Journal of Chromatography, 577 (1992) 9–18
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6316

High-performance liquid chromatographic separation of malondialdehyde–thiobarbituric acid adduct in biological materials (plasma and human cells) using a commercially available reagent

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(First received December 3rd, 1991; revised manuscript received February 6th, 1992)

ABSTRACT

The assay of malondialdehyde (MDA) is widely used in clinical chemistry laboratories to investigate lipid peroxidation in oxidative pathologies. In the present work, the thiobarbituric acid (TBA) reaction was carried out on plasma, human erythrocytes and fibroblasts. The reagents used were those of the fluorimetry MDA kit manufactured by Sobioda. We have defined the application of this kit to high-performance liquid chromatography. This adaptation satisfied the criteria of good analytical practice. The detection limit was 2.5 pmol per injection. The retention time of the MDA–TBA_2 peak (4.96 ± 0.07 min) led to excellent resolution of the complex. The within-assay (6–12%) and between-assay (11–12%) precisions were satisfactory. The analytical recovery of MDA after spiking samples of human plasma with tetraethoxypropane standards varied from 70 to 100%. The mean lipoperoxide concentration determined in 32 healthy adults (20–40 years) was 1.04 ± 0.23 μmol l^{-1} in plasma. Applied to the erythrocytes of fifteen laboratory workers, the method furnished physiological values of 0.59 ± 0.21 μmol l^{-1}. Concentrations were significantly higher in chronic renal dialysis patiens (4.15 ± 2.35 μmol l^{-1}). The MDA content of fibroblasts cultured in standard medium was 0.38 ± 0.04 μmol per g of protein and increased (5.78 ± 1.38 μmol per g of protein) if the cells were grown in an iron-enriched medium. This accurate high-performance liquid chromatographic method for detection of MDA is the first one which can be applied to plasma, red blood cells and cultured cells. This technique will prevent false positives and should make inter-laboratory comparisons possible.

INTRODUCTION

Free radicals can be generated in living organisms by different pathological pathways, including ultraviolet radiation [1], phagocytosis, ischaemia [2], cell activation due to a defect in the biocompatibility of various compounds such as haemodialysis membranes [3], etc. Therefore a number of different cell types, *e.g.* polymorphonuclear cells, monocytes or macrophages and endothelial cells, can release oxygen free radicals [4]. The superoxide anion ($O_2^{\cdot}$), one of the main oxygen free radicals generated by cells, participates in chain reactions that produce hydroxyl ($OH^{\cdot}$), peroxyl ($ROO^{\cdot}$), alkoxyl ($RO^{\cdot}$) and hydroperoxyl ($HOO^{\cdot}$) radicals, which can damage cells. These reduced oxygen species react with membrane lipids and nuclear contents, deplete cells of key metabolic intermediates and can ultimately cause cell death [5].

Oxidation of lipids can be measured at different stages, including losses of unsaturated fatty acids [6], measurement of primary peroxidation products, and measurement of secondary carbonyls and hydrocarbon gases [7]. Between these stages carbon- and oxygen-centred radicals can

0378-4347/92/$05.00

be detected and identified by their electron spin resonance spectra [8]. Of the available methods, the most accurate assays of lipid peroxidation are the most chemically sophisticated ones, but they require the most sample preparation.

The determination of lipid peroxidation in biological materials thus most often uses less sophisticated methods. The spectrophotometric or fluorimetric determination of malondialdehyde (MDA) after formation of an adduct with thiobarbituric (TBA) is the most widely used method [9–11]. However, in the course of the TBA test, a variety of chromogens may form by reacting with substances such as bile pigments, amino acids or sugars [12]. A modification of the TBA reaction [13] which removes non-specific chromogenic reaction products involves incubation at room temperature for 16–20 h and a column chromatographic step.

In order to overcome this obstacle, a number of authors have used separation methods such as high-performance liquid chromatography (HPLC) [14–16] or gas chromatography (GC) [17]. These methods are not rapid and are not well suited for clinical chemistry laboratories. The use of a reliable, rapid and moderately priced MDA assay method, the results of which can be verified with a separation method, would satisfy the criteria of good analytical practice.

After verification of the analytical characteristics (linearity, recovery in spiked samples, coefficient of variation) of the Sobioda (Grenoble, France) MDA fluorimetry kit [18], we report on the use of this kit in an HPLC separation method. This original work will enable fluorimetric results to be verified if necessary, and will remove the false positives resulting from the formation of chromogens other than the MDA–TBA_2 adduct. The use of a commercial kit will also lead to a better standardization of techniques, thereby facilitating inter-laboratory comparisons.

We have adapted this methodology to different human material, plasma, erythrocytes and fibroblasts, with the aim of developing a technique which will lead to a better understanding of the effects of lipid peroxidation on cells.

EXPERIMENTAL

Apparatus

HPLC apparatus. The chromatographic equipment was from Kontron Instruments (Rotkreuz, Switzerland) and consisted of a solvent delivery pump (LC pump Model T414), a Rheodyne Model 7161 injection system with a 50-μl sample loop (Rheodyne, Cotati, CA, USA), a guard column (3 cm × 0.46 cm I.D.) packed with 10-μm Spheri-10 RP_{18} from Applied Biosystems (Foster City, CA, USA), an analytical stainless-steel column (25 cm × 0.43 cm I.D.) packed with 5-μm Ultrasphere ODS from Beckman (San Ramon, CA, USA), and a multi-wavelength detector system (HPLC detector 430). The system was entirely controlled by a Kontron computer (Data System 450).

The spectrophotometric monitor, with a 3-μl flow cell volume and 1-cm optical pathlength, was set at 532 nm. The recorder sensitivity was 0.01 absorbance unit full scale (a.u.f.s.). Recorder chart speed was 1.7 cm min^{-1}. The flow-rate of the mobile phase was 1.0 ml min^{-1}. The injection was made with a 100-μl syringe from Hamilton (Bonaduz, Switzerland).

Fluorescence intensity was measured with a Perkin-Elmer fluorimeter (Perkin-Elmer, Bucks, UK).

Test tubes Polypropylene screw-cap tubes (7 ml) (Sobioda) were free from trace elements.

Reagents

Reagents kit. The reagents used were those of the MDA kit (Sobioda). They included solution 1 (thiobarbituric acid, TBA), solution 2 (perchloric acid) and a calibration solution consisting of 20 mmol l^{-1} 1,1,3,3-tetraethoxypropane (TEP) in ethanol. The working solution was TBA–perchloric acid (2:1, v/v). The stock standard solution was diluted with deionized water to obtain a concentration of 5 μmol l^{-1}. The working solution and standard solution were prepared fresh daily.

Additional chemicals. The mobile phase was prepared daily by mixing 580 ml of phosphate buffer (50 m*M*, pH 6.0) with 420 ml of HPLC-

grade methanol (Prolabo, Paris, France). The buffer was then filtered through 0.22-μm membrane filters (Millipore, Saint Quentin, France). Before use, the mixture was deaerated by ultrasound treatment.

The sample to be injected was neutralized with 0.7 *M* potassium hydroxide (Prolabo). The quality control serum used was lyophilized Probioqual AB 43 serum (Biomérieux, Lyon, France). Trichloroacetic acid (TCA) (20%), ferric iron chloride and tris(hydroxymethyl)aminomethane were obtained from Merck. Disodium ethylenediaminetetraacetic acid (EDTA) and butylated hydroxytoluene (BHT) were obtained from Sigma (St. Louis, MO, USA).

Sample preparation

Blood collection. Venous blood was collected in 5-ml trace element-free heparinized tubes. After centrifugation (1600 *g*) for 10 min, the supernatant plasma was carefully removed to avoid contamination with platelets and stored as rapidly as possibly after sampling at −20°C until analysis.

Human fibroblasts preparation. Cells were harvested by trypsination (6% trypsin Boehringer, Mannheim, Germany), rinsed and homogenized with a Potter apparatus in hypotonic Tris–HCl buffer (0.04 m*M*, pH 7.3)–1 m*M* EDTA. MDA was determined in the lysate.

Human erythrocytes preparation. To a 100-μl aliquot of fresh erythrocytes were added 50 μl of cold water and 10 μl of a 2% BHT solution, followed by deproteinization with 150 μl of cold TCA (20%). After centrifugation (2000 *g*) for 10 min the supernatant was carefully removed and either analysed immediately or frozen at −80°C and analysed within five days.

HPLC procedures

In each analytical run, we assayed reagent blanks, TEP working standard solutions, plasma, fibroblast or erythrocyte homogenates and a quality control specimen. Plasma and quality control sera were diluted 1:4 in deionized water.

When native TBA reactants were measured, we added to each tube 10 μl of 10 m*M* EDTA or 10 μl of a 2% (w/v) solution of BHT in 100% ethanol (Normapur), in order to inhibit the progression of lipid peroxidation. Then, into each polypropylene test tube, 0.100 ml of assay specimen was vortex-mixed with 0.750 ml of kit working solution. The tubes were tightly capped and placed in a 95°C water bath for 60 min. They were then chilled in an ice-water bath. The tubes were centrifuged and maintained at 4°C until HPLC analyses. Reagent blank, standard blank and an assay blank were left at room temperature for 60 min.

The above technique was modified for the assay of MDA in erythrocytes: 0.100 ml of 0.6 *M* hydrochloric acid and 0.600 ml of reagent 1 of the MDA kit were added to 0.100 ml of supernatant. After homogenizing, the mixture was placed in a 95°C water bath for 1 h. Subsequent steps were as described above.

The MDA–TBA adduct is unstable at neutral pH, and so each sample was separately neutralized within 10 min of injection. About 0.100 ml of 0.7 *M* potassium hydroxide was added to 0.200 ml of sample to bring the pH of the reaction mixture to 6.0. This was verified for each series of samples. After neutralizing, the samples were immediately centrifuged at 1600 *g* for 3 min and then analysed.

HPLC assay. The HPLC apparatus was equilibrated by pumping mobile phase at 1 ml min^{-1} for at least 30 min, until recorder baseline was stable. We then sequentially injected 50 μl of each blank, TEP standard, quality control and plasma or cells extracts. The absorbance of each sample was recorded at the column outlet at 532 nm for 10 min. A calibration curve was prepared by plotting the peak heights of the blank and TEP standard samples. The concentrations of the plasma lipoperoxides were determined as MDA (μM) from the calibration curve. Before the next run, the injection port and sample loop were rinsed with 100 μl of buffer.

Fluorimetric TBA test

Thiobarbituric acid reactants (TBARs) in plasma and the fibroblast lysate were determined according to the Sobioda MDA kit instructions which we have previously validated [13]. Mea-

surement was by fluorimetry (excitation wavelength 532 nm, emission wavelength 553 nm).

Erythrocyte TBARs were determined by modifying the technique described in the kit. As in the case of the HPLC method, 0.100 ml of 0.6 *M* hydrochloric acid and 0.600 ml of solution 1 of the MDA kit were added to 0.100 ml of supernatant. After homogenizing, the analysis was the same as for plasma.

Analytical performance

Detection limit. The detection limit (DL) was determined as described by Gatautis and Pearson [19]. A sample containing MDA at a concentration three to five times that of the reagent blank (S) was measured ten times and the detection limit was calculated using the formula:

$$DL = [2 \times S.D. \times (c)]/S$$

where S.D. is the corresponding standard deviation and (c) the concentration of the solution.

Linearity. Linearity was established by using the correlation coefficient according to EEC instructions (Additive to Directive 75/318/EEC, August 1989). The standard calibration solutions (0.1, 0.2, 0.4, 0.5, 1 and 2 μmol l^{-1}) were determined in triplicate. Linear regressions and the correlation coefficient were then calculated. A similar study was carried out with a high concentration range (1–20 μmol l^{-1}). In plasma linearity was established by using different dilutions of the assay.

Precision. In order to determine repeatability and reproducibility of the method, aliquots of three laboratory plasma samples containing different MDA levels were frozen (−20°C). They were blood donor plasma (normal), plasma from haemodialysed patients (high) and the diluted Biomérieux control serum (low). Samples were never frozen more than once. Samples were measured twice daily for ten days in order to calculate the within-run precision using pairs of values and the between-run precision with the VALTEC protocol [20]. The repeatability of the assay with red cells was calculated by carrying out ten assays on the same day. Red cells from haemodialysed patients (high level) and from healthy blood donors (normal level) were selected.

Accuracy. Accuracy was determined by assessing the recovery in spiked samples. Known quantities of the 5 μmol l^{-1} standard solution were added to a plasma previously diluted 1:4. After homogenizing, TBA reactants were measured as described above.

Normal and pathological values

Normal values were established in 32 healthy subjects (laboratory workers, fourteen males, eighteen females) between 20 and 40 years of age. The method was also used on plasma samples from subjects with pathologies that could have involved radical phenomena: seventeen haemodialysed patients, eight chronic kidney failure patients, twelve children with cystic fibrosis and two stage IV acquired immune deficiency syndrome (AIDS) patients on bleomycin for six weeks. The red cells from eleven laboratory workers and eleven haemodialysed patients were also studied.

We also performed twelve different experiments on human skin fibroblasts maintained in standard medium (10 μmol l^{-1} iron) or in iron-overloaded medium (100 μmol l^{-1} iron as ferric chloride hexahydrate).

Statistics

The statistical study [mean, standard deviation, coefficient of variation (C.V.), regression, Student's t-test for paired data] was carried out with a PCSM program (Data System). Correlations were studied by applying a linear regression according to Pearson.

RESULTS

Chromatograms

The HPLC technique we used separated (Fig. 1) and quantified MDA. The retention time of the MDA–TBA adduct was 4.96 ± 0.07 min (C.V. 1.4%) at a solvent flow-rate of 1.0 ml min^{-1}, which remained constant throughout the day. If the column was regenerated after each series, the retention time remained stable for 1500 injections. Different methods of neutralization were investigated in order to improve peak symmetry and prolong column life. The reagent

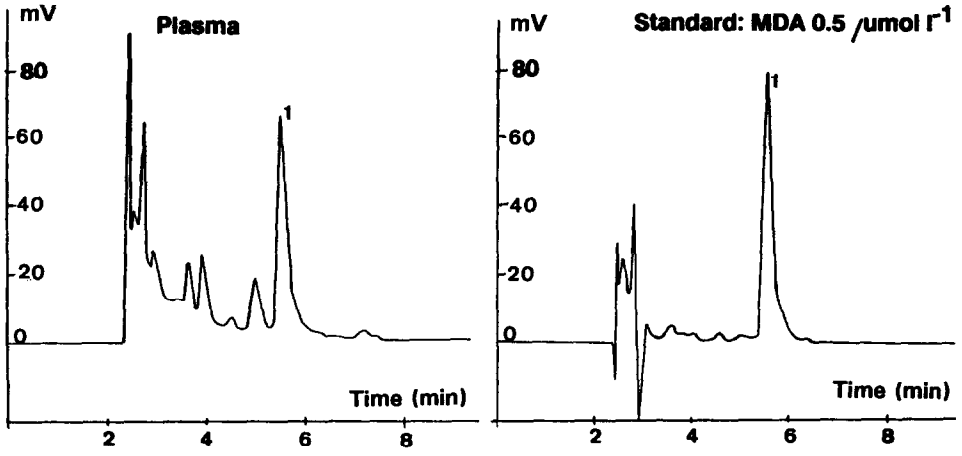

Fig. 1. Separation of malondialdehyde (MDA) in plasma and standard. Column: Ultrasphere ODS (5 μm) (25 cm × 0.43 cm I.D.). Eluent: methanol–50 μ*M* phosphate buffer pH 6.0 (42:58, v/v). Flow-rate: 1.0 ml min^{-1}. Peak 1 = MDA (detection at 532 nm).

chosen was 0.7 *M* potassium hydroxide, which led to better repeatability of the pH at the end of neutralization, the best peak symmetry and a longer column life.

Effect of antioxidant and iron-chelating agent addition

The conditions for assaying TBA reactants included an acid mixture at 95°C. These conditions favour the propagation of lipid peroxidation. When the analysis is carried out, it is important to use tubes that do not release transition elements and to use deionized water. In this case, the addition of BHT or EDTA at the concentrations previously described did not change the results (Table I).

Linearity

The concentrations of the TBA–acid mixture played a fundamental role in determining the linearity of the method when used with plasma samples. Linearity was correct only for a sample/reagent ratio of 1:7.5 (v/v), and only when plasma was diluted 1:2 or more. The regression equation was $y = 1.0043\ x + 1.97$ ($r = 0.9931$). This role was minimized when a standard solution was used. The correlation coefficient of the regression line ($r = 0.996$; $p = 0.0001$) for a TEP range from 0 to 2 μmol l^{-1} was excellent and thereby enabled MDA to be assayed in samples of human plasma, red blood cells and fibroblasts. Linearity remained satisfactory when TEP concentrations were ten times higher ($r = 0.997$; $p < 0.0001$).

TABLE I

INFLUENCE OF ANTIOXIDANTS (BHT AND EDTA) ON THE CONCENTRATION OF MDA IN PLASMA

Column packed with Ultrasphere ODS (5 μm) (25 cm × 0.43 cm I.D.). Eluent: methanol–50 μ*M* phosphate buffer pH 6 (42:58, v/v). Flow-rate: 1.0 ml min^{-1}. Assays were performed by adding 10 μl of 2% BHT to each analytical run (with BHT) *versus* 10 μl of water (without BHT) or 10 μl of EDTA (with EDTA) *versus* 10 μl of water (without EDTA).

	MDA concentration (μmol l^{-1})	
	Individual values	Mean ± 1 S.D. ($n = 5$)
Without antioxidants	0.534; 0.584; 0.649; 0.472; 0.326	0.513 ± 0.123
With BHT	0.433; 0.581; 0.569; 0.455; 0.316	0.470 ± 0.108
With EDTA	0.655; 0.611; 0.525; 0.468; 0.314	0.514 ± 0.133

Detection limit

The detection limit was 0.05 μmol l^{-1}, *i.e.* 2.5 pmol by injection. This shows the excellent sensitivity of the proposed method. It is sufficient to enable the method to be applied to cell homogenates. The sensitivity decreased if the water used to prepare the standards and samples was not deionized.

Accuracy

The standard recovery varied from 70 to 100%. It was satisfactory only when the final MDA overload concentration was lower than 0.80 μmol l^{-1}(Table II).

TABLE II

RECOVERY OF STANDARD ADDED TO THREE DIFFERENT PLASMA SAMPLES

N, normal level; H, high level; L, low level. Column packed with Ultrasphere ODS (5 μm) (25 cm × 0.43 cm I.D.). Eluent: methanol–50 μM phosphate buffer pH 6 (42:58). Flow-rate: 1.0 ml min^{-1}. The assay was performed by adding increasing increments of tetraethoxypropane to a constant concentration of a plasma dilution sample. The amount of MDA detected was determined using a standard curve. The average concentration of sample was used for calculation of recovery. Results of three representatives experiments are shown.

MDA overload (μmol l^{-1})	MDA detected (μmol l^{-1})	Recovery (%)
0	0.40 (H)	–
0	0.32 (N)	–
0	0.26 (L)	–
0.10	0.50 (H)	100
0.10	0.42 (N)	100
0.10	0.34 (L)	80
0.20	0.56 (H)	80
0.20	0.49 (N)	95
0.20	0.42 (L)	80
0.50	0.76 (H)	72
0.50	0.37 (N)	74
0.50	0.62 (L)	72
0.80	1.08 (H)	85
0.80	0.90 (N)	72
0.80	0.87 (L)	76
1.00	1.21 (H)	81
1.00	0.96 (N)	70
1.00	ND	ND

Precision

Within-assay precision (C.V. 6–12%) and between-assay precision (11–12%) over ten days were good (Table III). During the assay period, plasma samples were stored at −20°C, with no freeze–thaw cycles in trace element-free tubes. This prevented any *in vitro* lipoperoxidation. For red blood cell homogenates, the sample must be analysed within 4 h: derivatization (high level: n = 10; mean = 2.15 ± 0.17 μmol l^{-1}; C.V. = 8%; normal level: n = 10; mean = 0.42 ± 0.04 μmol l^{-1}; C.V. = 10%).

Physiological and pathological values

Plasma. The normal range (1.04 ± 0.23 μmol l^{-1}) determined for 32 normal subjects (laboratory workers) showed that there was no sex-related difference in MDA plasma concentrations (Table IV). The concentration of MDA in pathological plasma (Table IV) was higher as we have found previously using fluorimetric TBARs determination.

Red blood cells. Normal values (0.59 ± 0.21 μmol l^{-1}; range 0.30–0.96 μmol l^{-1}) were determined for eleven laboratory workers. The MDA values in eleven haemodialysed patients were two to ten times higher (4.15 ± 2.53 μmol l^{-1}; range 1.65–8.61 μmol l^{-1}) (Table V).

Human diploid fibroblasts. The MDA values

TABLE III

WITHIN-ASSAY AND BETWEEN-ASSAY PRECISION (n = 10) ON PLASMA

Column packed with Ultrasphere ODS (5 μm) (25 cm × 0.43 cm I.D.). Eluent: methanol–50 μM phosphate buffer pH 6 (42:58, v/v). Flow-rate: 1.0 ml min^{-1}. WR = within-run precision; BR = between-run precision.

Plasma		MDA (mean ± 1 S.D., n = 10) (μmol l^{-1})	C.V. (%)
Low	WR	0.46 ± 0.028	6
	BR	0.46 ± 0.052	11
Normal	WR	0.96 ± 0.09	10
	BR	0.96 ± 0.12	12
High	WR	2.16 ± 0.26	12
	BR	2.16 ± 0.28	12

TABLE IV

PHYSIOLOGICAL AND PATHOLOGICAL VALUES OF MALONDIALDEHYDE (MDA) IN PLASMA AS DETERMINED BY HPLC

Values are expressed as mean ± 1 S.D. *versus* normal values. No significant difference was found between males (M) and females (F).

	n	MDA (μmol l^{-1})
Healthy subjects	31	1.04 ± 0.23
	15 F	1.13 ± 0.25
	16 M	0.98 ± 0.20
Human disorders		
Chronic renal failure patients		
Non-haemodialysed	8	1.77 ± 0.46[a]
Haemodialysed	17	1.72 ± 0.24[a]
Cystic fibrosis	12	1.34 ± 0.46[b]
HIV		
Bleomycin treated	2	62.4; 33.6

[a] $p = 0.003$
[b] $p < 0.05$.

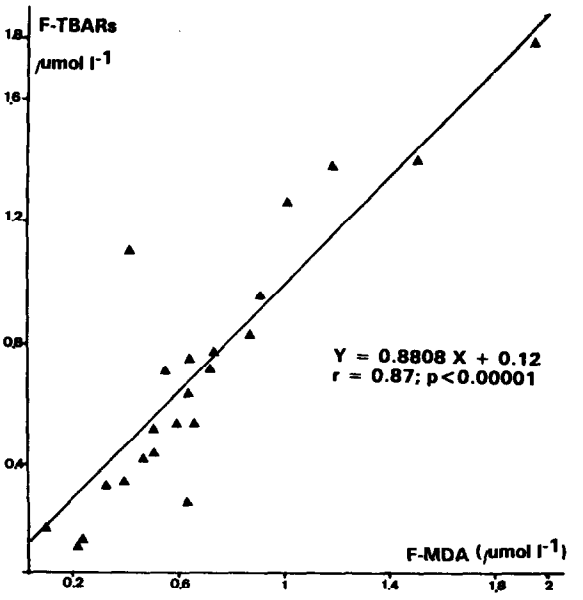

Fig. 2. Relationship between thiobarbituric acid reactants: fluorimetric evaluation with Sobioda MDA kit (F-TBARs) and HPLC evaluation (F-MDA) in human skin fibroblasts. The line represents the linear regression between the two parameters ($r = 0.87$; $p < 0.0001$).

determined in the lysates of fibroblasts grown for three subcultures in medium overloaded with iron (100 μmol l^{-1}) were fifteen times higher than those determined in control cells grown in standard medium (10 μmol l^{-1} iron) (Table V).

Correlation between HPLC determination of MDA and concentrations of TBARs by fluorimetry

The correlation between values determined by fluorimetry and those determined by HPLC was excellent when the sample was fibroblast homog-

TABLE V

MALONDIALDEHYDE (MDA) CONCENTRATIONS MEASURED IN DIFFERENT CELLS

Normal human erythrocytes (eleven laboratory workers); pathological human erythrocytes (eleven haemodialysed patietns); human skin fibroblasts ($n = 12$) grown in standard medium (10 μmol l^{-1} iron); human skin fibroblasts ($n = 12$) grown for three subcultures in iron-overloaded medium (100 μmol l^{-1}).

Human cells	*n*	MDA concentration	
		Mean ± 1 S.D.	Range
Normal erythrocytes	11	0.59 ± 0.21 μmol l^{-1}	0.30–0.96
Haemodialysed erythrocytes	11	4.15 ± 2.53 μmol l^{-1}	1.65–8.61[a]
Human skin fibroblasts	12	0.38 ± 0.04 μmol g^{-1} protein	0.34–0.45
Iron-overloaded fibroblasts	12	5.78 ± 1.38 μmol g^{-1} protein	4.02–8.91[a]

[a] $p < 0.001$ *versus* normal values.

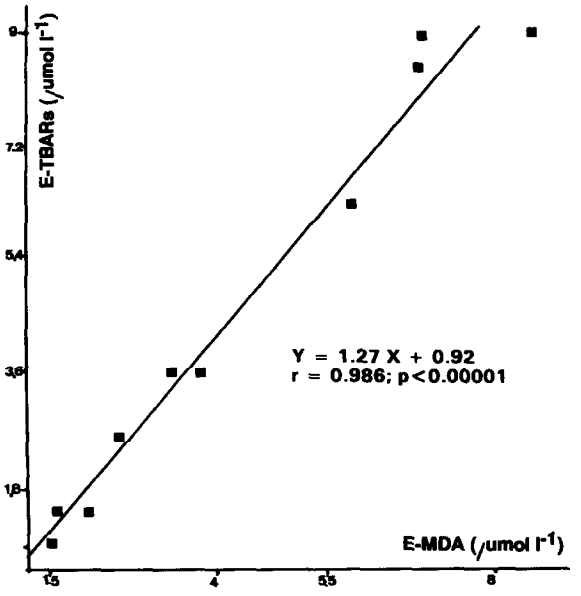

Fig. 3. Relationship between thiobarbituric acid reactants: fluorimetric evaluation with Sobioda MDA kit (E-TBARs) and HPLC evaluation (E-MDA) in human erythrocytes (n = 11). The line represents the linear regression between the two parameters (r = 0.98; p < 0.00001).

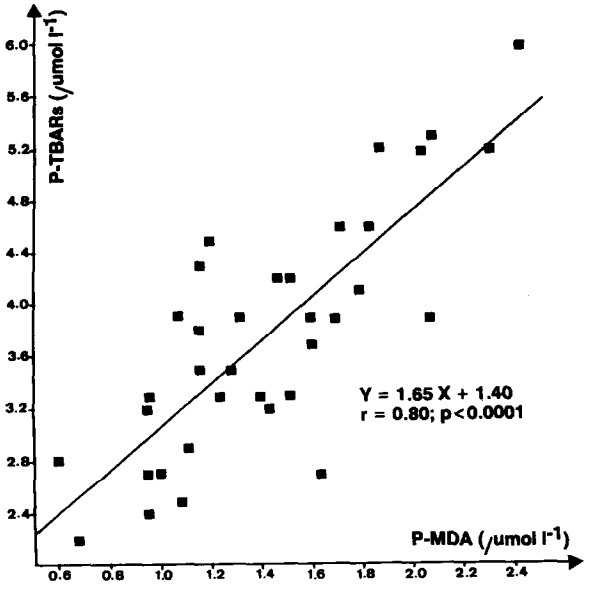

Fig. 4. Relationship between thiobarbituric acid reactants: fluorimetric evaluation with Sobioda MDA kit (P-TBARs) and HPLC evaluation (P-MDA) in human plasma (n = 32). The line represents the linear regression between the two parameters (r = 0.80; p < 0.0001).

enates (n = 23, r = 0.9976, p < 0.0001) (Fig. 2) or protein-free erythrocytes (n = 10, r = 0.986, p < 0.0001) (Fig. 3). The correlation between the values measured with the two methods was lower in the case of plasma (n = 32, r = 0.8, p < 0.001) (Fig. 4).

DISCUSSION

The measurement of oxidative stress status (OSS) in humans requires non-invasive methods. Study protocols are generally limited to urine and blood samples in which the individual's OSS is determined with reliable and routinely applicable methods. The measurement of OSS in isolated cells requires a very sensitive method. The assay of MDA by HPLC satisfies the criteria of accuracy, specificity and sensitivity and is the method of choice for evaluating the oxidative stress status [21]. The present work reports a method which can be used with plasma, red cells and fibroblasts. To our knowledge, no previous work has dealt with this determination applied to these different biological materials.

We obtained excellent resolution of the MDA–TBA complex. The eluent plays a critical role in the performance of HPLC. It should give a good resolution with reasonable time and flow-rate, and should not induce alteration in the column. The isocratic mode was selected to reduce the delay between two runs and to increase column life.

Wong *et al.* [14] used methanol–pH 6.8 phosphate buffer (4:6, v/v). In this case, the retention time was 4.2 min at a mobile phase flow-rate of 2.0 ml min^{-1}. Our modification enabled a lower flow-rate of 1.0 ml min^{-1} to be used at the same time as obtaining excellent resolution of the MDA–TBA_2 peak. This optimization led in some samples to the separation of additional adducts with shorter retention times than the MDA–TBA adduct and which we have not yet identified. No peak with a retention time longer than that of MDA–TBA has thus far been isolated.

The linearity of the method applied to a standard solution of TEP is close to that of Bird *et al.* [22], but is not found in the biological samples.

Plasma samples must first be diluted 1:4 in order for the method to comply with linearity criteria and to obtain acceptable recoveries of MDA. Our results confirm the findings of Petruska *et al.* [23], showing that optimal conditions for samples or cell numbers must be determined in order to remain within the linear range of the assay.

The method requires no prior extraction of the adduct, thereby decreasing sample preparation time. The absence of this critical step leads to satisfactory criteria of reproducibility and repeatability. The detection limit is 2.5 pmol per injection, similar to other HPLC methods previously described [16,23,24]. It should be noted that gas chromatography [25] can give lower detection limits (0.04 pmol). Csallany *et al.* [26] used an HPLC method with Spherogel-TSK G1000 PW particle size exclusion with direct detection of MDA and reported a detection limit of 14 pmol (1 ng) of MDA per injection.

When applied to human plasma, the method furnishes physiological values close to those published by authors [27] using the TBA test followed by HPLC separation. These values are lower than those determined fluorimetrically, confirming the fact that other aldehydes behave as TBA reactants and thus are also measured in non-separative methods [28]. The correlation found between the two methods when assaying plasma is satisfactory, and hyperlipidoperoxidation was found in pathologies involving radical phenomena, *e.g.* renal dialysis or chronic pulmonary infections.

This method enables MDA to be assayed in erythrocyte membranes. The MDA levels measured in healthy subjects are very low. Hyperlipidoperoxidation is found in the chronic haemodialysed subject. The same methodology is thus applicable to erythrocytes and plasma, which should lead to a better determination of the oxidative status of a subject.

Our preliminary research on isolated cells confirms the value of an MDA assay in pathologies of iron metabolism. We did not test the method with isolated leucocytes, but its sensitivity makes this application feasible.

In summary, MDA is a breakdown product resulting from lipid peroxidation. An accurate method for its detection by HPLC in plasma, red blood cells or fibroblasts is described. Even though fluorimetry remains a practical method because of the rapidity with which derivatized samples are quantified, the adaptation of an HPLC method by laboratories will prevent false positives. The use of a commercial reagent should also make inter-laboratory comparisons possible and thus lead to a better understanding of oxidative pathologies.

ACKNOWLEDGEMENT

This work was supported by Région Rhones Alpes, Research Program G.B.M. 1991.

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