Malondialdehid Ölçümünde iki Yöntemin Karşılaştırılması

Comparison of two Methods for Malondialdehyde Measurement

Malondialdehid Ölçümü / Malondialdehyde Measurement

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Özet

Amaç

Bu çalışmada; malondialdehid düzeylerinin iki ayrı metod ile ölçülmesi ve bu metodların karşılaştırılması amaçlanmıştır.

Gereç ve Yöntemler

60 gönüllünün serum malondialdehid düzeyleri spektrofotometrik tiyobarbitürik asit testi ve 2,4-dinitrofenilhidrazin ile türevlendirilmesi sonrası yüksek basınçlı sıvı kromatografisi ile ölçülmüş ve bu iki metot karşılaştırılmıştır.

Bulgular

Yüksek basınçlı sıvı kromatografisi (1.85 ± 0.09 µmol/L) ve spektrofotometre (2.47 ± 0.18 µmol/L) ile ölçülen serum MDA seviyelerinde istatistiksel olarak anlamlı fark bulundu ($P < .001$). İki ayrı yöntemle yapılan ölçümler arası tutarlılık değerlendirildi ve sınıflar arası korelasyon katsayısı 0.365 olarak bulundu ($P = .042$). Ölçümler arasında istatistiksel olarak önemli, zayıf-orta derecede korelasyon bulunmuştur ($r = 0.284$, $P = .028$).

Sonuç

2,4-dinitrofenilhidrazin ile türevlendirme sonrası yüksek basınçlı sıvı kromatografisi ile malondialdehid ölçüm metodu, konvansiyonel spektrofotometrik tiyobarbitürik asit metoduna göre lipit peroksidasyonunun daha doğru ve duyarlı değerlendirilmesini sağlamıştır.

Anahtar Kelimeler

Malondialdehid, Lipid Peroksidasyonu, Oksidatif Stres, Yüksek Basınçlı Sıvı Kromatografisi, Spektrofotometrik Yöntem.

Abstract

Aim

In this study, we aimed to measure malondialdehyde levels by two different methods and compare these methods.

Material and Methods

Serum malondialdehyde levels of 60 volunteers were measured with thiobarbituric acid test and high pressure liquid chromatography after derivatization of malondialdehyde with 2,4 dinitrophenylhydrazine and these two methods were compared.

Results

A statistically significant difference ($P < .001$) has been found in serum malondialdehyde levels measured by high pressure liquid chromatography (1.85 ± 0.09 µmol/L) and spectrophotometry (2.47 ± 0.18 µmol/L). Cohesion between measurements made by two different methods have been evaluated and the interclass correlation coefficient has been found as 0.365 ($P = .042$). Statistically significant, weak-mild degree correlation has been found between measurements ($r = 0.284$, $P = .028$).

Conclusions

High pressure liquid chromatography method for malondialdehyde measurement after derivatization with 2,4 dinitrophenylhydrazine provided a more accurate and sensitive assessment of lipid peroxidation than the conventional spectrophotometric thiobarbituric acid method.

Keywords

Malondialdehide, Lipid Peroxidation, Oxidative Stress, High Pressure Liquid Chromatography, Spectrophotometric Method.

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Introduction

Reactive oxygen species and particularly free radical induced lipid peroxidative tissue damage have been implicated in the pathogenesis of various diseases including diabetes, atherosclerosis, cancer and Parkinson's disease.[1-5,7-9] Lipid peroxidation by reactive oxygen species involves the oxidative deterioration of polyunsaturated fatty acids in biomembranes and generates a variety of aldehyde products including malondialdehyde (MDA).[5] Production of MDA has been shown to be a relevant indicator to demonstrate the occurrence of in situ lipid peroxidation.[15] MDA is a three-carbon compound formed by scission of peroxidized polyunsaturated fatty acids, mainly arachidonic acid. The analysis of MDA is most frequently done by spectrophotometric assays based on its reaction with thiobarbituric acid (TBA) at 100 0C in acidic media and measuring absorbance of the reaction mixture at 532 nm.[5,17,18] Although it is simple and inexpensive but non-specific since TBA reacts with many other carbonyl-containing compounds such as carbohydrates, pigments, amino acids[5,8,19] and.[20] This interference in the TBA assay causes considerable variability in the results.[9,14-16] Alternatively derivatization of MDA with 2,4-dinitrophenylhydrazine (DNPH) and conversion into its pyrazole and hydrazone derivatives has been found to allow specific estimation of MDA, especially if combined with separation by HPLC.[5,13,17,18] The aim of this study was to compare two methods of MDA measurement, HPLC and TBA assay, in sera of healthy volunteers.

Material and Methods

All reagents and chemicals used in this experiment were analytical grade of highest purity. All organic solvents were HPLC grade. 1,1,3,3-tetraethoxypropane (TEP), trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA) and dinitrophenylhydrazine (DNPH), sodium hydroxide (NaOH), perchloric acid, hydrochloric acid, sulfuric acid, methanol and ethanol were obtained from Merck (Germany). Acetonitrile was obtained from Sigma-Aldrich (Germany). Blood was collected by veni puncture into separated-gel tubes from 60 non smoker volunteers aged between 18 and 45 years. After centrifugation (400g 10 min at 4 ^{0}C) serum was immediately stored at -80 ^{0}C.

Dinitrophenylhydrazone Method by HPLC

50 µL of 6 M NaOH was added to 0.250 mL serum then incubated in a 60 ^{0}C water bath for an hour. The hydrolyzed sample was acidified with 0.125 mL of 35% (v/v) perchloric acid. After centrifugation for 10 minutes, 0.250 mL supernatant was mixed with 25 µL of 5 mM DNPH solution and incubated in dark for half an hour. 100 µL of the reaction mixture was directly injected into HPLC system.[11] MDA standard was prepared by dissolving 25 µL 1,1,3,3-tetraethoxypropane (TEP) in 100 mL of water to give a 1 mM stock solution. Working standard was prepared by dilution of 1 mL stock solution in 50 mL of 1% sulfuric acid and incubation for 2 hours at room temperature. The resulting MDA standard of 20 µmol/L was further diluted with 1% sulfuric acid to yield the final concentration of 10, 5, 2, 1 ,0.5, 0.2 µmol/L to get the standard curve for the estimation of total MDA. 0.250 mL of standard was mixed with 25 µL DNPH solution and incubated in dark for 30 minutes. 100 µL of the reaction mixture was directly injected into HPLC system. The samples were analyzed on an Agilent 1100 series HPLC apparatus (Germany). The analytical column was 125 X 4 mm ODS-2 C18 reserve phase column with particul size of 5µm (Thermo, England). The mobile phase was acetonitrile-distilled water (34:66, V/V) containing 0.2 % (V/V) acetic acid. All separations were performed at isocratic conditions with a flow rate of 1 mL/min and UV detector was set at 310 nm. MDA peaks were determined according to its retention time and confirmed by spiking with added exogenous standard. Concentrations of MDA were calculated from standard curve prepared from TEP and expressed as µmol/L.

Thiobarbituric acid Method by Spectrophotometry

0.5 mL of serum was shaken with 2.5 mL of 20% TCA in a 10 mL centrifuge tube. The mixture was warmed for 15 minutes in a boiling water bath followed by rapid cooling. After centrifugation at 5000 rpm for 10 min. 2 mL of the supernatant was added to 1 mL of 6.7% TBA and absorbance readings were performed at 532 nm. MDA standard was prepared by dissolving 50 µL of TEP to 25 mL of 40% ethanol to give 8360 µmol/L stock solution. Working solution was prepared by taking 100 µL of stock solution and adding this to 20 mL of 40% ethanol the resulting MDA standard

Figure1. Standard curve of MDA used for HPLC Method.

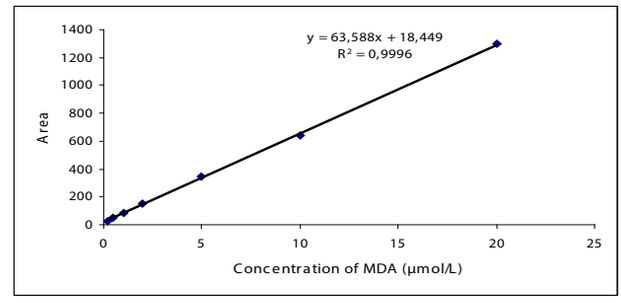
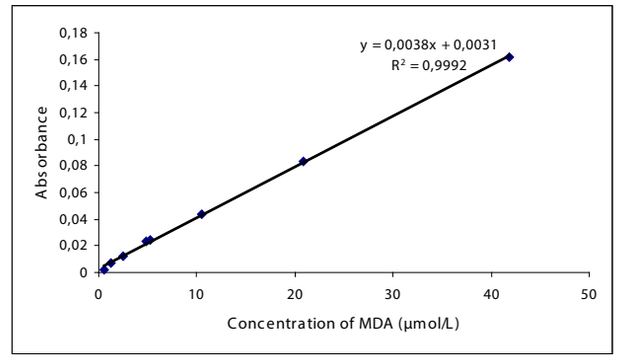


Figure 2. Standard curve of MDA used for TBA method.



of 41.8 µmol/L was further diluted with distilled water to yield 20.9, 10.45, 5.22, 4.86, 2.43, 1.22 and 0.61 µmol/L to get the standard curve of MDA.

Validation Study

The intra-day MDA assay precision was determined by ten replicate analyses of the serum sample on the same day. The inter-day assay precision was determined by analyzing the serum sample on ten different days. Intra and inter-day assay precision was determined for both HPLC method and spectrophotometric TBA assay. The data were presented as mean±standard deviation.

Statistical Analysis

Statistical analyses were performed using a software program (SPSS 15.0 for windows, Chicago, IL, USA). Data were expressed as mean± S.D. The two different methods for measuring serum MDA levels were compared by t-test. Linear regressions between the methods were also calculated. A p value of less than 0.05 was considered as statistically significant.

Results

The standard curve gave a linear response for MDA concentrations and HPLC peak areas in the range of 0.2 to 20 µmol/L as shown in figure 1. A standard curve was also prepared for TBA and gave a linear response for MDA concentrations in the range of 0.61 to 20.9 µmol/L as shown in figure 2.

An important difference between serum MDA levels was observed. The mean±SD values of serum MDA levels with HPLC and TBA method were 1.87 ± 0.06 µmol/L and 2.63 ± 0.15 µmol/L respectively, at a significance of $P <$ .002. In all of the measurements, MDA levels of spectrophotometric method have been found higher than HPLC method. The paired differences between two measurements ranged from 8% to 61% with a 33.7% difference in median values.

The analytical conditions applied (alkaline hydrolysis of protein bound MDA, protein precipitation and derivatization of MDA with DNPH) yielded clear supernatant and thus no further extraction was necessary. Very good resolution was obtained, with no interfering peaks, which allowed a straightforward determination of the MDA as shown in figures 3 and 4.

While coefficient of variation (CV) of intra and inter-assay in the HPLC method was 3.3% and 4.3%, it was 5.5% and 4.4% for spectrophotometric TBA method respectively. Recovery of MDA was 97.8% in HPLC method (Figure 5) and 90% in spectrophotometric method.

Discussion

Determination of serum MDA levels is still the most commonly applied assay for lipid peroxidation in biomedical fields, because MDA is one of the major aldehydes formed after breakdown of lipid hydroperoxides. Therefore, it is considered to be a good biomarker of the free radical involvement damage in pathologies associated to oxidative stress.[5-9]

Although determination of MDA after reaction with thiobarbituric acid is considered to have important limitations, even in its chromatographic approach, it is the method most

Figure 3. HPLC chromatogram of 5 µmol/L MDA standard.

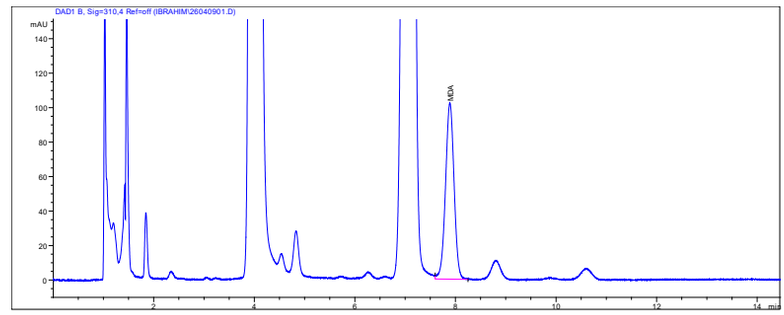


Figure 4. HPLC chromatogram of serum with 1.85 µmol/L MDA concentration.

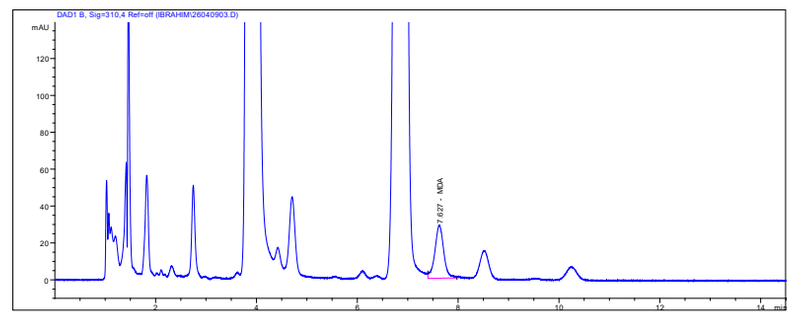
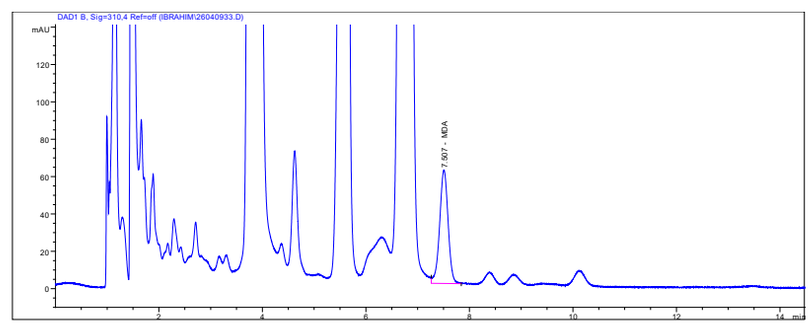


Figure 5. HPLC chromatogram of the previous sample (Figure 4) after adding a standard of 20 µmol/L MDA for recovery calculations



widely used in research laboratories. However, Pilz et al reported an alternative methodology based on derivatization of MDA with DNPH, that allowed determination of free and bound MDA in serum and plasma with good results, HPLC based determination of the MDA hydrazone, has proved to be precise and reproducible, as well as sensitive enough to reflect differences in the oxidative status in vivo.[11] In this method centrifugation of sample, followed by release of protein-bound MDA by alkaline hydrolysis of supernatants, and derivatization of MDA is required without any further extraction steps before direct injecting into the chromatographic system. Rapid separation by HPLC and monitorization of the well-resolved MDA peak by UV detection, good recovery, reproducibility and high sensitivity makes this method easy and suitable to quantify MDA in biological samples.[5,7,8,11,14]

In our experiment MDA was determined by two methods as a comparison study to find the most accurate, sensitive and specific method. We used HPLC system to find MDA after derivatization with DNPH and as dithiobarbituric acid adduct in human serum. Comparative evaluation for the determination of MDA was made between the conventional spectrophotometric and HPLC procedure. Our HPLC method was validated for the measurement of free MDA in the human serum.

As expected, MDA levels were significantly different. It is clear that there is also a difference between the methods used. This difference could be explained by the occurrence of chemical interference.

The conventional spectrophotometric procedure requires TBA to be added to the sample and heated at 100 0C to form the MDA-TBA complex. During the formation of this complex unsaturated fatty acids in biological sample also react with TBA to form colored complexes, which absorb light at or near 535 nm[5-12] Hence, spectrophotometric detection includes both MDA-TBA complex and other complexes which absorb at the same wavelength a higher amount of MDA is obtained.

Our aim in this experiment was to develop a method for measurement of MDA simple enough for routine determination. The samples were derivatized with DNPH since the procedure proceeds rapidly under certain pH and temperature and resulting derivatives were unique for a given aldehyde. In previous studies, it has been reported that these derivatives are stable in the absence of light and could be separated by HPLC to get a specific signal for MDA. These are important advantages for analyzing of MDA content in complex biological samples in relation to the commonly used TBA assay.[1,4,5,7-9]

We found mean 1.87 ± 0.06 µmol/L of total MDA in human serum by using HPLC method as MDA-DNPH, This result is closed to the previous results reported by different authors with slight differences, these differences may be related to the factors such as age and nutrition of healthy volunteers.[11]

On the other hand we found 2.63 ± 0.15 µmol/L of total MDA in human serum by spectrophotometric method as MDA-TBA. This result is about 20% lower than the reported values.

MDA-DNPH method by HPLC represent acceptable recovery levels about 98.8%, other studies have also reported good recovery levels for this method, while MDA-TBA method represent a lower recovery levels about 90%. In this experiment, we found MDA determination by HPLC is more sensitive during run to run and within run measurements

In conclusion, the HPLC method after derivatization with DNPH provided a more accurate and sensitive assessment of lipid peroxidation than the conventional spectrophotometric TBA method. The short retention time of MDA (approximately 7.5 minutes) reduced the total analysis time. About 0.5 mL of sample volume is required to measure the MDA-(TBA) 2 complex spectrophotometrically, whereas only 0.25 mL is enough to detect MDA directly by HPLC. This method also minimized chemical materials loss which makes it preferable over other methods.

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