.0, 10.0 and 20.0 μ*M* MDA. Plasma samples from two normal subjects (one spiked with a known amount of MDA and one neat, aliquoted and stored at −70 °C) were included in every run as quality control samples.

2.4. Preparation of internal standard

We used methyl malondialdehyde (Me-MDA) as the internal standard. The procedure for synthesizing the sodium salt of Me-MDA was as reported by Claeson et al. [18]. Briefly, 0.5 g DMP and 0.2 g sodium hydroxide were dissolved in 0.7 ml HPLC grade water and incubated at 70 °C for 25 min. The liquid obtained was evaporated under reduced pressure until white crystals formed. Traces of NaOH were washed away carefully with a mixture of acetone and ethanol (1:1, v/v). The crystals were then dried over P_2O_5 in a desiccator. A 200 m*M* solution in water was prepared as stock solution.

2.5. Sample preparation for MDA determination in DNPH-HPLC method

A 50-μl sample (standard and plasma) was spiked with 50 μl Me-MDA in a 1.5-ml Eppendorf tube. Then 400 μl 1.3 *M* NaOH was added to a final

concentration of 1 *M* NaOH. The sample was then incubated for 60 min at 60 °C and cooled on ice for 5 min. The hydrolysed sample was then acidified with 200 μl 35% perchloric acid to precipitate protein and centrifuged for 10 min at 14 000 *g*. The supernatant was decanted into a fresh 2-ml Eppendorf tube. The sample was then derivatised with 25 μl DNPH reagent for 10 min at room temperature. Derivatised samples were protected from light from this step onwards. The aqueous phase was extracted twice with 1 ml hexane and mixed gently for 5 min. The organic extracts were combined, evaporated under a stream of nitrogen and reconstituted in 200 μl mobile phase. A 90-μl volume was injected into the HPLC system for analysis.

2.6. HPLC analysis

The HPLC analysis was carried out with a Waters HPLC system which includes a model 510 pump, a 712 WISP autoinjector and a model 481 LC spectrophotometer set at 310 nm. A Phenomenex, Synergi™ MAX-RP general reversed-phase, 150× 4.60 mm column was used for separation. The mobile phase was prepared by mixing 380 ml acetonitrile with 620 ml HPLC grade water and 2 ml of glacial acetic acid and degassed under reduced pressure. The flow-rate was 1.4 ml per min and the resulting backpressure was 1.9×10^4 kPa.

Chromatograms were registered and peak heights determined with a Shimadzu Class VP 4.3 integration system.

2.7. Lipid peroxidation (LPO) assay kit and the TBA assay

MDA levels were also measured using the LPO assay kit and the TBA assay. The LPO assay kit was from Calbiochem (Cat. No. 437634). Procedures for the experiment were in accordance with the kit instructions. The TBA assay was carried out as previously described [19].

2.8. Dilution effect

Plasma samples were diluted with water to obtain the following plasma concentrations: 75, 50, 25, 12.5 and 5%. MDA levels were then determined according to the methods described above. However, the hydrolysing step was carried out in accordance with the initial protocol of Pilz et al. [13], that is, 250 μl diluted plasma was hydrolysed with 50 μl 6 *M* NaOH for 30 min at 60 °C, and acidified with 125 μl 35% perchloric acid.

3. Results

3.1. HPLC separation of the DNPH derivatives

Fig. 1 shows the chromatographic separation of DNPH-derived MDA and Me-MDA in water and plasma. In the reagent blank, both MDA and Me-MDA peaks are absent as seen in Fig. 1A. Fig. 1B shows the presence of endogenous MDA eluting at 12.5 min in a plasma sample. Fig. 1C shows the chromatogram when the same plasma sample was spiked with Me-MDA, which elutes at 22.5 min. As seen in the chromatogram, the MDA and Me-MDA peaks are well separated and symmetrical. Fig. 1D is the same sample as in C, spiked with 20 μ*M* MDA.

3.2. Dilution effect

As shown in Fig. 2A, the recovery of Me-MDA increases with increasing plasma dilution. As the amount of Me-MDA added to each sample is the same, an increase in plasma concentration may decrease the availability of the Me-MDA to be derived with DNPH. Similarly, when the plasma is diluted, in the range of 100–60% plasma, the effect on the level of MDA is minimal. Further dilution gradually improves the recovery of MDA, with dilutions <20% plasma continuing to produce near linear recovery (Fig. 2B). This shows that for the same plasma sample, a higher MDA level is obtained if the initial mix is more dilute. Similar trends were observed in LPO and TBA assays (data not shown).

3.3. Hydrolysis efficiency

We tested the efficiency of hydrolysis of bound MDA under increasing alkaline conditions. Both MDA and Me-MDA levels decreased with increasing NaOH concentration (Fig. 3A). From 0.5 to 1.5 *M* NaOH, the extent of the decrease was similar for

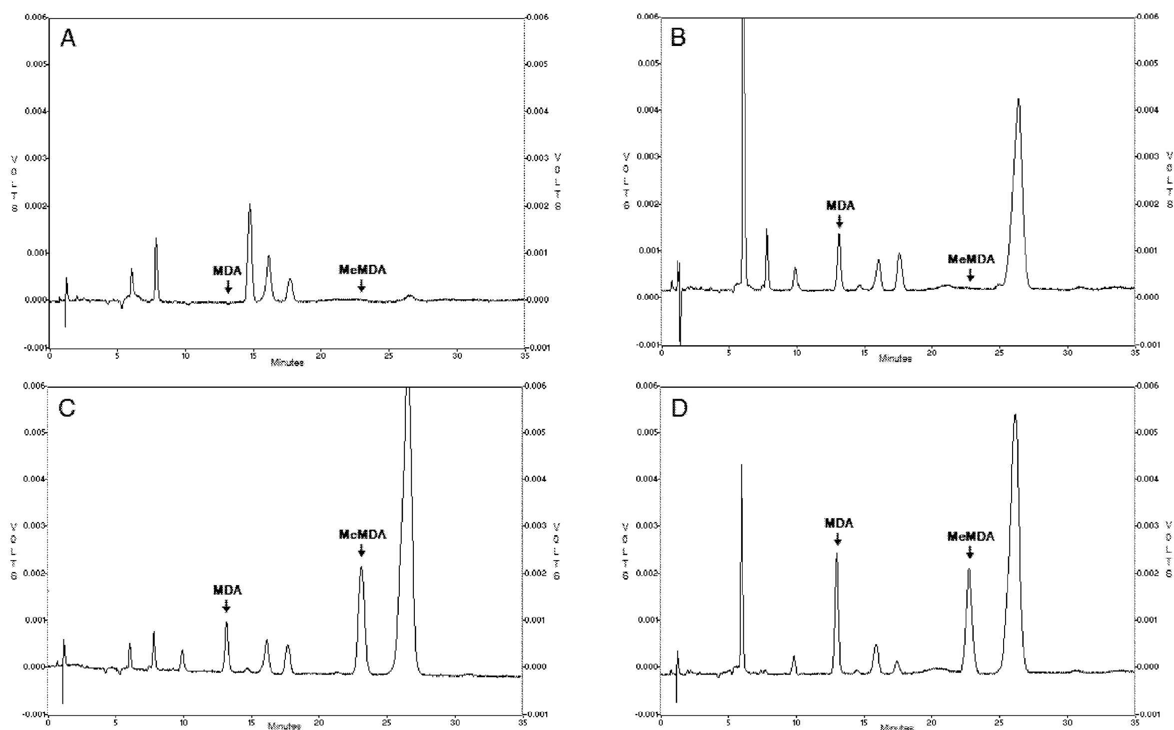

Fig. 1. Chromatographic separation of DNPH-derived MDA and Me-MDA in water and plasma samples that were processed as described in Section 2.5. In (A), the reagent blank, both MDA and Me-MDA peaks are absent. (B) Presence of endogenous MDA in a plasma sample. (C) Plasma spiked with Me-MDA demonstrates that the MDA and Me-MDA peaks are well separated. (D) Sample is the same as in (C), but spiked with 20 μ*M* MDA. In (B,C,D), a large endogenous peak is present. This is likely to be a by-product of the derivatization step. The peak appearing after MDA in (A) is seen only in the reagent blank, but is consistently quenched when plasma samples are analysed. During 2 h of observation, no further peaks appeared.

both MDA and Me-MDA, and is reflected in similar MDA/Me-MDA peak height ratios. However at high pH (2 *M* NaOH), the decrease in MDA was greater than in the methyl form as seen by a lower peak height ratio (Fig. 3B).

3.4. Effect of hydrolysis time

With increasing time of hydrolysis, MDA levels rose steadily until 60 min. The levels then declined with further prolongation of the incubation time (Fig. 4).

3.5. Validation of the method

Since the peak shapes were symmetrical as seen in the chromatograms in Fig. 1, peak height was used to determine the MDA level. Ratios of peak height of MDA/Me-MDA measured in spiked plasma (using plasma diluted to 10%) were linear over a range of 0–100 μ*M* ($y=0.035x(\pm 0.011)-0.018(\pm 0.024)$; $R^2=0.997(\pm 0.003)$, for y=ratio of peak height of MDA/Me-MDA, x=MDA concentration in μ*M*). In our system, the detection limit was 2.1 μ*M* with signal-to-noise ratio >2. Comparing the slopes of the calibration curves of pure MDA standards in plasma and in water acidified with sulphuric acid to pH 1.0, the recovery was 88.5% over the whole range. We determined the precision profile of the method by multiple analysis of a neat plasma and a spiked plasma sample stored in aliquot. The within-run variations of two samples (five replicates each) were 3.1 and 2.2%, while between-run variations for two different samples on eight different days were 3.7 and 6.3% (Table 1).

We carried out two assays on separate occasions to

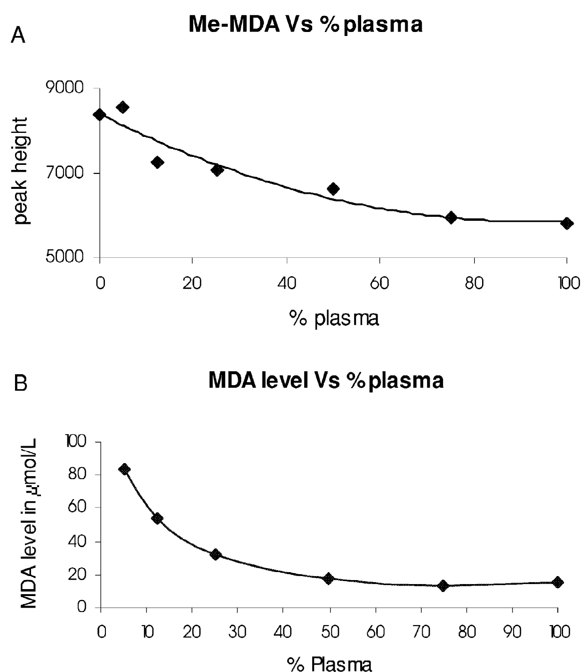

Fig. 2. (A) Recovery of Me-MDA added to the plasma increases as the dilution of plasma increases. (B) As dilution of plasma increases, measured MDA levels increase. Data were from two different experiments carried out on different occasions. They produced identical results.

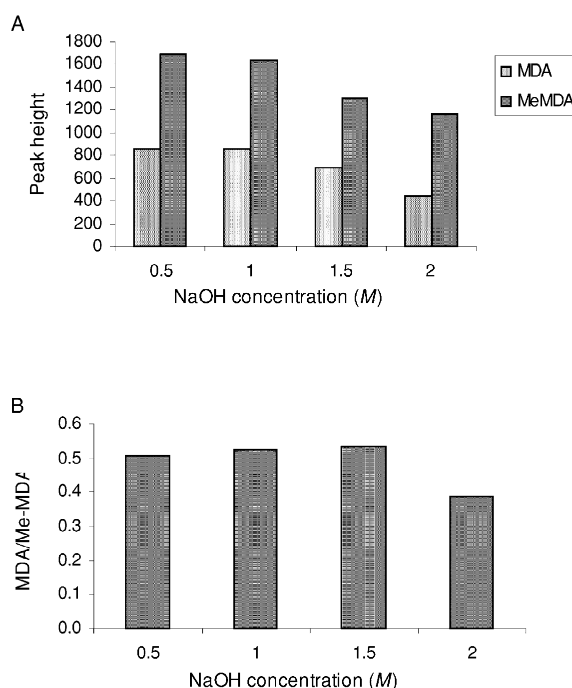

Fig. 3. (A) Both MDA and Me-MDA levels decrease with increase in NaOH concentrations. (B) The MDA/Me-MDA height ratio does not differ from 0.5 to 1.5 *M*, but is markedly decreased at 2.0 *M*.

determine the effects of dilution, NaOH concentration and hydrolysis time. Identical results were obtained in each case.

Stock solutions of MDA and Me-MDA stored at 4 °C were stable during the whole 2 months of assay development as reflected in the consistent satisfactory correlation coefficients of the standard curves.

4. Discussion

Determination of MDA is commonly used for monitoring lipid peroxidation in biological samples. However, the estimation of MDA in plasma is difficult due to the complex matrix. The TBA method, although easy to use, is not specific and often gives results that are not reproducible [20]. The initial objective of our study was to develop a suitable assay for MDA detection, which could include an internal standard without the problems of the TBA assay. We preferred derivatisation of MDA with DNPH because the reaction proceeds rapidly under mild acidic conditions at room temperature [13], and this minimizes the formation of undesired artefactual aldehydes which may occur with the TBA method. Moreover, the resulting derivatives are unique for a given aldehyde, enabling the use of Me-MDA as an internal standard. Derivatives of both

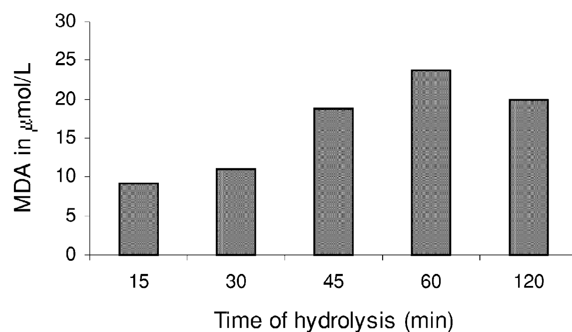

Fig. 4. MDA level increases with hydrolysis time up to 60 min followed by a decline at 120 min of hydrolysis.

Table 1
Precision profiles for the MDA-DNPH assay

Precision	n	Mean (μM)±SD	CV
Within-run			
Sample 1	5	12.1±0.4	3.1
Sample 2	5	21.3±0.5	2.2
Between-run			
Sample 1	8	9.3±0.3	3.7
Sample 2	8	31.7±2.0	6.3

MDA and Me-MDA are poorly soluble in aqueous solutions thus allowing a quantitative recovery by extraction with hexane. In addition, they are stable, can be separated well by HPLC, and give distinct signals as shown in the chromatograms. Our assay proved to be specific and the incorporation of a suitable internal standard rectified experimental variations and enhanced the robustness and reproducibility of the method. We did not quantify free MDA as the reported level is below the lowest calibrator of the assay [13].

The need to work with small paediatric samples in our laboratory led us to explore the possibilities of working with diluted samples. As shown in Fig. 2B, when the plasma was diluted, there was minimal effect on the MDA level in the range of 100–60% plasma. Further dilution gradually improved the recovery of MDA and dilutions with <20% plasma show near linear recovery. This established that for the same plasma sample, a higher MDA level is measured if it is more dilute in the reaction mixture. We also observed a similar trend in the TBA and LPO assays, which use different derivatising reagents (data not shown). This suggests that the amount of diluent present influences the accessibility of bound MDA to hydrolysis, and that dilution renders the bound MDA more accessible. Alternatively, it is possible that a plasma constituent(s) inhibits the release of bound MDA during hydrolysis, and that this inhibition is reduced with increasing dilution.

Similarly, we found increasing levels of Me-MDA with increasing dilution of a plasma sample (Fig. 2A). Since equal amounts of Me-MDA are added to each sample, the increasing plasma concentration must, in some way, hinder its accessibility to DNPH. It is possible that with increased plasma there is binding of the free Me-MDA or interference in the reaction with DNPH. The mechanism requires further investigation, but our findings document the importance of maintaining a fixed dilution factor all the way throughout a study, and, in particular, ensuring that the final concentration of plasma is about 10%.

When we used a 10 times diluted mix in contrast to the 1.2 times dilution used by Pilz et al. [13], we found that the linearity of the resulting calibration curve over a wide range was excellent ($R^2>0.99$) and that the recovery of 88.5% was well above that of 50% reported by Jentzsch et al. [19]. Although sample preparation involved a series of steps, i.e. alkaline hydrolysis, precipitation, decanting of supernatant to fresh tubes, and extraction twice with hexane and reconstitution with mobile phase, the assay was highly reproducible; the intra-assay CV was less than 4% and the inter-assay CV below 7%, findings which validate the inclusion of Me-MDA as the internal standard.

To determine the best hydrolytic conditions, we subjected the same plasma sample to differing levels of alkaline pH. With an increase in alkaline conditions, the Me-MDA levels decreased steadily. Since Me-MDA is added as free aldehyde, raising the pH could change the aldehyde molecules into forms not detectable by our assay system. This is also true with free MDA molecules as we observed very low recovery in MDA spiked reagent blanks (data not shown). On the other hand, many researchers have reported that a higher pH is more efficient in releasing MDA from its bound form [12,13,15,16]. So a balance between improved hydrolysis efficiency and decrease in free molecule degradation needs to be considered when choosing the NaOH concentration. The time course experiments further demonstrated that a longer incubation time with NaOH resulted in the hydrolysis of more MDA. Our data suggest that all the bound MDA is hydrolysed by 1 h, and that incubation for a longer time at the alkaline pH produces degradation of the free MDA molecules resulting in a decrease in measured MDA levels at 120 min (Fig. 4).

There is considerable variation in the published levels for plasma MDA. Recent studies have reported modifications that produce lower MDA levels in the belief that these are the outcomes of improved

specificity. An example of this is the inclusion of antioxidants like butylated hydroxytoluene (BHT) to avoid lipid oxidation during the assay [3,7,11,16,19,21]. However, the study by Pilz et al. demonstrated clearly that the reduced MDA level is merely the effect of ethanol alone and not of the BHT [13]. Therefore, we did not include BHT or ethanol in our study, and with the exclusion of BHT and ethanol, and the use of a more diluted plasma sample, our results show a reverse trend. When we used the original protocol reported by Pilz et al. [13] in our initial experiments, we obtained MDA values similar to their values. When we increased the dilution of the reaction mix from 1.2 times to 10 times, the mean level measured from 20 healthy volunteers (11 males and nine females, age 17–75, non smokers and not taking any vitamin or other food supplements) was 13.8 μ*M* (±1.32). This is almost 6.5-fold higher than the mean MDA value measured by Pilz et al. [13] (2.16 μ*M*).

We then compared MDA values obtained by different GC–MS methods published by other authors [11,16,17] (Table 2). Basal MDA levels measured by Rauli et al. [17] were about sevenfold higher than those measured by Yeo et al. [11]. Extrapolating from our data, the main contributor to the increase in MDA values obtained in Rauli's method is likely to be the use of more diluted plasma (three times compared to 1.04 times). The dilution factors used by both Rauli et al. and Cighetti et al. [16] were similar (three times and 2.5 times) and the inclusion of the alkaline hydrolysis step greatly improved the availability of MDA in Cighetti's method.

Since both TBA and LPO showed similar trends in dilution patterns, we suggest that there is an intrinsic limitation to most MDA assay systems published to date, and that this is related to the amount of the MDA that remains bound. Addition of an efficient hydrolysis step to release the bound MDA has proved to be important [12,13,16]. Our finding of the effect of plasma dilution constitutes another major parameter to the release of the bound aldehyde. The possible contribution of an increase in water molecules involved in this mechanism also requires further investigation.

In conclusion, we report a method for MDA measurement in human plasma that is simple and robust. Inclusion of a suitable internal standard greatly enhanced the reproducibility and reliability of the assay. The use of only 10% plasma in the reaction mix results in improved recovery which is linear. Furthermore, this reduced the volume of sample required and facilitated measurement in pediatric samples. We believe that this method could be applied in any setting where MDA is used as a marker of lipid peroxidation and that the improved recovery of bound MDA may help to better identify differences in MDA levels not distinguishable by other assay methods.

Table 2
Human plasma MDA values reported using a GC–MS method

MDA (μ*M*)	Dilution	Specification of the assay (hydrolysis method/derivatised)	Reference
0.03	1.04	H_2SO_4/ (pentafluorophenylhydrazine)	[11]
0.20	3.0	No hydrolysis step/ (pentafluorobenzylhydroxylamine)	[17]
1.30	2.5	1 *M* NaOH/(phenylhydrazine)	[16]

Acknowledgements

We are grateful to Dr Xingli Wang for reviewing the manuscript and to Natalia Duarte and Paul Stathakis for technical assistance.

References

[1] V. Cavalca, G. Cighetti, F. Bamonti, A. Loaldi, L. Bortone, C. Novembrino, M. De Franceschi, R. Belardinelli, M.D. Guazzi, Clin. Chem. 47 (2001) 887.
[2] B. Halliwell, S. Chirico, Am. J. Clin. Nutr. 57 (1993) 715S.
[3] J. Templar, S.P. Kon, T.P. Milligan, D.J. Newman, M.J. Raftery, Nephrol. Dial. Transplant. 14 (1999) 946.
[4] H. Esterbauer, K.H. Cheeseman, Methods Enzymol. 186 (1990) 407.
[5] H. Esterbauer, R.J. Schaur, H. Zollner, Free Radic. Biol. Med. 11 (1991) 81.
[6] J. Liu, H.C. Yeo, S.J. Doniger, B.N. Ames, Anal. Biochem. 245 (1997) 161.
[7] N. Volpi, P. Tarugi, J. Chromatogr. B 713 (1998) 433.
[8] J.A. Knight, R.K. Pieper, L. McClellan, Clin. Chem. 34 (1988) 2433.
[9] H.H. Draper, E.J. Squires, H. Mahmoodi, J. Wu, S. Agarwal, M. Hadley, Free Radic. Biol. Med. 15 (1993) 353.

[10] G.A. Cordis, N. Maulik, D.K. Das, J. Mol. Cell Cardiol. 27 (1995) 1645.
[11] H.C. Yeo, H.J. Helbock, D.W. Chyu, B.N. Ames, Anal. Biochem. 220 (1994) 391.
[12] Y.L. Hong, S.L. Yeh, C.Y. Chang, M.L. Hu, Clin. Biochem. 33 (2000) 619.
[13] J. Pilz, I. Meineke, C.H. Gleiter, J. Chromatogr. B 742 (2000) 315.
[14] J.P. Steghens, A.L. van Kappel, I. Denis, C. Collombel, Free Radic. Biol. Med. 31 (2001) 242.
[15] D. Gerard-Monnier, I. Erdelmeier, K. Regnard, N. Moze-Henry, J.C. Yadan, J. Chaudiere, Chem. Res. Toxicol. 11 (1998) 1176.
[16] G. Cighetti, S. Debiasi, R. Paroni, P. Allevi, Anal. Biochem. 266 (1999) 222.
[17] S. Rauli, M.D. Puppo, F. Magni, M.G. Kienle, J. Biochem. (Tokyo) 123 (1998) 918.
[18] K. Claeson, G. Thorsen, B. Karlberg, J. Chromatogr. B 751 (2001) 315.
[19] A.M. Jentzsch, H. Bachmann, P. Furst, H.K. Biesalski, Free Radic. Biol. Med. 20 (1996) 251.
[20] F. Nielsen, B.B. Mikkelsen, J.B. Nielsen, H.R. Andersen, P. Grandjean, Clin. Chem. 43 (1997) 1209.
[21] D. Lapenna, G. Ciofani, S.D. Pierdomenico, M.A. Giamberardino, F. Cuccurullo, Free Radic. Biol. Med. 31 (2001) 331.