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Malondialdehyde levels can be measured in serum and saliva by using a fast HPLC method with visible detection

Determinarea printr-o metodă HPLC-VIS rapidă a concentraţiilor serice şi salivare ale malondialdehidei

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Abstract

Oxidative stress appears when the amount of free radicals that are formed in a living organism exceed its spin-trapping ability. One of the most dangerous free radicals that are formed in the human body is the hydroxyl radical. It can alter several biomolecules, including the unsaturated fatty acids; this process is known as lipid peroxidation and can lead to cell necrosis and generation of several harmful byproducts including malondialdehyde, which serves also as a biomarker of oxidative stress. A new HPLC method with visible detection was developed for the detection of malondialdehyde in human serum and saliva samples. The method was verified in terms of specificity, linearity, limits of detection (0.35 ng/ml), limit of quantification (1.19 ng/ml), recovery (90.13±10.25 - 107.29±14.33) and precision (3.84±1.49% - 6.66±1.76%). An analysis time of only 1 minute was obtained and no interferences from the matrices were observed. Statistical analysis (Pearson correlation test) showed a moderate correlation (R = 0.5061, p = 0.0099) between serum and saliva concentrations (N = 25). The possibility of measuring salivary concentrations of malondialdehyde extents the applications of oxidative stress/lipid peroxidation estimations to categories of population unreachable before (pregnant women, small children, etc); repeated sample studies are also easier to make.

Keywords: *malondialdehyde, serum, saliva, HPLC.*

Rezumat

În urma dezechilibrului apărut între sistemele prooxidante/antioxidante aflate în celule, în favoarea prooxidanților, apare fenomenul numit stres oxidativ. Unul dintre cei mai reactivi radicali liberi este radicalul hidroxil. Biomoleculele cel mai des afectate de acest radical sunt acizii graşi polinesaturaţi, fenomen numit peroxidare lipidică. Peroxidarea lipidelor poate duce la necroza celulară şi la eliberarea mai multori molecule secundare periculoase ca malondialdehida, care este un bun biomarker al stresului oxidativ. În cadrul acestui studiu am reuşit dezvoltarea unei metode rapide de determinare a malondialdehidei serice şi salivare. S-au verificat specificitatea, linearitatea, limita de detecţie (0.35 ng/ml), limita de cuantificare (1.19 ng/ml), recuperarea (90.13±10.25 –107.29±14.33) şi precizia (3.84±1.49% – 6.66±1.76%) metodei. Timpul de analiză a fost de doar un minut. Analiza statistică a

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demostrat o corelaţie moderat pozitivă (R = 0.5061, p = 0.0099) între concentraţia malondialdehidei serice şi salivare (N = 25). Corelaţia moderată poate să fie un punct de pornire în folosirea malondialdehidei salivare ca marker al peroxidării lipidice, recoltarea probelor fiind mai puţin invazivă ca în cazul sângelui. De asemenea, se poate aplica la categorii de persoane cu restricţii la prelevarea probelor de sânge (sugari, femei însărcinate, etc.). Recoltarea salivei permite de asemenea efectuarea cu uşurinţă a unor studii ce necesită prelevare repetată timp îndelungat.

Cuvinte cheie: *malondialdehida, ser, saliva, HPLC.*

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Introduction

Oxidative stress is a highly studied process in which the amount of the generated free radicals exceeds the spin-trapping ability of a living organism. One of the most important free radicals that contributes to the oxidative stress is the hydroxyl radical (HO·). It is considered the most reactive free radical species in the human organism and has a half-life of about 10^{-9} s. It can be formed through water irradiation with UV light or higher energy electromagnetic or particle beams, by the well-known Fenton reaction and also by low pH decomposition of peroxynitrite. Fenton reaction involves the reaction of H_2O_2 with metals in their low oxidation status, Fe^{2+} being the most commonly encountered [1-4].

Because of the high reactivity of the hydroxyl radical, there are no natural antioxidant defenses against it, therefore all biological molecules can be used as targets for the hydrogen abstraction process. DNA, proteins and lipids are the most commonly encountered biomolecules that are modified/damaged by the hydroxyl radical. One of the most studied reactions of the hydroxyl radical with a biomolecule is the lipid peroxidation that involves the unsaturated fatty acids. This process leads to the damage of the cell membrane and if it can not be repaired, it will lead to cell necrosis. One of the most studied biomarkers of the lipid peroxidation process is malondialdehyde (MDA). It can be considered not only a biomarker but even an "ultimate toxicant" of the hydroxyl radical [3,5-8].

Measurement of malondialdehyde has been done in several animal and human biological samples. The most commonly and widely available method is based on the reaction of malondialdehyde with thiobarbituric acid when a red condensation product with a maximum of absorption at 532 nm is formed [9-13]. It can be detected spectrophotometrically after extraction with organic solvents (n-butanol, isobutyl alcohol) [14], it can be analyzed by commercial kits [8,15-17]. The major problems with this method are the lack of sensitivity and the occurrence of byproducts because the byproducts absorb light near 532 nm (maximum of absorption of MDA-$(TBA)_2$) [18, 19]. HPLC methods with VIS or fluorescence detection were tried but neither the analysis time nor method sensitivity are advantageous [20]. Direct detection of malondialdehyde has been tried with limited utility [21, 22].

MDA is the main product of lipid peroxidation and it is found as an oxidative stress indicator. The aim of this study was to establish a new, simple and inexpensive method for MDA measurement, which can be easily used for testing the oxidative status in a non-invasive (saliva) sample. In case of positive correlation between serum and salivary MDA concentration, this new method has the potential to become a new standard technique for the assessment of oxidative stress in clinical practice. Several studies described MDA determination from biological samples, but most of them have reduced sensi-

tivity and specificity, they also have long analysis time [23,24]. The purpose of this work is to develop a fast and highly sensitive HPLC method with visible domain detection for measurement of malondialdehyde in human serum and saliva samples. The method will be based on the reaction of malondialdehyde with thiobarbituric acid. Validation of the method for saliva samples would bring the possibility of estimating the oxidative damage status in special populations, too (pregnant women, small children, etc), where the blood sampling procedure for such experiments could be contraindicated. Checking for a possible correlation between serum and saliva samples is another purpose of this work.

Material and methods

All common chemicals and reagents were purchased from local providers and were used without any further purification: acetonitrile (VWR International S.A.S., Fontenay-sous-Bois, France), methanol (Sigma Aldrich Chemie GmbH, Steinheim, UK), anhydrous Na_2HPO_4 (Merck KGaA, Darmstadt, Germany), 96% H_2SO_4 solution (Chemical Company, Iasi, Romania), 85% H_3PO_4 solution (Merck KGaA, Darmstadt, Germany). Ultra-pure water was obtained using a Milli-Q purification system (Merck Millipore Corporation, USA). Malondialdehyde is an unstable compound, therefore 1,1,3,3-Tetramethoxypropane (TMP, 99%, Sigma-Aldrich, China) was used for preparation of the standard solutions [25,26]. Thiobarbituric acid (98%) was purchased from Sigma-Aldrich, Germany.

Approval of the Ethics Committee for scientific research of University of Medicine and Pharmacy Tîrgu Mureş was obtained for the experiments involving biological material (No. 38/21.03.2016); these experiments were made according to international regulations.

HPLC analysis was carried out on a Merck HPLC system consisting of: quaternary pump Merck Hitachi L-7100, auto sampler Merck Hitachi L-7200, column thermostat Merck Hitachi L-7360, DAD detector Merck Hitachi L-7455, interface Merck Hitachi L-7000, solvent degasser Merck Hitachi L-7612, software D-7000 HSM-Manager, Supelcosil™ LC-18 (3 µm) SUPELCO Column 3.3 cm x 4.6 mm, Supelco Column Saver 0.5 µm Filter.

Final chromatographic condition:

Mobile phases: acetonitrile (A), phosphate buffer (20 mM, pH=6) (B); isocratic elution was used: A 12% and B 88%; injection volume: 100 µl (loop mode), analysis time 1 minute. A constant flow rate of 1 ml/min was employed, and the eluent was monitored at 532 nm by a diode array detector; spectral scans were collected over the range of 300 to 600 nm.

Sample, calibration and spiked samples treatment

Whole blood was collected from healthy volunteers in serum separator tubes. After the collection, the blood was allowed to clot by leaving it undisturbed at room temperature for 15 minutes. The clot was removed by centrifugation at 1,500 x g for 10 minutes. The resulting supernatant (serum) was immediately transferred into clean tubes and stored at -20°C until analysis.

Saliva was obtained from volunteers in the morning in a "non-stimulated fashion by spitting", at the same time with blood sampling procedure. Oral hygiene was performed 1 hour before the saliva sampling. About 3 ml of saliva were sampled in a clean plastic tube. The collected samples were centrifuged at 1,500 x g, and the supernatant was stored at -20°C for up to one week.

Preparation of MDA standard:

30 µl TMP were accurately diluted to 100 ml with ultra-pure water. The concentration of MDA stock solution was 0.13 mg MDA/ml

(1.8 mM). The standard solutions were prepared from MDA stock solution by pipetting 0.01-5 ml of the stock solution into a 50 ml calibrated flask and diluted to volume with ultra-pure water. 490 µl water, serum or saliva were spiked with 10 µl of standard solutions with different concentrations of MDA. The final concentration of the MDA in the reaction mixture was between 0.52 ng MDA/ml – 262 ng MDA/ml water, serum or saliva. The preexisting content of saliva and serum should be considered to the added spiking amount, as well.

After homogenization, 1.5 ml acetonitril was added in order to precipitate the proteins. Then, the samples were centrifuged at 1500 x g for 5 min. 1.5 ml TBA 4 mg/ml and 2.5 ml H_2SO_4 50 mM were added to 1 ml supernatant. The tubes were vortexed, then the mixture was heated immediately for 30 min at 95˚C, then cooled on ice. An aliquot was analyzed by injection into the HPLC system. All standard and spiking solutions were prepared the same day the analysis took place and the samples were analyzed shortly after the derivatization process.

Statistical analysis was performed with GraphPad Prism 5, GraphPad InStat software and Microsoft Excel 2003. All data are presented as mean ± SD. Pearson test for correlation and Kolmogorov-Smirnov test for data distribution were used. The level of significance was set at p below 0.05.

Results

Optimization of the HPLC method

Several pH values, combination of mobile phases (methanol and acetonitril), and HPLC columns (columns with length of 3.3, 15 and 25 cm; particle sizes of 3 and 5 µm; regular C18 and RP-selectB fillings) were tried. With the use of the DAD detector, several wavelengths were followed for the best signal to noise ratio. The lack of any interference from serum, at 532 nm, allowed the use of a short HPLC column and elution of the peak of interest at a short time interval after the interferences brought by the injection (injection peak) ceased. The pH of 6 was found to be the optimal solution in terms of retention time and the percentage of organic solvent that was needed in the mobile phase (an organic phase percentage of about 10 is the best option regarding method cost and column life expectancy).

Specificity

The reaction of malondialdehyde with thiobarbituric acid has been widely employed in the spectrophotometric detection of malondialdehyde in several biological samples. However, in order to ensure a good specificity of the method, the following blank samples were injected: thiobarbituric acid without malondialdehyde, malondialdehyde without thiobarbituric acid, serum and saliva samples without thiobarbituric acid. All blanks were prepared according to the sample preparation procedure with the sample or

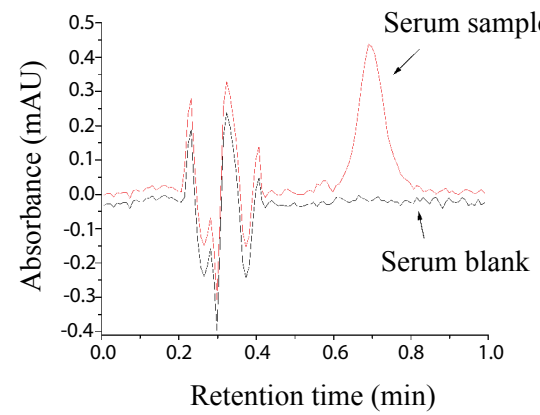

Figure 1. Chromatogram of a blank serum sample (containing malondialdehyde without thiobarbituric acid and the same sample with thiobarbituric acid).

reagent being replaced with purified water. Absolutely no interferences were recorded with the peak of the derivatized malondialdehyde when the blanks were injected into the HPLC system (**Fig 1**). The online diode array detector provided further evidence of specificity as a high peak purity (>99%) was obtained in all cases.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were calculated on the basis of a signal-to-noise ratio of 3:1 and 10:1, respectively. LOD and LOQ values were determined in water samples (impossible to measure in biological samples due to the fact that all biological samples contain MDA) was 0.35 ng/ml and 1.19 ng/ml, respectively. Because chromatograms obtained for water and biological matrices are similar and no hindrance in the detection was brought by the use of the biological matrices, it is reasonable to presume that these LOD and LOQ values are valid for biological samples, too.

Linearity

Solutions of malondialdehyde with seven different concentrations (N= 3) were used for the estimation of the linear response between the concentration of malondialdehyde (1.31 ng/ml – 262 ng/ml) and the peak area. Three sets of calibration standards were analyzed for the intraday linearity estimation.

Table 1. Method linearity parameters

	Slope	Intercept	Coefficient of correlation (R^2)	Coefficient of variation (%)
Water	0.0026	0.006	0.9998	1.24-10.01
Serum	0.0029	0.023	0.9997	2.28-8.20
Saliva	0.0026	0.022	0.9984	1.59-9.05

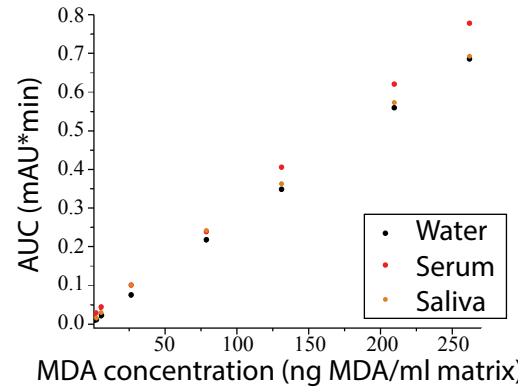

Figure 2. Calibration curves obtained for water and spiked matrices.

The residuals that did not correlate with the concentrations and had lower values than 10% proved the existence of an acceptable linear response in the detection of malondialdehyde. Coefficients of variations in the calibration curves did not exceed the 10% value.

Accuracy and precision

Accuracy was determined by spiking water, human serum and saliva (5 different concentrations: 2.62 - 13.1 - 52.4 – 104.8 – 157.2 ng/ml, n = 5 replicates) with known amounts of malondialdehyde. Accuracy values in the range of 90.13±10.25 –107.29±14.33% for each of the three matrices were obtained.

The precision of the assay was good on each of the three different matrices, with a mean relative standard deviation 3.84±1.49%, 5.04±2.44%, 6.66±1.76% in water, serum and saliva, respectively.

Correlation between serum and saliva concentrations of malondialdehyde

25 pairs of saliva and serum were simultaneously obtained from volunteers and analyzed. Statisti-

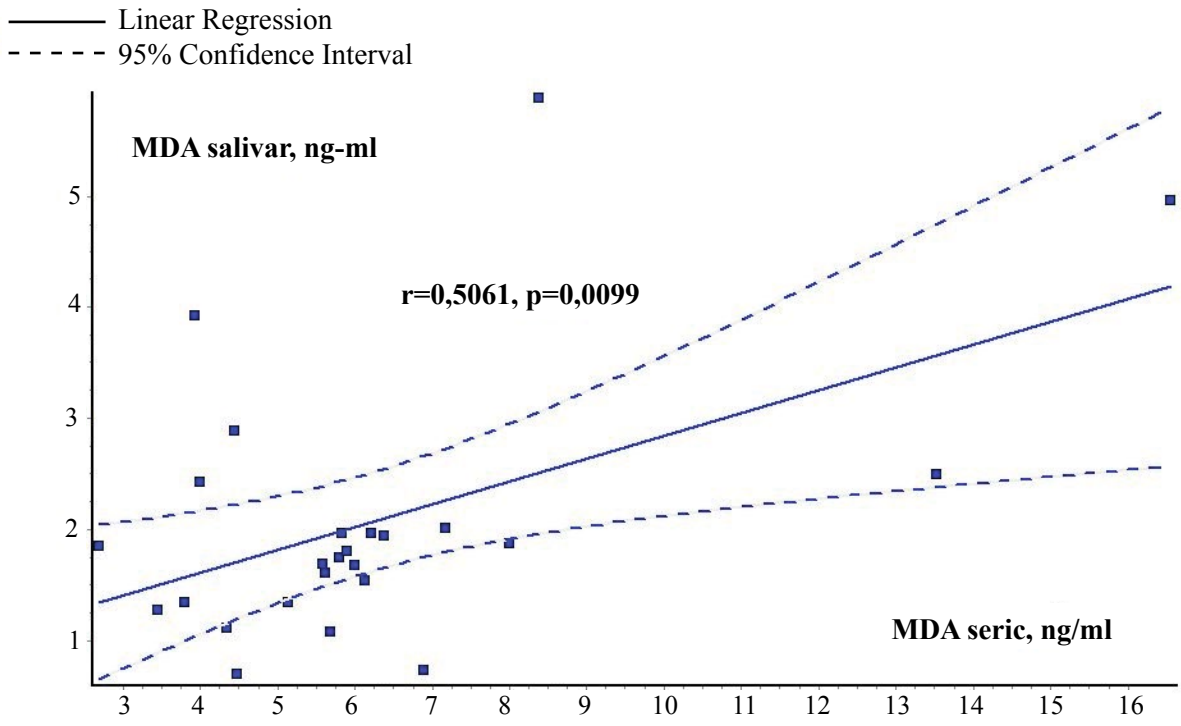

Figure 3. Correlation between serum and salivary MDA concentration

cal analysis (Pearson correlation test) showed a moderate correlation (R = 0.5061, 95% confidence interval: 0.1385-0.7511) between serum and saliva concentrations at a p value (two tailed) of 0.0099. The wide 95% confidence interval and the coefficient of correlation of only 0.5061 could be a result of the limited sample size that was used for this preliminary testing.

Discussions

The new method has good specificity, accuracy, precision and detectability and is usable for accurate measurement of low concentrations of malondialdehyde. The method is suitable for measurement of malondialdehyde in saliva or serum samples with higher selectivity compared to the spectrophotometric method which does not allow for blank compensation of reagent color or serum color at the same time [27]. The very short analysis time (1 minute), obtained using simple HPLC equipment, is another advantage of the method. Compared with other chromatographic methods [19,28-30] for the measurement of malondialdehyde, it can be seen that higher specificity (not only a retention time, as for direct detection, but also the ability to form a color is followed), detectability (lower LOD and LOQ values are attainable with the derivatization process rather than with direct UV detection due to elimination of the noise brought by the UV absorbance of the many compounds present in serum by reading at 530 nm) and analysis time are obtained. HPLC measurement of malondialdehyde in biological samples, after derivatization with TBA, was also tried using a fluorescence detector [31]; this, however, resulted in a method in which a longer

column was employed (longer analysis time), and a narrow concentration range in the calibration curve was obtained together with a lower detectability compared to our method.

The work we presented here has two shortcomings: the first one is the relatively low number of samples that has been used to correlate serum and saliva concentrations, the second one being the lack of data about the interference brought by colored substances traditionally used as antioxidants (antocyanins from red wine and other fruits, betanin from beetroot, and others). These possible sources of errors should be further investigated. However, the presence of colored substances with retention times similar to MDA should be easily investigated by making a serum blank without the derivatization reagent.

Measurements of salivary concentrations of MDA were made previously with the purpose of evaluating the status of periodontal disease [27, 31]. The values measured by us are similar to those found for healthy volunteers when an HPLC-fluorescence detection was employed [31].

Increasing the number of healthy volunteers and also similar studies on patients with different diseases will improve the power of the statistics and will clarify the situations in which saliva can be used as a blood replacement in the study of oxidative stress. However, the promising correlation that was obtained between serum and saliva concentrations opens the possibility of making oxidative stress assessments in situations that were previously impossible or almost impossible to make (pregnant women, small children, etc). Repeated sample studies are also easier to be made when only saliva is needed instead of serum.

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