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Research Article

Effects of Analytical Procedures on the Repeatability of Malondialdehyde Determinations in Biological Samples

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ABSTRACT

Background: Malondialdehyde (MDA) is a commonly used biomarker of oxidative stress in clinical studies and has been measured in many pathological conditions during last decades. Different analytical methods have been reported for determination of MDA in biological samples in which MDA was adducted with thiobarbituric acid (TBA) to produce more sensitive chromophore and also convert it to a fluorescent compound. In spite of the routine applications of this derivatization and subsequent analysis of MDA in biomedical studies, its reliability, repeatability and reproducibility is questionable. The aim of this work is to investigate the effects of some factors on the repeatability of MDA determinations in standard solutions and also in plasma samples using spectroscopic method.

Methods: MDA-TBA adduct is prepared in standard solutions and the effects of pH, temperature, reaction time, open, closed and reflux systems and the ratio of MDA and TBA is investigated by measuring the absorbance of the solution at 532 nm. These effects are also investigated in human plasma samples.

Results: The best results are obtained at pH 2.5, temperature of 70 °C, reaction time of 150 minutes, reflux system and ratio of 2.

Conclusion: Using the optimized conditions are resulted in better repeatability.

Introduction

Oxidative stress is a general term in biology and used to describe an imbalance between activities of oxidants and antioxidants of the biological system. It has been measured using various biomarkers and malondialdehyde (MDA) is the most frequently used one as a disease biomarker for more common health problems in medical sciences.[1-5] Although the results of case-control comparisons using statistical analyses showed significant increase in the case groups, however there are some non-significant changes and also some significant decreased values for MDA of case groups against control groups.[6-10] In addition there are very wide variations in the reported MDA values in both case and/or healthy control groups. The variations are wider when various analytical methods are considered [6]. A number of reasons for describing these variations which were discussed with further details along with some other aspects were reviewed in a recent work.[11]

Various electrochemical, separation and spectroscopic methods were applied for analysis of MDA in various biological matrices. Two more recent review articles[11,12] were summarized the progresses and some draw-backs of MDA analysis in biological samples.[11] Formation of an adduct with thiobarbitoric acid (TBA) and its spectroscopic, liquid chromatographic and electrophoretic determination is the most commonly used analytical procedure for determination of MDA in biological samples. The main problem of these methods is their low repeatability which is caused by different factors.[13,16] Firstly, reaction temperature; most of the reported methods were applied temperatures higher than 70 °C. The boiling point of MDA, 109 °C, provides the possibility of evaporation at higher temperatures and alteration of its concentration.

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Moreover at high temperatures, reactants and products may be decomposed. Originally, MDA is unstable and be easily converted to alcohols and acids even at room temperature.[17-19] Secondly, MDA reacts with itself to produce polymeric forms,[20] in addition, the product of reaction, *i.e.* MDA-TBA adduct, also can react with polymeric form of MDA and other species. It seems that at higher temperatures the mentioned by-reactions are carried out in higher rates. As a third reason, in acidic medium TBA could be hydrolyzed to thiourea, malonic acid and barbituric acid.[21] There are some other reasons which are not discussed here and readers may refer to the mentioned literature.

Following our brief editorial,[22] in the present work, the effects of a number of parameters on the repeatability of MDA and TBA reaction are reported. Based on different derivatization conditions in aqueous solutions and human plasma including reflux, open and closed systems, reproducibility of the results are checked. Also, effects of temperature, pH and reaction time were investigated in details.

Materials and Methods

All UV spectra were recorded using a spectrophotometer CECIL CE7250 (UK). TBA, 1,1,3,3-tetramethoxypropane (TMP; as a reagent of MDA) and HCl were purchased from Merck (Germany). Double distilled water was used in preparation of aqueous working solutions. A micro centrifuge with 6200 rpm, Micro one Tomy (Japan), was used for protein precipitation of plasma samples. Plasma samples were obtained from Tabriz Blood Transfusion Research Center and kept at -20 °C till analysis time.

Derivatization in aqueous medium

After addition of 1.25×10^{-4} M TBA and 6.25×10^{-4} M TMP to a reaction flask, the pH of solution is adjusted to 2.5. Then the refluxed reaction system was set by increasing the temperature of the mixture in an oil bath to the temperature of interest for 3 hr. Polymerization rendered a stable red colored adduct from which excess monomers were washed away by HCl (0.6 M), hot water, ethanol and 5 mL diethyl ether. Likewise, the precipitated solid was dried at 60 °C for 3 hrs and applied for further reactions. To increase the purity of the produced adduct, the solid is dissolved in 20 mL of 0.6 M HCl and then refluxed for 40 min at 100 °C. Afterwards, the produced adduct is washed and dried in an oven at 60 °C. A schematic representation of reaction between TBA and MDA in acidic medium is shown in Figure 1.

10 mg of produced solid is weighted, dissolved in a 250 mL volumetric flask and then 1 mL 0.1 M sodium hydroxide and 1 mL 0.6 M HCl (according to a published procedure [23]) are added to the mixture up to 250 mL. 1.25 mL of the prepared solution is added to a 100-mL volumetric flask. Subsequently, absorbance of the solution is measured at 532 nm.

Derivatization in human plasma

Initially, 250 μmol TBA is added to 10 mL of plasma and then pH adjusted to 2.5 by HCl (1.0 M). A similar procedure used for aqueous solution is applied to plasma samples. The effect of temperature is investigated in reflux condition at 70, 85 and 100 °C. At each temperature, sampling and absorbance measurements are done with 30 min time intervals. Also, this reaction is repeated in two non-refluxed closed and open-door flask systems at 70, 85 and 100 °C.

Results and Discussion

Optimization of pH of the reaction solution

Optimization of the reaction parameters are investigated concerning both repeatability and sensitivity in optimization process. Owing to the production of MDA-TBA adduct in the acidic condition, the effect of pH is tested over the pH range of 1.0 to 3.0. It is found that the absorption repeatability increases along with the increasing pH value of the reaction solution. However, the repeatability become very low, *i.e.* 29% in pH 1.0.[23] According to our observations, at the reaction temperature of 100 °C and TBA/MDA ratio of 2, the optimized pH of reaction is 2.5, therefore we adjusted pH values at 2.5 in further experiments.

Optimization of ratio of TBA-to-MDA

The effect of TBA/MDA ratio on the concentration of the produced adduct is studied. As demonstrated in Figure 1, the stoichiometric ratio of TBA/MDA is 2 (RSD= 5.2%) but results reveal that maximum absorbance of the adduct at 532 nm is in the TBA/MDA ratio of 5 (RSD=4.6%).

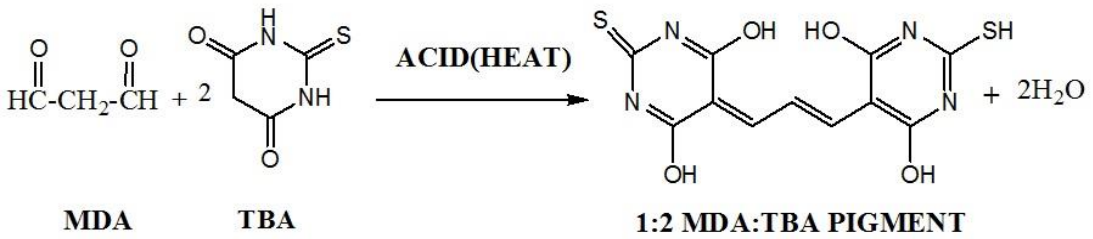

Figure 1. Schematic representation of reaction between TBA and MDA.

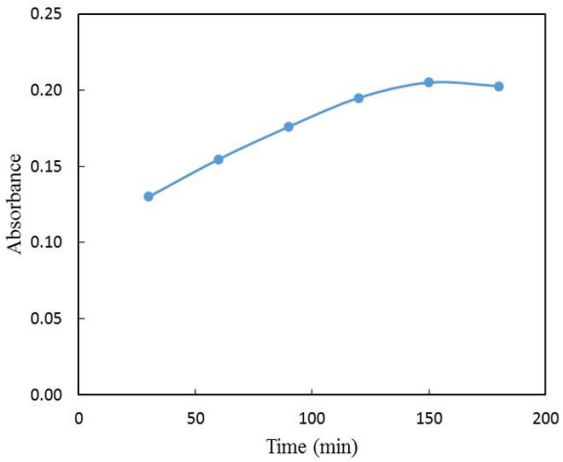

Figure 2. Effect of time of reaction on the process (pH 2.5, TBA/MDA ratio of 5, 70 °C, refluxed system).

Although the favored value of the UV absorbance is observed in TBA/MDA ratio of 3, the measured absorbance is not repeatable (RSD=19.8%). It could be concluded that in higher concentrations of TBA, the competition among various TBA reactive substances (TBARs) are decreased, since there is sufficient TBA to react with all TBARs. This hypothesis was supported by others [24,25] where they declared that about 80 % of TBARs were not originated from lipid peroxidation.

Effect of time of reaction

Figure 2 depicts the effect of reaction time on the absorbance of the adduct at 532 nm. Results showed that the absorbance was increased with increasing reaction time from 30 to 150 min at 70 °C, and further increases in heating time did not change the absorbance. Therefore a heating time of 150 min was adopted as optimized reaction time for subsequent studies.

Effect of temperature

Temperature is considered as a major factor that affects the rate of a chemical reaction. Keeping the pH value of reaction solution at 2.5 and ratio of TBA/MDA at 5, the effect of temperature is investigated at three temperatures of interest, *i.e*. 70, 85 and 100 °C. As shown in Figure 3 by decreasing the temperature of reaction to 70 °C the absorbance increased remarkably. In addition, temperature of reaction affects both repeatability and sensitivity. As mentioned, high temperature resulted in loss of reaction components. Due to this fact that the boiling point of MDA is about 109 °C therefore at higher temperatures evaporation of MDA may cause the loss of analyte and subsequently to decrease in absorbance of the solution.

Effect of reaction procedure on the repeatability

The reaction between MDA and TBA in plasma samples was carried out in three modes of open, closed and also in refluxed systems. Unlike the open system, in closed system reaction was implicated in a volumetric flask capped with a glass. Figure 4 shows absorbance of the MDA-TBA adduct in reflux, open and closed systems in different of times. Due to solvent evaporation in open system at longer reaction times, absorbance of the solution could not be measured.

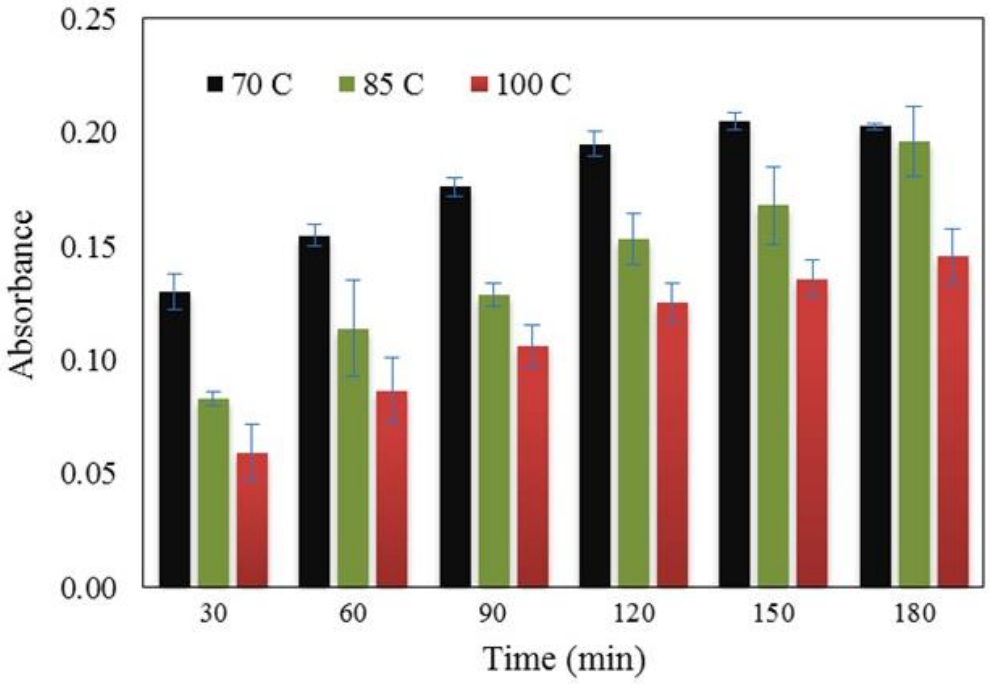

Figure 3. Effect of temperature on reaction between TBA and MDA (pH 2.5, TBA/MDA ratio of 5, refluxed system).

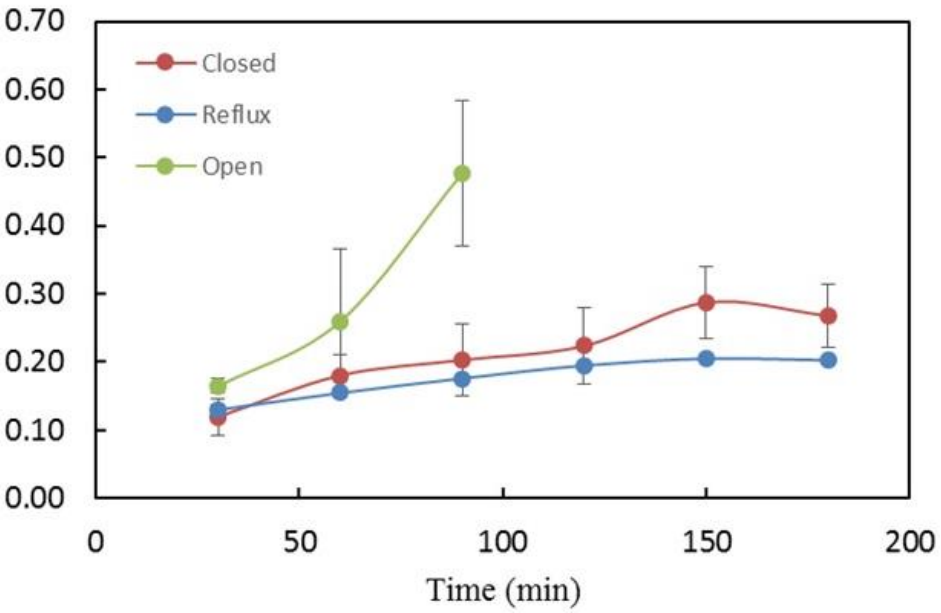

Figure 4. Effect of reaction procedure in repeatability (pH 2.5, TBA/MDA ratio of 5).

From Figure 4, it is clear that in spite of high absorbance in closed and open systems, the refluxed system showed better repeatability. In open and closed systems, increases in absorbance are resulted from evaporation of water. Poor repeatability as a main drawback of analytical methods[26] is resolved using refluxed system where the relative standard deviations (RSD) for all temperatures are between 0.7-6.1% (average, 2.8%). The the obtained RSDs for open and closed systems are 23% and 21%, respectively. According to our observations, the reflux procedure is recommended for determination of MDA in human plasma samples to provide acceptable repeatability of the analytical methods.

Conclusion

Analysis of MDA or in general term TBARs as biomarkers of oxidative stress is employed in many clinical and biomedical investigations. In the present work, MDA was analyzed in human plasma using reaction of TBA and MDA with a modified method. In order to optimize the reaction condition, some experimental factors, such as temperature, time and pH of the reaction solution, were investigated. Results revealed that to reach a more repeatable procedure, reaction should be performed in a refluxed system at 70 °C and pH 2.5. Using this reaction condition, MDA is analyzed in human plasma with favorable repeatability. By applying the refluxed system for implementation of reaction between TBA and MDA, the major problem, *i.e.* repeatability of the results, was resolved while further studies are needed to increase sensitivity of the modified method in biological samples. Coupling of separation techniques such as liquid chromatographic and/or capillary electrophoretic techniques with more sensitive detectors like fluorescence or mass spectroscopic detections could provide more selective and sensitive results. Employing the above mentioned derivatization condition will provide better repeatability and reproducibility data and recommended for providing more reliable data for clinical investigators.

Conflict of interests

The authors claim that there is no conflict of interest.

References

1. Kilic N, Taslipinar MY, Guney Y, Tekin E, Onuk E. An investigation into the serum thioredoxine, superoxide dismutase, malondialdehyde, and advanced oxidation protein products in patients with breast cancer. Ann Surg Oncol. 2014;21(13):4139-43. doi:10.1245/s10434-014-3859-3
2. Vanishree BJ, Kodliwadmath MV, Prasad CVB, Sonoli SS. Malondialdehyde, ascorbic acid and lipid profile in diabetic and hypertensive with myocardial infarction: A case control study. Biomedicine. 2011;31(2):231-4.
3. Antus B, Drozdovszky O, Barta I, Kelemen K. Comparison of airway and systemic malondialdehyde levels for assessment of oxidative stress in cystic fibrosis. Lung. 2015;193(4):597-604. doi: 10.1007/s00408-015-9739-1.
4. de Sousa RT, Zarate CA, Zanetti MV, Costa AC, Talib LL, Gattaz WF, et al. Oxidative stress in early stage bipolar disorder and the association with response to lithium. J Psychiatr Res. 2014;50:36-41. doi:10.1016/j.jpsychires.2013.11.011
5. Sönnerborg A, Carlin G, Akerlund B, Jarstrand C. Increased production of malondialdehyde in

patients with HIV infection. Scand J Infect Dis. 1988;20(3):287-90. doi:10.3109/00365548809032453
6. Khoubnasabjafari M, Ansarin K, Jouyban A. Comments on "An investigation into the serum thioredoxin, superoxide dismutase, malondialdehyde, and advanced oxidation protein products in patients with breast cancer". Ann Surg Oncol. www.surgonc.org/news-publications
7. Khoubnasabjafari M, Ansarin K, Jouyban A. Comments concerning "Comparison of airway and systemic malondialdehyde levels for assessment of oxidative stress in cystic fibrosis". Lung. 2015;193(5):867-8. doi:10.1007/s00408-015-9774-y
8. Khoubnasabjafari M, Ansarin K, Jouyban A. Reliability of malondialdehyde as a marker of oxidative stress in pediatrics. Pediatr Neonatol. 2016;57(5):450. doi:10.1016/j.pedneo.2015.10.015
9. Khoubnasabjafari M, Ansarin K, Vaez-Gharamaleki J, Jouyban A. Comments on "Salivary 8-hydroxy-2-deoxyguanosine, malondialdehyde, vitamin C, and vitamin E in oral pre-cancer and cancer: diagnostic value and free radical mechanism of action". Clin Oral Investig. 2016;20(2):395-6. doi:10.1007/s00784-015-1687-x
10. Khoubnasabjafari M, Ansarin K, Jouyban A. Variations of malondialdehyde in pre-eclampsia. Hypertens Pregnancy. 2016;35(3):346-9. doi:10641955.2016.1148163
11. Khoubnasabjafari M, Ansarin K, Jouyban A. Critical review of malondialdehyde analysis in biological samples. Curr Pharm Anal. 2016;12(1):4–17. doi:10.2174/1573412911666150505185343
12. Tsikas D. Assessment of lipid peroxidation by measuring malondialehyde (MDA) and relatives in biological samples: Analytical and biological challenges. Anal Biochem. 2017;524:13-30. doi:10.1016/j.ab.2016.10.021
13. Satoh K. Serum lipid peroxide in cerebrovascular disorders determined by a new colorimetric method. Clin Chim Acta. 1978;90(1):37-43. doi:10.1016/0009-8981(78)90081-5
14. Kikugawa K, Kojima T, Yamaki S, Kosugi H. Interpretation of the thiobarbituric acid reactivity of rat liver and brain homogenates in the presence of ferric ion and ethylenediaminetetraacetic acid. Anal Biochem. 1992;202(2):249-55. doi:10.1016/0003-2697(92)90102-d
15. Schoenmakers AW, Tarladgis BG. Reliability of the thiobarbituric acid test in the presence of inorganic ions. Nature. 1966;210(5041):1153. doi:10.1038/2101153a0
16. Stalikas CD, Konidari CN. Analysis of malondialdehyde in biological matrices by capillary gas chromatography with electron-capture detection and mass spectrometry. Anal Biochem. 2001;290(1):108-15. doi:10.1006/abio.2000.4951
17. Tarladgis BG, Watts BM, Younathan MT, Dugan L. A distillation method for the quantitative determination of malonaldehyde in rancid foods. J Am Oil Chem Soc. 1960;37(1):44-8. doi:10.1007/bf02630824
18. Almandos ME, Giannini DH, Ciarlo AS, Boeri RL. Formaldehyde as an interference of the 2-thiobarbituric acid test. J Sci Food Agric. 1986:37(1):54-8. doi:10.1002/jsfa.2740370108
19. Kumar A, Dhillon BS, Rao DN, Menon G, Shankar H, Dhaliwal LK, et al. Temporal trends of malondialdehyde in stored human plasma. Ind J Clin Biochem. 2012;27(4):405-9. doi:10.1007/s12291-012-0215-x
20. Gutteridge JMC. The use of standards for malondialdehyde. Anal Biochem. 1975;69(2):518-26. doi:10.1016/0003-2697(75)90155-4
21. Tarladgis BG, Pearson AM, Dugan LR. The chemistry of the 2-thiobarbitoric acid test for the determination of oxidative rancidity in foods. I. Some important side reactions. J Am Oil Chem Soc. 1962;39(1):34-9. doi:10.1007/bf02633347
22. Azizi S, Shahrisa A, Khoubnasabjafari M, Ansarin K, Khoubnsasabjafari M, Soleymani J, et al. A possible reason for the low reproducibility of malondialdehyde determinations in biological samples. Bioanalysis. 2016;8(21):2179-81. doi:10.4155/bio-2016-0228
23. Snnihuber RO, Yu TC. Characterization of the red pigment formed in the 2-thiobarbituric acid determination of oxidative rancidity. J Food Res. 1958;23(6):626-34. doi:10.1111/j.1365-2621.1958.tb17614.x
24. Halliwell B, Gutteridge JMC. Formation of a thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts the role of superoxide and hydroxyl radicals. FEBS Lett. 1981;128(2):347-52. doi:10.1016/0014-5793(81)80114-7
25. Warso MA, Lands WE. Presence of lipid hydroperoxide in human plasma. J Clin Invest. 1985;75(2);667-71. doi:10.1172/jci111745
26. Pacer Z, Veselkova A, Rath R. Oxidative stress. Experienta. 1965;21:19-23