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Rapid quantification of malondialdehyde in plasma by high performance liquid chromatography–visible detection

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Abstract

Background: Malondialdehyde (MDA) is one of the better-known secondary products of lipid peroxidation, and it is widely used as an indicator of cellular injury. The employment of the thiobarbituric acid reactive substances (TBARS) technique to measure MDA has received criticism over the years because of its lack of specificity. Thus, a specific and reliable method for MDA determination in plasma by high performance liquid chromatographic (HPLC)–VIS was validated; alkaline hydrolysis, *n*-butanol extraction steps and MDA stability were established.

Methods: The plasma underwent alkaline hydrolysis, acid deproteinization, derivatization with TBA and *n*-butanol extraction. After this, MDA was determined at 532 nm by HPLC–VIS. The method was applied to 65-year-old subjects from a retirement home.

Results: The assay was linear from 0.28 to 6.6 μM. The reproducibility of intra-run was obtained with CV% < 4% and the inter run with CV% < 11%. The accuracy (bias) ranged from 2 to −4.1%, and the recovery was greater than 95%. The limit of detection (LOD) and limit of quantification (LOQ) were 0.05 and 0.17 μM, respectively. For the stability test, every sample was stored at −20 °C. The plasma MDA was not stable when stored after the alkaline hydrolysis step, remained stable for 30 days after TBA derivatization storage and was stable for 3 days when stored after *n*-butanol extraction. The elderly subjects had MDA plasma levels of 4.45 ± 0.81 μM for women and 4.60 ± 0.95 μM for men.

Conclusion: The method is reproducible, accurate, stable, sensitive, and can be used in the routines in clinical laboratories. Besides, this technique presents advantages such as the complete release of protein bound MDA with the alkaline hydrolysis step, the removal of interferents with *n*-butanol extraction, mobile phase without phosphate buffer and rapid analytical processes and run times.

Keywords: MDA; HPLC–VIS; Human plasma; Alkaline hydrolysis; *n*-Butanol extraction

1. Introduction

Free radicals and reactive oxygen species (ROS) are continuously produced by cells as part of their metabolic processes [1]. The overproduction of reactive species results in oxidative stress, a combination of an increased formation of oxygen–nitrogen derived radicals and reduced antioxidant capacity, causing an imbalance that always results in the attack of cellular components, especially lipids [2].

Malondialdehyde (MDA) is one of the better-known secondary products of lipid peroxidation, and it can be used in biomaterials as an indicator of cell membrane injury [3], being that oxidative stress has been implicated in the pathogenesis of various diseases, including diabetes [4], cancer [5], atherosclerosis [6,4], renal [7] and Alzheimer diseases [8].

A variety of methods described in the literature allow the detection and determination of MDA in biological matrices. The main method utilized is the reaction of MDA with thiobarbituric acid (TBA). In 1968, Yagi et al. [9] applied the reaction of TBA with MDA and linked chromogens to lipoperoxides in biomaterials, resulting in the well know method, "thiobarbituric acid reactive substances" (TBARS). Its product can be detected by

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colorimetry (532–535 nm) or fluorimetry (excitation at 532 nm and emission at 553 nm) [10]. Although this method is easy and inexpensive, the use of the TBARS test has received wide criticism over the years. The major problem with this method is the lack of specificity, since TBA reacts with a variety of compounds, such as sugars, amino acids, a variety of aldehydes and bilirubin, producing interference with colorimetric and fluorimetric measurements of MDA [10,11].

Therefore, several high performance liquid chromatographic (HPLC) methods have been reported for the determination of MDA, with colorimetric and fluorimetric detection [12–16]. This technique is more specific, reliable and reproducible, as MDA is separated from other interfering substances.

In recent years, several innovations have been introduced to improve the specificity of the old procedures. One of these is the difference between detection of free MDA and total plasma MDA [13]. Free MDA is unbound to plasma proteins and it can be detected without any hydrolytic sample treatment. On the other hand, a significant amount of MDA may be bound to matrix molecules, and therefore undetectable without an adequate step to liberate it [17].

In this work, a rapid method with a plasma alkaline hydrolysis step is presented to quantify MDA by a chromatographic procedure based on an isocratic elution without phosphate in the mobile phase. Furthermore, sample and reaction stability were evaluated, time-dependent analysis conditions were established and a sample extraction step with *n*-butanol was added to improve the method.

2. Material and methods

2.1. Chemicals and reagents

HPLC grade methanol and *n*-butanol were purchased from Tedia Company (Fairfield, USA). Thiobarbituric acid and malondialdehyde bis (dimethylacetal) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All the other chemicals used were of analytical grade. Aqueous solutions were prepared in Milli-Q water (Millipore, Bedford, MA, USA).

2.2. Instrumentation and chromatographic conditions

The chromatographic equipment consisted of a gradient chromatography system Knauer® apparatus, WellChrom model, equipped with a quaternary pump, reservoir for solvents, dynamic mixer, an online vacuum solvent degasser with four canals, manual injector with loop of 20 μl and UV–VIS detector. Chromatographic control, data collection and processing were carried out using EUROCHROM 2000 SOFTWARE®, basic edition, 2.05 for Windows.

The separation was achieved using a reverse-phase column: Eurospher-100 150 mm × 4 mm with 5 μm particle size and a guard column Eurospher-100 5 mm × 4 mm with 5 μm particle size.

The mobile phase was a mixture of Milli-Q water and methanol (50:50, v/v). The flow rate was maintained isocratically at 0.6 ml/min, the absorbance of the eluent was monitored at 532 nm and the total run time was 8 min. The column was thermostated at 40 °C in a thermostatization system for chromatographic columns (Chromacon®).

2.3. Sample preparation

2.3.1. Standard solutions

Malondialdehyde bis (dimethylacetal) was used as standard. After water dilution, 3 mM MDA stock solution was stored in aliquots of 200 μl at −20 °C and remained stable for at least 18 months. To prepare MDA working standard solutions, 3 mM solution was diluted in water obtaining concentrations ranging from 0.28 to 6.6 μM. These solutions were prepared daily.

2.3.2. Blood samples

The Ethics Committee of the Center of Health Sciences approved the study and all participants gave informed consent.

Blood samples (5 ml) from humans were drawn by venipuncture into 7 ml evacuated tubes containing an EDTA solution as anticoagulant. Blood samples were maintained at 0–4 °C until centrifugation.

2.3.3. Sample procedure

The blood samples were centrifuged at 1500 × *g* for 10 min in refrigerated centrifuge (4 °C) and supernatant plasma was removed with care to avoid contamination with platelets.

A volume of 75 μl of plasma was added to 25 μl of standard or water and 25 μl of NaOH 3N and incubated at 60 °C for 30 min in a shaking water bath system. After this, 125 μl of H_3PO_4 6% and 125 μl of TBA 0.8% were added and the mixture was heated at 90 °C for 45 min. Then, the mixture was cooled, 50 μl of 10% sodium dodecyl sulfate (SDS) was added and extraction with 300 μl of *n*-butanol was carried out by vortex-mixed for 1 min and centrifuged at 3000 × *g* for 10 min. Twenty microliters of the butanol layer was injected into HPLC with a visible detector, using a reverse-phase column and eluted as described previously.

2.4. Assay validation

2.4.1. Linearity, precision, accuracy, recovery and sensitivity

Linearity is the ability to show that the results are directly proportional to the analyte concentrations in samples within a given range. This was determined by five analytical curves with 0.28, 0.56, 1.7, 3.4 and 6.6 μM of MDA spiked in plasma. Plasma without spiked MDA was considered to be a basal level. The curves were prepared on five different days, with five different plasma samples, and the linear regression was evaluated.

The precision of a method is determined by the extent to which the test results of multiple injections of standards agree. It can be subdivided into repeatability or intra-run precision and intermediate precision or inter-run. Accuracy is the extent to which the results generated approached the real value. The intra- and inter-run precisions and accuracy of the method were

evaluated on five separated days, with five different plasma samples. Three standard concentrations (0.28, 1.7 and 6.6 μM) were carried out in replicate and injected into HPLC in triplicates. The intra- and inter-run precisions were calculated by variation coefficient, in percentage. The accuracy was expressed as a percentage of bias.

Recovery is reported as the extraction efficiency of an analytical process, reported in percentage. Recovery was conducted by comparing peak areas of replicates from plasma spiked with MDA (0.28–6.6 μM) with those of aqueous standards. It was prepared on five different days, with five different biological samples.

The limits of detection (LOD) and quantification (LOQ) were calculated on the basis of a signal-to-noise ratio of 3:1 and 10:1, respectively [15].

2.4.2. Stability

For the assessment of MDA stability in plasma after storage, a fresh sample was drawn and separated in aliquots. A replicate was analyzed at the time of collection. Part of the sample underwent alkaline hydrolysis, was stored at −20 °C and was analyzed after 24 h. For yet another part of the plasma, the reaction progressed until reaching TBA derivatization, was stored at −20 °C and was analyzed over a 30-day period. Finally, all reaction steps were developed, stored at −20 °C and MDA plasma stability after extraction with *n*-butanol was evaluated at 1, 2, 3 and 4 days.

The MDA working standard solution stability was also tested. Solutions of 80.0, 20.6 and 3.4 μM were prepared at the time of the analyses and their peak areas were measured at the time of the test and after 6 h.

2.4.3. Ruggedness

The ruggedness of the method was tested in plasma samples by varying several chromatographic parameters, such as mobile phase pH, mobile phase composition, flow rate, temperature, chromatographic columns and analysts.

2.4.4. Application

Blood samples were collected from 45 women and 20 men from two retirement homes in Santa Maria. All subjects were 70 ± 10 years and they did not smoke or drink, and did not practice exercise. Moreover, a nutritionist controlled their diets.

2.4.5. Statistical methods

The analysis of the data was carried out using software SPSS 10.0 for windows with Student's *t*-test for independent samples. All results were expressed as mean ± standard deviation. A value of $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Chromatographic separation

The chromatographic separation of plasma MDA was satisfactory with a mobile phase of Milli-Q water:methanol (50:50). Typical chromatograms of extracted aqueous blanks and plasma spiked with MDA (concentration of 1.7 μM) are shown in Fig. 1. The relative retention time for MDA was 1.8 min and the total chromatographic run time was 8 min, demonstrating that the method is quick and a large number of analyses can be done during 1 day.

It is well known that high concentrations of phosphate salts in mobile phase, such as 50 mM [16,18,19] can precipitate on the chromatographic system or chromatographic column and increase chromatographic pressure or damage the system. Therefore, this problem was not observed because it was not used KH_2PO_4 in this methodology.

To confirm the best chromatographic conditions, several parameters were tested, including different KH_2PO_4 concentration, methanol percentage and temperature of the column (Fig. 2). It was observed that there was no difference in the peak area without KH_2PO_4. Furthermore, the methanol percentage variation was tested, from 35 to 50%, and the best peak area was found with 50% of methanol. The temperature of the column was checked between 25 and 45 °C. The peak area was notably increased when the temperature increased. This occurred up to 40 °C; above this, the peak area decreased and the efficiency of the column was altered.

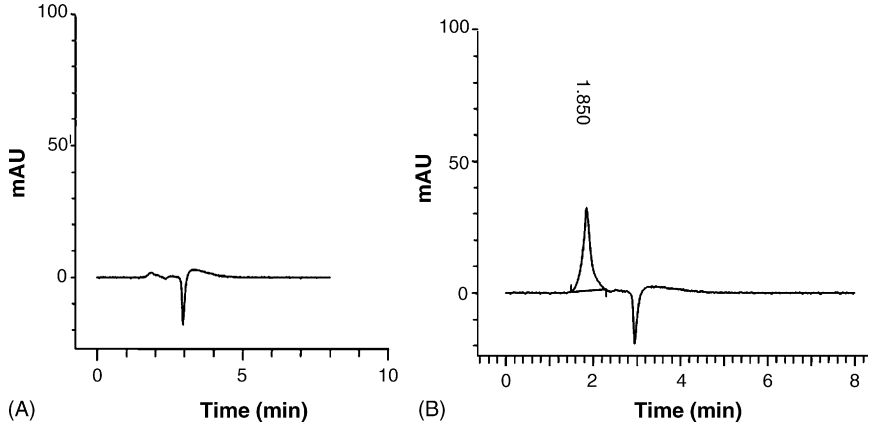

Fig. 1. It shows a typical MDA chromatogram. In (A) aqueous blank; in (B) plasma spiked with MDA 1.7 μM, both extracted with *n*-butanol.

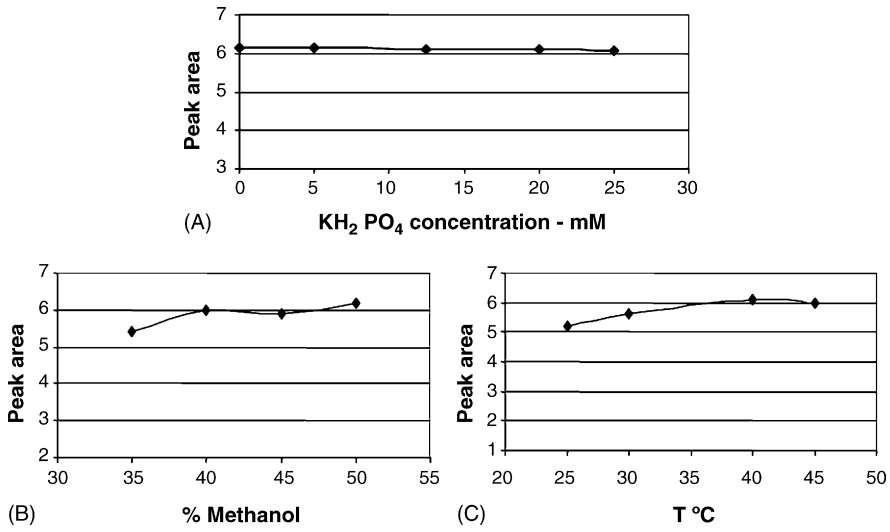

Fig. 2. Parameters analyzed to verify the best chromatographic conditions. (A) Change in KH_2PO_4 concentration. Peak areas did not show important difference, thus the best concentration to the chromatographic system is with water. (B) Variation of methanol percentage in mobile phase; 50% was the option. (C) Temperature was tested from 25 to 45 °C and the best peak area and column efficiency was obtained with 40 °C.

3.2. Validation

3.2.1. Linearity, precision, accuracy, recovery and sensitivity

After choosing the pre-treatment procedure and establishing the chromatographic conditions for the analysis, the method was validated [20,21]. Biological samples were collected and analytical plasmatic curves were prepared with pooled human plasma in duplicate, on five different days by different analysts. The concentration of plasma not spiked with MDA was determined as the basal level. The pooled human plasma was spiked with 0.28, 0.56, 1.7, 3.4 and 6.6 μM of MDA. Analytical aqueous curves were obtained with the same concentrations as for MDA.

Linearity and reproducibility were evaluated by linear regression. The equations obtained by the least squared regression were $y = 4.7412x + 2.0451$ for plasma curves and $y = 4.9665x - 0.0387$ for aqueous curves, and the values to r^2 were 0.9984 and 0.9995 for plasma and aqueous curves, respectively. Analytical curves (i.e., peak area of each concentration from spiked plasma against area from aqueous MDA standards) showed excellent linearity and parallelism between MDA aqueous standard solutions over the concentration range of 0.28–6.6 μM with a correlation coefficient > 0.995.

The intra-and inter-run precision (expressed in percentage by variation coefficient) and the accuracy (expressed as percentage of bias) based on peak area ratios are presented in Table 1. The overall intra-run precision was less than 4% and the inter-run precision for all concentrations was less than 15%. The assay bias ranged from 2.0 to −4.1%.

Table 1
Validation data precision and accuracy for plasma spiked with MDA ($n = 5$)

Concentration (μM)	Intra-run precision (%)	Inter-run precision (%)	Accuracy (% bias)
0.28	2.80	10.4	−3.2
0.56	3.74	7.7	−1.1
1.7	0.9	10.9	0.7
3.4	3.9	11.0	−4.1
6.6	3.64	9.8	1.8

The analytical plasma recovery was calculated in plasma spiked with 0.28, 0.56, 1.7, 3.4 and 6.6 μM of MDA, which had recoveries of 97.5, 98.9, 100.7, 95.9 and 102.7%, respectively. This demonstrated that MDA was preserved during all pre-treatment procedures.

LOD and LOQ were determined evaluating the signal-to-noise, resulting in 0.05 μM for LOD and 0.17 μM for LOQ.

3.2.2. Stability

For the stability of plasma samples stored at −20 °C after the different reaction steps, the results are shown in Fig. 3. Samples stored after alkaline hydrolysis were found to be unstable. There was a high loss of MDA when the plasma was stored after this step (about 50%). However, when the sample was stored after TBA derivatization, it was stable for 30 days. After extraction with *n*-butanol, the sample remained stable only for 3 days. All the samples above were stored at −20 °C.

In relation to the MDA working standard solution stability, the solutions did not prove to be stable. It was only possible to use these solutions at the time of their preparation. MDA was degraded after 6 h, even though the solutions were stored at −20 °C.

3.2.3. Ruggedness

Methodological robustness was considered satisfactory under different chromatographic conditions, such as chromatographic columns and mobile phase constituents of different production lots. Samples of different subjects were provided on different days and separate analysts carried out the procedure. The temperature ranged from 38 to 42 °C and the flow rate from 0.5 to 0.7, with satisfactory results. The results found during the validation showed that this method could be used suitably for plasma MDA quantification.

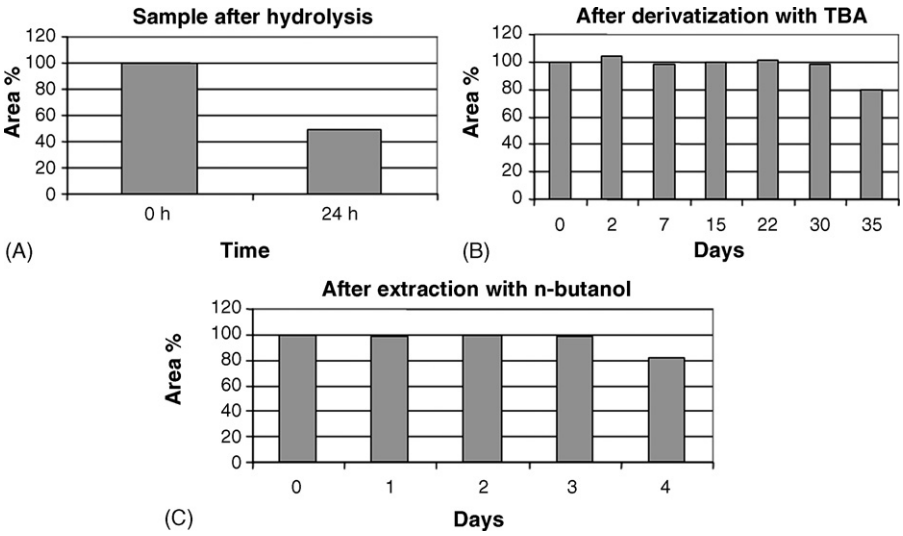

Fig. 3. Stability of the samples stored at −20 °C. Day 0 represents the MDA measured on time of the draw. In (A) MDA was measured after alkaline hydrolysis step, in 24 and 48 h of storage. In (B) samples were stored after TBA derivatization. In (C) there were extraction of the sample with *n*-butanol; after this, aliquots were stored and analyzed in 4 days.

3.3. MDA quantification and application

Although the determination of MDA is one of the most commonly used methods for monitoring lipid peroxidation in biological samples and various HPLC-based TBA methods exist, the results often vary with the method used. By using a modified HPLC-based TBA test including an alkaline hydrolysis of the plasma, the present study provided an MDA plasma level of 4.45 ± 0.81 μM for women and 4.60 ± 0.95 μM for men, considering the plasma dilution.

To ensure that no lipid oxidation occurs during the assay, it is common to add BHT, an antioxidant, to the plasma before TBA incubation. However, the study by Pilz et al. [17] demonstrated clearly that the reduced MDA level is merely the effect of ethanol alone and not of the BHT. In this work, it was tested a plasma aliquot with ethanol and others with and without BHT. The results obtained were 6.0 μM for samples without BHT, 2.8 μM for sample with BHT and 2.8 μM with ethanol, showing that the reduction in the level of MDA is due to the effect of ethanol, probably by the deproteinization effect. Therefore, it was not include BHT in this study.

Alkaline hydrolysis is a critical step, being that MDA can be both free and bound to proteins, and the majority of the research has measured only free MDA. In a recent study, it was found that the alkaline hydrolysis of plasma with NaOH led to a nearly two-fold increase in the MDA-TBA level [13]. Here, both free and bound MDA were analyzed, and the results obtained were 2.2 and 5.5 μM to free and bound MDA, respectively, showing that the bound MDA level is actually two-fold bigger than the free MDA level. The concentration of NaOH and the hydrolysis time were also tested. For the NaOH concentration, there was no significant difference in 3 and 6N, therefore 3N was chosen. The hydrolysis time ranged from 30 to 60 min, without a difference in the MDA level.

3.4. Advantages of the method

In the present method, MDA measure can be carried out 30 days after derivatization with TBA if the samples are kept at adequate temperature (−20 °C). Stability is an important methodological aspect in human disease and oxidative stress studies, as it is possible to carry out posterior analyses, without false results.

In addition, the alkaline hydrolysis step was important because it led to a more complete and uniform release of protein bound MDA and it gave the real value of MDA in the subjects.

The method was improved with the *n*-butanol extraction step before injection into the chromatographic system. The extraction removed the interferents and extended the lifetime of the column by removing contaminants from the incubation mixture.

An important modification of the method was the mobile phase without using the KH_2PO_4. The employ of water and methanol in mobile phase prevented the damage of the chromatographic system.

Plasma MDA quantification is of interest because the alteration of their levels may reflect similar variations in less accessible tissues, and it is well known that MDA is a good marker of oxidative stress in a large number of diseases.

4. Conclusion

An analytical method for plasmatic MDA quantification by HPLC–VIS was optimized and validated using TBA derivatization. TBA is not expensive, and visible spectrophotometric detection is widely utilized. Moreover, the optimized method is very simple. The results obtained for elderly subjects were considered to be reference values for this age band.

The method presented advantages such as the complete release of protein bound MDA with the alkaline hydrolysis step, extraction with *n*-butanol, which extracted interferents, and the optimization of chromatographic conditions, including no salt

concentrations in the mobile phase. Moreover, the stability of plasmatic MDA was studied at different steps of the methodology, showing that the plasma sample after derivatization with TBA is stable for 30 days, which is important for studies on oxidative stress for posterior analyses with real values.

The analytical process is rapid and the run time of each sample is just 8 min. The method is stable, accurate, reproducible, sensitive and can be used in routines in clinical laboratories.

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