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Free and Total Malondialdehyde Measured as 2,4-dinitrophenylhydrazine Adduct by HPLC-UV in Hemodialysis Patient Serum

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Abstract

Objective: Malondialdehyde (MDA) is an index of oxidative stress. In biological matrices MDA exists in both free (f-MDA) and bound (b-MDA) forms. In this report, a method to detect f-MDA and t-MDA was developed in human serum, based on MDA derivatization with 2,4-dinitrophenylhydrazine (DNPH) and HPLC separation.

Methods: This method gave high sensitivity, with Limits of Detection (LOD) and Limits of Quantification (LOQ) of 3.5 pmol/ml and 10 pmol/ml, respectively; while recoveries of t-MDA from spiked matrices (R%) reached 98.1 ± 1.8 and 96.51 ± 1.8 for f-MDA. Considering the lower levels of f-MDA in biological fluids, this method improves derivatization conditions (DNPH medium solution, time and temperature) and yielded 47 ± 12 pmol/ml of f-MDA in serum of 17 controls. Our method succeeded to determine f-MDA and t-MDA in hemodialysed patients.

Results: Results indicated that f-MDA levels increased, b-MDA even doubled respect to controls. After dialytic treatment, b-MDA did not change, while f-MDA decreased up to pre-dialytic values, before the following cycle. Data suggest that patients on chronic Hemodialysis (HD) presented a remarkable oxidative stress status highlighted by lipid peroxidation increase.

Conclusion: In conclusion, this method proposes a simple and sensitive alternative to preexisting protocols that could be suitable for a non-invasive evaluation of oxidative stress in human diseases.

Keywords: MDA, Malondyaldeide; Oxidative stress; Lipid peroxidation; Free and bound MDA

Abbreviations

MDA: Malondyaldeide; OS: Oxidative Stress; LP: Lipid Peroxidation; f-MDA: Free Malondyaldeide; b-MDA: Bound Malondyaldeide; HD: Hemodialysis; LDL: Low Density Lipoprotein; HPLC: High-Pressure Liquid Chromatography; UV: UV Detector; GC/MS: Gas Chromatography/Mass Spectrometry; LC/MS: Liquid Chromatography/Mass Spectrometry; SEM: Standard Error of the Mean

Introduction

Oxidative Stress (OS) is characterized by damages of cellular and extracellular macromolecules (protein, lipids and nucleic acid) that might cause tissue injury when natural defenses of the organism (enzymatic, non-enzymatic or of dietary origin) are overwhelmed by an excessive generation of Reactive Oxygen Species (ROS) [1,2]. ROS could attack lipids to initiate lipid peroxidation reacting with double bonds of Polyunsaturated Fatty Acids (PUFAs) to yield lipid hydro peroxides [3]. The break-down of such hydro peroxides in the biological system produces aldehydes, including Malondialdehyde (MDA) and 4-Hydroxynonenal (4-HNE) [4,5]. MDA, commonly used as an index of oxidative stress, turns out to be a terminal compound of lipid peroxidation [6,7]. This process has been linked with various pathological conditions such as atherosclerosis, cancer, cardiovascular, cardiovascular and liver diseases [8-10] but also has been implicated in a range of degenerative diseases, including diabetes [11], cardiovascular disease, Parkinson's disease, Alzheimer's disease [12], and psychiatric disorders, including schizophrenia [13]. Haemodialysis has also been reported to induce repetitive bouts of OS. A number of mechanisms have been postulated to be responsible for OS increase in patients undergoing Hemodialysis (HD). Some studies hypothesized an activation of polymorph nuclear leukocytes on the surface of dialysis membranes to produce ROS thus increasing OS [14,15]. Other authors suggested the loss of antioxidants through the dialyzer membranes during HD [16] or uremia to be responsible for OS increase [17]. In biological matrices, MDA exists in two different forms: the free (f-MDA) is an index of recent damage that binds to SH and NH_2 groups of macromolecules, such as proteins, nucleic acids and lipoproteins [4-18], while bound (b-MDA) is a marker of older injury [4,19]. Considering its role in pathophysiology encountered in HD patients, controversial data have been reported on the removal of tMDA by HD; the majority of authors observed a rapid decrease in tMDA after HD sessions [20], while others did not [21]. The significant decrease in plasma tMDA observed by some authors after

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HD sessions is thought to be due to MDA low molecular weight and water solubility [20]. On the contrary, bMDA has an high molecular weight and thus cannot cross the dialysis membrane, but it is excreted in healthy subjects [8]. The MDA derivatization with Thiobarbituric Acid (TBA) was the first method applied to detect MDA in plasma and serum [22,23]. Unfortunately, this method is not specific because TBA is very reactive toward other compounds besides MDA (nucleic acids, amino acids, proteins and phospholipids) and different species derived from oxidation processes [24,25]. To overcome these drawbacks, other methods utilizing high-performance capillary electrophoresis [7], HPLC-MS/MS [26], HPLC-FL [26], GC-MS [18], HPLC [6,26] for MDA evaluation in biological matrices have been tested. The last two techniques imply MDA derivatization with 2, 4-Dinitrophenylhydrazine (DNPH) to its respective Hydrazone (MDA-DNPH) and present clear advantages: 1) it improves the specificity of the methods, 2) the reaction of the carbonylic compound with hydrazine proceeds at room temperature under mild acid pH condition leading to the formation of a stable derivate specific for a given aldehyde and 3) the product is easily separable through HPLC [27-31], GC-MS [18] and LC-MS [32]. In the present work, we modified the original HPLC method developed by Pilz et al. [31] optimizing chemical and physical parameters of DNPH derivatization reaction which increased sensitivity up to a LOD of 3.5 pmol/ml for f-MDA. This improved method was tested for the serum determination of both f-MDA and t-MDA in patients subjected to Hemodialysis (HD). These patients were selected because of their high risk of complications associated with OS-such as atherosclerosis, inflammation, immune-dysfunction and cancer. All-cause mortality rates among hemodialysis patients are undoubtedly higher than the general population. Aside from the common risk factors, elevated oxidative stress along with states of chronic inflammation has been shown to play a crucial role in accelerated atherosclerosis and raised mortality. OS is present from the initial stages of chronic kidney failure and worsens as the disease progresses. It is further aggravated by the HD therapy itself. A variety of factors contributes to the excessive OS witnessed in HD patients, including the decreased elimination of toxins that leads to uremia, the lack of antioxidants caused not only by a strict diet but also by an increased loss during dialysis sessions, and the process of leukocyte activation during which oxidizing agents are released [33,34]. The aim of present study was to improve the current procedure of DNPH-based MDA derivatization and subsequently to optimize the downstream analysis using HPLC with UV detector. This technique was applied to investigate the potential effect of HD on lipid peroxidation in patient with End-Stage Renal Disease (ERSD).

Methodology

Chemicals

All reagents were of analytical grade and the organic solvents were of HPLC grade. Acetonitrile (ACN), n-hexane, Trichloroacetic Acid (TCA), Hydrochloric acid 36% (HCl) and glacial acetic acid were purchased from Panreac Quimica S.L.U. (Barcelona, Spain). 1,1,3,3-Tetramethoxypropane (TMP) and 2,4-Dinitrophenylhydrazine (DNPH) were supplied by Sigma-Aldrich (St Louis, MO). All other reagents used in this work were from Merck (Romania). HPLC-grade water was used.

Standard and derivatization agent preparation

MDA standard solution was prepared adding 17 µl of TMP to 10 ml of 0.1M HCl. The resulting solution was incubated at 40°C for 1 hour to allow the TMP hydrolysis into MDA. The concentration of MDA in the standard solution was determined by measuring its absorbance at 245 nm (ε=13,700 mol-1cm-1). MDA standard solutions were stored at -10°C in dark conditions. The derivatization agent was prepared dissolving 1 mg DNPH in 1 ml ACN and stored at 1-2°C in a dark place. Both aliquots were freshly prepared every week.

Sample collection and experimental subjects

A total of 15 patients (10 men and 5 women) with a mean age of 50.2 ± 10 years receiving maintenance HD treatment were recruited among those attending the Nephrology and Dialysis Unit of Azienda Ospedaliera S. Maria di Terni (Italy) and referred as HD group. Treatment was based on alternate-day HD for an average of 84 months, each time for 4-hours (08.00 to 12.00 hours). 17 healthy and non-smoking subjects (13 men and 4 women) a, with no clinical history of renal diseases, were included in the study as control group (CTR). Patients with systemic diseases, such as liver disorders, tumors, collagen-vascular disease were excluded from the present study, as well as smokers. Patients did not receive any lipid-lowering drug 6 months before the recruitment and none of them presented acute recent illness. A polysulfide membrane dialyzer was used for all subjects; blood flow and heparinization were the same in each dialysis session. Peripheral venous blood samples were withdrawn in Vacuette Z after an overnight fasting. Serum Clot Activator and EDTA/K3 tubes were used for MDA and biochemical parameters evaluation, respectively. The blood samples were centrifuged and serum was stored at -80°C until analysis. Serum samples were collected before and after a session of HD and after the interdialytic period. All biochemical parameters were measured by standard laboratory techniques, using ADVIA 1800 (Chemistry System, Siemens). The investigation conformed to the principles outlined in the Declaration of Helsinki, and informed consent was obtained from all subjects enrolled in the study.

Determination of f-MDA in human serum

0.7 ml of serum were cooled down to 1-2°C and then mixed with 0.7 ml of ACN. After vortexing, the mixture was centrifuged at 12,000xg for 15 min. 1 ml of supernatant was transferred in a new 10 ml glass tube with a conical stopper and then it was acidified up to pH 2 with 0.1 ml of HCl 1M. Next, 0.1 ml of DNPH solution (1mg/ml ACN) was added to the mixture; the glass tube was incubated at 50°C for 2 hours in dark conditions. The aqueous phase was extracted twice with 4 ml of n-hexane with shaking for 5 minutes. The organic extracts were evaporated under argon stream at 40°C. Reconstitution was performed in 40 µl of mobile phase and 20 µl of the resulting solution were injected into the HPLC column.

Determination of t-MDA in human serum

50 µl of NaOH 6 M were added to 0.25 ml of serum for alkaline hydrolysis of proteins bounded to MDA. The solution was then incubated at 60°C in a water bath (30 minutes in the dark). At the end of alkaline saponification, the resulting solution was cooled down to 1-2°C and then mixed with 300 µl of 20% cooled TCA. After 15 min of centrifugation at 12,000 g, 450 µl aliquots of supernatant were transferred in a new 10 ml glass tube with a conical stopper, neutralized to pH 6-7 with 5 µl of NaOH 6 M and then acidified with 10 µl of HCl 1 M to pH 2. After that, 535 µl of KH_2PO_4 (0.05 M, pH 2) and 0.1 ml of DNPH solution (1mg/ml ACN) were added to the mixture; the solution was vigorously mixed and kept at 50°C for 2 hours in dark conditions. 20 µl of the resulting solution were injected into the HPLC column.

HPLC analysis and method validation

Analytical HPLC separation was performed by Agilent 1100 system (S.C. Agilrom Scientific S.R.L) equipped with a high-pressure gradient

quaternary pump, a manual sample injection valve (loop 20 ml). An Agilent 325 Dual Wavelength UV-Vis detector set to 307 nm was used. Data were recorded using Agilent ChemStation sofware (S.C. Agilrom scientific S.R.L). A Genesis C_{18} column (4 µm diameter, 150 mm×4.6 mm), equipped with column saver pre-column filter (0.5 µm) was used for separation. A column heater maintained the temperature at 40°C. The mobile phase was prepared by mixing 500 ml HPLC-grade water with 500 ml ACN and 2 ml glacial acetic acid. The flow rate was 0.6 ml/minute. The following parameters were evaluated in order to validate the developed method: specificity, linearity, sensitivity, and precision and recovery rate. The specificity of the chromatographic method was estimated comparing the retention times of MDA-DNPH peaks in processed MDA serum samples before and after the addition of MDA standard solutions (spiked serum samples). The linearity of the method was assessed referring to the calibration curves at five concentration points of MDA standard solutions. The ranges were 5-100 pmol/ml and 0.2-4.0 nmol/ml for f-MDA and t-MDA, respectively. The calibration curves were obtained by plotting the responses of MDA-DNPH adducts peak areas against the nominal standard MDA concentrations. Regression equations and correlation coefficients were calculated using GraphPad Prism 4 software. The sensitivity of the method was evaluated by quantifying both LOD and LOQ of standard MDA concentrations. A signal-noise ratio of 3 and 10 times the average noise level was calculated for LOD and LOQ standard MDA concentration, respectively. Moreover, the latter one was calculated from heights of noises on both sides of MDA-DNPH peak in three standard MDA solutions [26]. The intra-day variation was established performing analyses in triplicate with standard MDA solutions at three different concentrations, whereas inter-day repeatability was accounted repeating the analyses of the same samples for 6 days. The MDA recovery from serum was assessed by adding standard MDA at various concentrations to CTR serum. The concentration in spiked serum samples was determined performing the assay in triplicate.

MDA quantitation of serum samples

The MDA concentration in serum samples was established by quantifying the chromatogram-derived MDA-DNPH adduct peak areas using Agilent ChemStation software. The peak areas, expressed as Absorbance Unit (AU), were converted to MDA concentration (pmol/ml or nmol/ml) using the corresponding calibration standard curves.

Statistical analysis

Data are presented as mean ± SEM and SD. Variables were compared by one-way ANOVA and differences were considered significant at a $p<0.05$. Linear correlation was performed using GraphPad Prism 4.

Results and Discussion

MDA occurs as a result of the oxidative deterioration of PUFAs and it has been shown to be involved in the aging process, atherosclerosis, chronic inflammation, degenerative diseases as well as in various cancers [33,34]. Therefore, serum MDA has been used as a lipid peroxidation biomarker that acts as an indicator of free radical damage in these conditions, with lot of work being put towards the improvement of existing methods during the last few decades. Plasma MDA detection was first performed using TBA derivatization. However, it is somewhat negatively viewed because of its low specificity and tendency to produce artifacts. The TBA derivatization process itself further generates some UV absorbing and fluorescent components that are not related to MDA [24,25]. However, The TBA procedure is an accessible and rapid test that continues to be one of the most popular methods of free radical damage evaluation, due to its simplicity. MDA levels can also be measured by LC-MS. However, there are a number of advantages of using HPLC over LC-MS. HPLC is largely automated, highly reproducible and very specific while requiring minimal training. This makes it a streamlined and cost-effective methodology. Still, what HPLC gains in speed, it loses in precision. HPLC can be less sensitive than LC-MS, particularly for f-MDA levels estimation. Although HPLC is a reliable method, it needs higher quantities of plasma compared to LC-MS. Its throughput is also inferior to LC-MS, the time needed for analysis usually being around 15-30 minutes per sample [32]. Another widely used technique that measures MDA levels is GC-MS. Both methods offer high reliability and specificity. However, despite its correlation with LC-MS, GC-MS might seem cheaper in terms of materials and maintenance, though it is still more expensive than HPLC. Furthermore, the GC-MS equipment is rather sophisticated, making this technique less likely to be adopted for common usage in regular laboratories [18,35].

HPLC analyses of t-MDA and f-MDA

The present method improves the determination of f-MDA and t-MDA in human serum samples after derivatization with DNPH and HPLC separation of hydrazone derivates. For t-MDA, serum samples were saponified with NaOH 1 M and the proteins were precipitated with 20% cooled TCA solution. Cooled ACN was used as precipitant agent for f-MDA. Derivatization of both f-MDA and t-MDA was performed by incubation of samples for 2 hours at 50°C with DNPH dissolved in ACN. Furthermore, t-MDA analysis was performed loading samples directly into HPLC system, whereas preliminary extraction in hexane was carried out for f-MDA determination. The preparation of DNDP with ACN allowed a high molar absorptivity at 307 nm of this derivate suiting for analysis using HPLC with UV detector. Anyway however derivative MDA was so strongly retained from hydrophilic bonds of the C_{18} column but the utilization of ACN and acetic acid as mobile phase let the very good chromatographic separation of MDA adduct from other endogenous species present in human serum. The typical chromatograms for DNPH-derivatized f-MDA and t-MDA at 307 nm are shown in (Figure 1). The peak corresponding to DNPH-derivatized t-MDA was eluted after 8.65 minutes (Figure 1a) and confirmed by spiking samples with standard MDA (10 nmol/ml) (Figure 1b). The chromatographic run showed the presence of endogenous f-MDA into human serum samples (Figure 1c), as confirmed by peaks of spiking MDA adduct (250 pmol/ml) that were eluted at a retention time similar to f-MDA (Figure 1d). The specificity of the present method was ensured comparing the peak retention times of processed and spiking MDA adducts (Figures 1b and 1d).

Kinetics of MDA derivatization with DNPH

The main reaction parameters, among which incubation temperature, incubation time of DNPH derivatization and derivatization reagent preparation, were experimentally evaluated to optimize the MDA-DNPH adduct production. It was pointed out that an incubation temperature of 50°C and the incubation time of 2 hours, used in our method for the derivatization of MDA with DNPH, allowed an higher yield (2.94 and 9.11 times greater) of the MDA-DNPH adduct compared to the method developed by Pilz et al. that provided 25°C and 10 minutes as parameters analyzed above [31] (Table 1). The effect of derivatization agent preparation on the MDA-DNPH adduct yield was also studied, as shown on (Table 2). In our method, the use of ACN as solvent of derivative agent improved 2.21 times the formation of MDA-DNPH hydrazone with respect to HCl. The incubation temperature was 50°C in both assays. Considering that

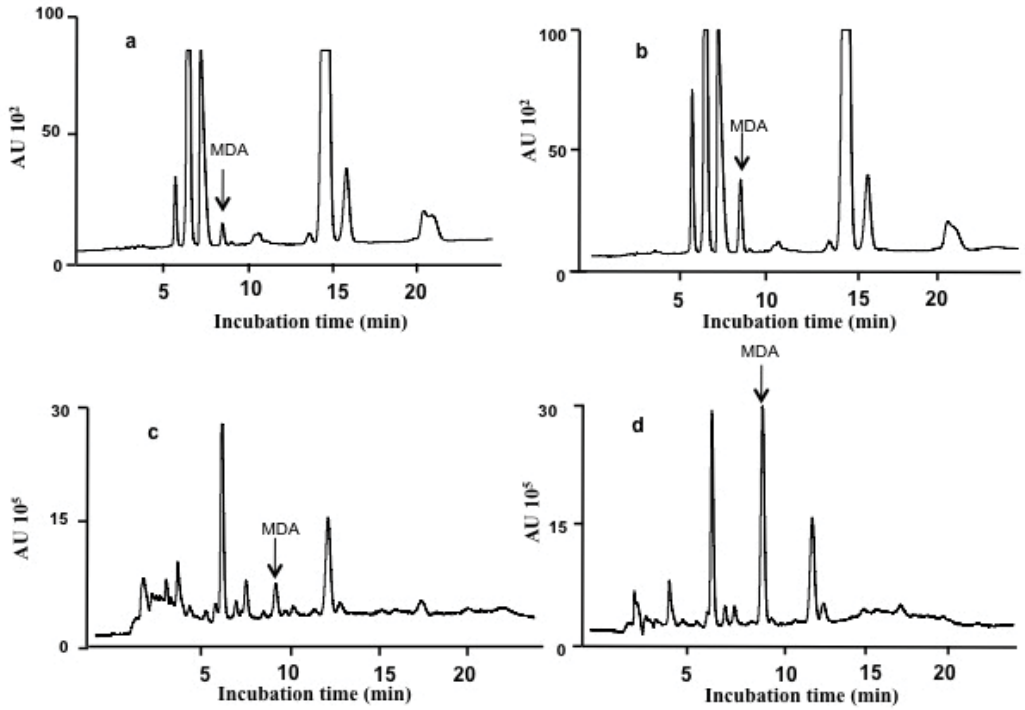

Figure 1: HPLC chromatograms of DNPH-derived f-MDA (Panels a and b) and t-MDA (Panels c and d) in human serum. Panels b and d represent serum samples spiked with 250 pmol/ml and 10 nmol/ml MDA standard concentrations, respectively. Arrows indicate MDA peaks.

	CTR serum level	Modified derivatization method Absorbance (AU)	Pilz et al. [17]derivatization method Absorbance (AU)
f-MDA	47.37 ± 6.07 pmol/ml	14,204 ± 1,822	4,832 ± 825
t-MDA	1.061 ± 0.152 nmol/ml	70,835 ± 10,180	24,085 ± 257

Table 1: Effect of incubation temperature on the MDA-DNPH amount during the derivatization reaction. Peak areas were expressed as absorbance units (AUs) and converted to MDA concentration (pmol/ml and nmol/ml for f-MDA and t-MDA, respectively) using the corresponding calibration standard curves. Absorbance unit full scales (AUFS) were 0.005 and 0.02 for f-MDA and t-MDA, respectively. Values are expressed as means ± SEM of serum samples from three healthy subjects.

	CTR serum level	Modified derivatization method Absorbance (AU)	Pilz et al. [17] derivatization method Absorbance (AU)
f-MDA	47.53 ± 7.71 pmol/ml	31,686 ± 3,351	14,373 ± 2,341
t-MDA	1.061 ± 0.152 nmol/ml	150,835 ± 16,180	68,5615 ± 1,057

Table 2: Effect of derivatizing agent preparation on MDA-DNPH hydrazone formation. Comparisons between our protocol and that developed by Czauderna were made. The peak areas were expressed as absorbance units (AUs) and converted to MDA concentration (pmol/ml and nmol/ml for f-MDA and t-MDA, respectively) using the corresponding calibration standard curves. The absorbance unit full scales (AUFS) were 0.005 and 0.02 for f-MDA and t-MDA, respectively. Values are expressed as means ± SEM of serum samples from three healthy subjects

the incubation time represents one of the main factors to take into account for an high yield of DNPH-derivatized MDA, an high yield in terms of MDA-DNPH adduct has been reported as revealed by chromatographic signal. The dependence of chromatographic signal on the reaction time at 50°C is shown in (Figures 2a and 2b), which referred to 50 pmol/ml or 2 nmol/ml concentrations of MDA solution, respectively. The reactions proceeded rapidly during the first 60 min followed by a slight drop of the signal until 90 minutes. We choose 120 minutes as optimal incubation time since it allowed an increase in MDA-DNPH adduct production of 3.8 times if compared to other derivatization methods [31].

Method validation

Standard calibration curves were built by plotting peak areas at 307 nm for different MDA standard concentrations (Figure 3). Regression lines were found: Y=0.7412x - 0.0389 for f-MDA and Y=0.8784x - 0.0115 for t-MDA with correlation coefficients (r) >0.9999. The LOD (LOQ) were 3.5 (10) pmol/ml related to MDA determination. The sensitivity of the proposed method was significantly higher than the previous one [31] (detection limits: 25 (75) pmol/ml). Our method decreased the LOD (LOQ) values 2.4 (2.8) times lower and as consequence the sensitivity 2.42 times when compared to the method developed by Czauderna et al. (detection limits 8.5 (28.5) pmol/ml) [26]. The advantages of obtaining a better sensitivity in our method compared to the classical ones was particularly important in determining the MDA whose value was 22.95 ± 0.92 time lower than in healthy subject. Intra-day and inter-day values were below 1% and 3.7%, respectively, thus giving us confidence about the reproducibility of the method (Table 3). The reliability of the current method was assessed by the addition of MDA standard solution to the serum from CTR subjects. The results summarized in (Table 4), the recoveries (R%) of standard MDA added to serum samples (referred to both f-MDA and t-MDA procedures) were significantly higher than those reported by Pilz et al. (98.1 ± 1.8 and 96.51 ± 1.8 for t-MDA and f-MDA against 93.6 ± 3.5 for both f-MDA and t-MDA sets) [31].

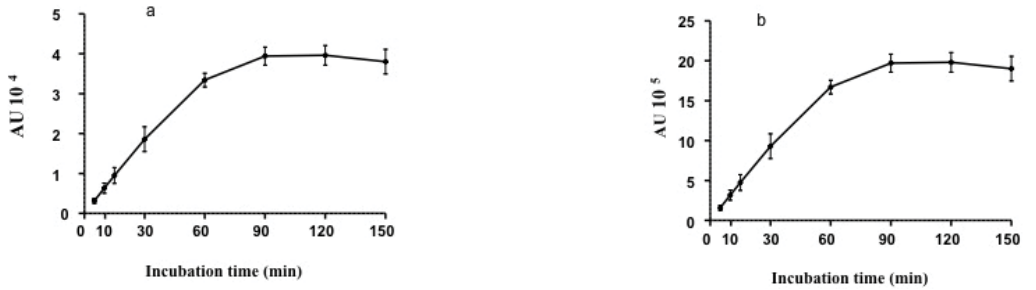

Figure 2: Effect of incubation time on MDA derivatization with DNPH at 50°C. The concentrations of MDA standard solution were 50 pmol/ml and 2 nmol/ml in panel a and b, respectively. The bars represent SEM values.

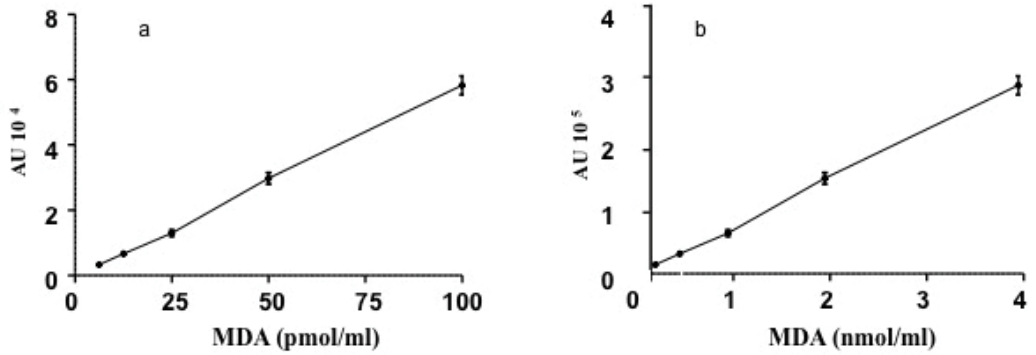

Figure 3: Calibration curves of peak area ratio. Panel a: DNPH-derived MDA and MDA concentration (pmol/ml) referred to f-MDA protocol. Panel b: DNPH-derived MDA and MDA concentration (nmol/ml) referred to t-MDA protocol. Peak areas are expressed as Absorbance Units (AUs). Each point represents the mean ± SEM of three experiments.

Assayed sample	Concentration	Intra CV (%)	Inter CV (%)
standard MDA f-MDA determination	25 pmol/ml	0.75	1.21
	50 pmol/ml	1.32	2.32
	75 pmol/ml	0.93	0.89
standard MDA t-MDA determination	0.5 nmol/ml	0.72	1.18
	1 nmol/ml	0.96	0.94
	2 nmol/ml	1.43	1.47

Table 3: Reproducibility for intra-day and inter-day standard MDA determination. Concentration of standard MDA were 25,50,75 pmol/ml and 0,5,1,2 nmol/ml for f-MDA and t-MDA, respectively. Mean values are expressed by Intra CV (%) (within-day percentage variation coefficient), based on three replicates of each MDA standard solution. Mean values expressed by Inter CV (%) (between-day percentage variation coefficient) were calculated on 6 days.

Serum Samples	Replicates	MDA standard	R (%)
f-MDA procedure	3	10 pmol	96.14 ± 1.87
	3	25 pmol	95.41 ± 2.33
	3	40 pmol	94.45 ± 2.42
t-MDA procedure	3	0.5 nmol	96.15 ± 1.82
	3	1.0 nmol	97.23 ± 2.35
	3	2.0 nmol	95.45 ± 1.45

Table 4: R% (percentage of the mean recovery ± SEM) of the MDA standard added to serum samples. Recoveries were calculated as follows: $R(\%)=({}^{1}S_n - {}^{0}S_n) \times 100\%/S$. S, amount of added standard MDA; ${}^{0}S_n$ and ${}^{1}S_n$, measurements performed before and after addition of the standard MDA, respectively. The concentration of serum f-MDA and t-MDA are 48.15 pmol/ml and 1.22 nmol/ml, respectively.

MDA levels of healthy control subjects

In serum from 17 CTR subjects, t-MDA and f-MDA mean values were 1.075 ± 0.201 nmol/ml and 47 ± 12 pmol/ml, respectively. Remarkably, these values were comparable to the majority of literature data available [5,36]. Serum t-MDA and f-MDA values here reported are in agreement with those reported in other works, where the measurements of DNPH-derivatized MDA levels were carried out by HPLC-UV method (<50 pmol/ml for f-MDA and a range of 1.012-2.132 nmol/ml for t-MDA) [27-31]. Regarding f-MDA levels, significant changes were observed when our method was compared to those using GC/MS and LC/MS assessments (values ranged from 110 to 440 pmol/ml with respect to 47 ± 12 pmol/ml exhibited in our method) [18,35,37,38]. Likely, GC/MS and LC/MS techniques improved the precision of f-MDA quantitative evaluation.

Biochemical characteristic in hemodialysis patients

The optimized method here proposed could successfully be applied for the quantification of f-MDA and b-MDA serum levels in patients with an End Stage Renal Disease (ERSD), receiving regular HD that exacerbates OS and consequently lipid peroxidation [39-42]. Kinetics of clinical parameters during HD is reported in (Table 4). Creatinine and urea were twelve and four times higher in Pre-HD^0 and Pre-HD^1 patients, compared to CTR, respectively ($p<0.01$). After the HD session, a drastic reduction in urea levels occurred, while the decrease of creatinine concentration remaining significantly higher respect to CTR ($p<0.01$). The increase of these renal markers could be due to chronic uremia, a peculiar state in patients with ESRD, whereas their marked decrease after HD session was the result of the ultrafiltration occurred during the dialysis treatment. Sustained modifications of specific endogenous metabolites were observed during HD. Indeed, uric acid (6.12 ± 1.13 mg/dl Pre-HD^0 level) was eliminated during HD, reaching the concentration of 3.24 ± 0.97 mg/dl which was similar to the value of CTR group (3.21 ± 0.89 mg/dl). After the interdialytic interval, the Pre-HD^1 uric acid concentration returned to Pre-HD^0 levels. Bilirubin and albumin levels were measured as endogenous metabolites and showed changes during HD. In fact, bilirubin underwent a slight significant increase ($p <0.05$) while albumin decreased significantly ($p<0.01$) during HD cycles compared to CTR. The reduced albumin plasma concentration could be attributed to the significant hematocrit decrease ($p<0.01$) due to the erythropoietin deficit in ESRD patients respect to CTR. Plasma C-reactive protein (CRP) levels were two times higher in the patients during pre- and post-dialysis treatment ($p<0.01$) and after the interdialytic interval when compared to CTR. This is in agreement with previous reports, suggesting that a dialysis membrane-induced inflammation might lead to hepatocyte-driven synthesis of acute phase proteins as PCR in HD patients [43,44]. Triglycerides were significantly higher in both Pre-HD^0 and Post-HD^0 patients ($p<0.01$), but also before the next dialysis treatment (Pre-HD^1) ($p<0.01$) with respect to CTR. In agreement with other authors, these triglyceride plasma increments, together with unchanged cholesterol levels, could reflect an abnormal lipid profile during the HD cycles. In turn, this established hypertriglyceridemia could induce an increase in Plasma Unsaturated Fatty Acids (PUFA) which is the substrates for peroxidative reactions [45-47].

Variation in MDA levels in patient before and after hemodialysis

To investigate whether the proposed method could be used for the non-invasive analysis of oxidative stress in human serum, the serum levels of both t-MDA and f-MDA were measured, allowing us to calculate b-MDA amounts in HD and CTR patients. t-MDA was significantly higher in HD patients before (Pre-HD^0) and after (Post-HD^0) dialysis session but also at the end of the interdialytic interval (Pre-HD^1) ($p<0.001$) respect to CTR. The Post-HD^0 t-MDA level, although markedly reduced compared to Pre-HD^0 and Pre-HD^1 values ($p<0.01$), remained above the normal range after dialysis ($p<0.001$). These results are in agreement with those reported by other authors using the main methods such as Thiobarbituric Acid Reactive Substances (TBARS), DNPH derivatization-HPLC, GC-MS and LC-MS techniques [47,48,41,36,38]. An additional original feature of our method was the measurement of both b-MDA, an index of an older injury, and f-MDA, a marker of recent or potential damage [37]. We noticed a significant increase of both MDA forms; in particular, f-MDA and b-MDA levels were 31.1 and 1.72-fold higher in Pre-HD^0 patients with respect to CTR ($p<0,001$). These data strongly demonstrated a remarkable OS-derived lipid peroxidation in the serum of HD patients with ESRD. However, a significant increase of f-MDA levels ($p<0.01$) were observed during HD treatment after correcting for creatinine values (14.66 ± 3.93 nmol/mg in Pre-HD^0 and 24.56 ± 2.89 nmol/mg in Post-HD^0, (Table 5). Several authors suggest that lipid peroxidation and the consequent production of MDA during HD is driven by the activation of polymorpho nuclear leukocytes that generate ROS on the surface of the dialyzer membrane [48,49]. Ratio between f-MDA and b-MDA underlines the high amount of f-MDA respect to b-MDA in HD patients ($p<0.001$), (Table 6). High f-MDA concentrations suggests the occurrence of recent lipid peroxidation in Pre-HD^0 in patients respect to CTR [37,48]. A slight increase in f-MDA in HD patients (2.5-fold) respect to CTR subjects was instead reported from De Vecchi et al. [37]. This discrepancy could be attributed to the diversity of the used method. To evaluate the profile of f-MDA concentration during HD treatment, we compared the f-MDA levels in preHD^0, postHD^0 and preHD^1 phases. As observed in (Table 6) and (Figure 4), the HD treatment caused a significant reduction (54.61 ± 9.44%, $p<0.001$) of f-MDA levels in PreHD^0 (1.46 ± 0.36 nmol/ml) respect to Post-HD^0 (0.65 ± 0.08 nmol/ml). Serum f-MDA, being a small water-soluble molecule, was cleared soon after HD [37,49]. Considering that MDA is a toxic molecule and its interaction with DNA and protein is potentially mutagenic and atherogenic [22], the HD-induced f-MDA clearance could reduce the inflammatory and oxidative damage-driven atherosclerotic cardiovascular complications in ESRD patients on chronic dialysis [33,34]. This role of f-MDA as the main dialyzable form was also reported by Steghens et al. [40]. When the serum samples were analyzed after the interdialytic period, the f-MDA levels increased significantly ($p<0.001$, twice than Post-HD^0 and neared to the pre-HD^0 values) (Table 6 and Figure 4). Serum b-MDA did not vary significantly neither during the HD session nor during the interdialytic period, whilst it doubled with respect to CTR

Biochemical parameters	Pre-HD^0	Post-HD^0	Pre-HD^1	CTR
	(n=15)	(n=15)	(n=15)	(n=17)
Haematocrit (%)	35.53 ± 3.47*	37.73 ± 2.32*	36.09 ± 2.97*	43.88 ± 2.75
Triglycerides (mg/dl)	281 ± 42*	271 ± 45*	266 ± 39*	149 ± 43
Cholesterol (mg/dl)	228 ± 51	232 ± 47	218 ± 61	216 ± 35
Urea (mg/dl)	119 ± 77$^{*\$}$	40.66 ± 10.97	120.31 ± 33.76$^{*\$}$	32.29 ± 7.77
Uric acid (mg/dl)	6.12 ± 1.13$^{*\$}$	3.24 ± 0.97	5.98 ± 1.12$^{*\$}$	3.21 ± 0.89
Creatinine (mg/dl)	11.31 ± 1.1$^{*\$}$	3.24 ± 0.97*	10.59 ± 2.36$^{*\$}$	0.91 ± 0.19
Bilirubin (mg/dl)	0.83 ± 0.08**	0.82 ± 0.07**	0.81 ± 0.06**	0.68 ± 0.21
Albumin (gr/dl)	3.58 ± 0.49*	3.61 ± 0.66*	3.29 ± 0.39*	4.21 ± 0.31
CRP (mg/L)	1.22 ± 0.27*	1.19 ± 0.28*	1.01 ± 0.27*	0.54 ± 0.14

Table 5: Biochemical characteristics of HD and CTR patients. Pre-HD^0, samples withdrawn before a dialysis session; Post-HD^0, samples withdrawn after a dialysis session; Pre-HD^1, samples withdrawn at the end of the longest interdialytic interval and before the next dialysis treatment. Data are reported as mean ± SEM; $^*p<0.05$ and $^{**}p<0.01$ were referred to CTR; $^\$p<0.01$ was referred to Post-HD^0. CRP, C-reactive protein.

	Pre-HD^0	Post-HD^0	Pre-HD^1	CTR
	(n=15)	(n=15)	(n=15)	(n=17)
t-MDA (nmol/ml)	3.316 ± 0.239$^{*\circ}$	2.520 ± 0.294*	3.085 ± 0.285$^{*\circ}$	1.771 ± 0.247
b-MDA (nmol/ml)	1.852 ± 0.288*	2.068 ± 0.365*	1.868 ± 0.305*	1.079 ± 0.221
f-MDA (nmol/ml)	1.464 ± 0.361$^{*\$}$	0.654 ± 0.08*	1.316 ± 0.386$^{*\$}$	0.047 ± 0.012
Ratio f-MDA/b-MDA	0.841 ± 0.288$^{*\circ}$	0,362 ± 0.088*	0.785 ± 0.333$^{*\circ}$	0.043 ± 0.012
f-MDA/creatinine (nmol/mg)	14.66 ± 3.93$^{\varepsilon}$	24.56 ± 2.89$^{\varepsilon}$	13.25 ± 4.28$^{\varepsilon}$	-

Table 6: Total, free and bound malondialdehyde levels in HD and CTR patients. Pre-HD^0, samples withdrawn before a dialysis session; Post-HD^0, samples withdrawn after a dialysis session; Pre-HD^1, samples withdrawn at the end of the longest interdialytic interval and before the next dialysis treatment. Data are reported as mean ± SEM; *p<0.001 respect to CTR; $^{\circ}$p<0.01 respect to Post-HD^0; $^{\$}$p<0.001 respect to Post-HD^0. $^{\varepsilon}$p<0.01 respect to Post-HD^0.

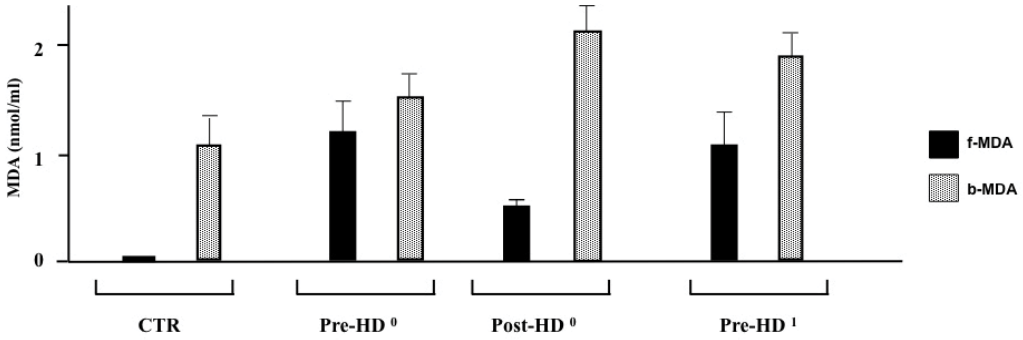

Figure 4: Serum levels of f-MDA and b-MDA in 10 patients and 17 CTR. Pre-HD^0, samples withdrawn before a dialysis session; Post-HD^0, samples withdrawn after a dialysis session; Pre-HD^1, samples withdrawn at the end of the longest interdialytic interval and before the next dialysis treatment; controls, healthy individuals. Columns represent the mean values ± SEM.

(p<0.001). Referring to b-MDA as a marker of an older injury [4,37], this behavior could be indicative of a well-established status of lipid peroxidation, confirming the presence of a sustained oxidative stress during the HD therapy.

Conclusion

The aim of the study was to develop an improved HPLC method for the detection of f-MDA and t-MDA in human serum, using DNPH as a derivatization agent. Although there are many methods reported for the determination of MDA, such as capillary electrophoresis, ultraviolet assay, GS-MS, LC-MS or LC-MS/MS, the HPLC technique is the most widely used. The method we proposed has reached a sensitivity index close to the detection limits of GC-MS and LC-MS techniques which require extensive sample preparation and sophisticated equipment, thus making them difficult to use in an ordinary laboratory. In addition, the method we presented is inexpensive and easier to use in a laboratory. Although MDA absorbs ultraviolet light, a chemical derivatization of the MDA molecule was developed in order to make the molecule more stable and increase the analytical sensitivity. The first and most common analytical method for MDA evaluation is based on its derivatization with TBA. However, it was proved that the TBA method is not specific for MDA and requires a rather high derivatization temperature, which makes it difficult to be used as a routine technique for the analysis of MDA in biological samples. Over the years, other derivatization reagents were developed for MDA evaluation, such as Phenylhydrazine (PH), 2,4,6-tri-chlorophenylhydrazine, 2,4-Dinitrophenylhydrazine (DNPH), Diaminonaphthalene (DAN), dansyl hydrazine, rhodamine B hydrazide, 3-nitrophenylhydrazine and pentafluorophenyl hydrazine. Quantification of MDA as a marker of lipid peroxidation in human serum samples after derivatization with DNPH and HPLC separation of hydrazone derivatives is a relevant technique in many research fields. We developed an improved method in which the main reaction parameters (incubation temperature, incubation time of DNPH derivatization and preparation of the derivatization reagent) were experimentally evaluated in order to optimize the MDA-DNPH adduct production. Using this method, we obtained f-MDA and t-MDA through acidic precipitation and alkaline hydrolysis followed by another acidic precipitation. We used ACN as a solvent for the derivatization agent (instead of HCl) and observed that the formation of MDA-DNPH hydrazone has increased by 2.21 times. By extending the incubation time, as opposed to other derivatization methods, we managed to increase the production of MDA-DNPH adduct by 3.8 times. In this study, we applied the present method to analyze the biological samples of hemodialysed patients during an entire dialysis cycle. Considering that these patients presented a marked oxidative stress status highlighted by the increased ROS-determined lipid peroxidation, we believe that our simple yet sensitive assay could be suitable for evaluating f-MDA and b-MDA as lipid peroxidation markers in pathophysiological processes associated with elevated OS, such as ischemia-reperfusion disorders, inflammatory events especially atherosclerosis, the aging process, chronic inflammation, degenerative diseases as well as various forms of cancer.

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Conflicts of Interests

Authors declare that there is no conflict of interest.

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