Fırat Tıp Dergisi 2006;11(2): 88-92

Experimental Research

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Measurement of Total Malondialdehyde in Plasma and Tissues by High-Performance Liquid Chromatography and Thiobarbituric Acid Assay

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ABSTRACT

Objective: Malondialdehyde, a product of lipid peroxidation, is a good marker for development of oxidative stress.

Mateials and Methods: In this study MDA was determined in the tissues and plasma by means of thiobarbituric acid test and directly by reverse phase high-pressure liquid chromatography method after derivatisation with 2,4-dinitrophenylhidrazine.

Results: We have found a significant difference between two methods by Mann-Whitney U test ($p<0.001$). There was the formation of non-lipid-related, malondialdehyde like, TBA-reactive substance that leads to overestimation of the extent of lipid peroxidation. On the contrary, by direct HPLC method, there was a decrease of MDA from plasma and tissue serving as control.

Conclusion: Our results indicated that TBA test is not a specific and reliable index of lipid peroxidation. *©2006, Fırat Üniversitesi, Tıp Fakültesi*

Key words: *Malondialdehyde; High pressure liquid chromatography; Plasma; Tissue*

ÖZET

HPLC ve TBA Yöntemi ile Plazma ve Dokularda Total MDA Ölçümü Olgu Sunumu

Amaç: Bir lipit peroksidasyon ürünü olan malondialdehit ölçümünün oksidatif stresin değerlendirilmesinde önemli bir belirteç olduğu bilinmektedir.

Gereç ve Yöntem: Çalışmamızda malondialdehit seviyesi karaciğer, böbrek, beyin ve plazmada hem tiyobarbitürik asit yöntemiyle hem de 2,4 dinitrofenilhidrazin ile derivatize edilerek HPLC metoduyla ölçülerek sonuçlar karşılaştırılmıştır.

Bulgular: Mann Whitney-U testi ile yapılan istatistik analiz sonucu tiyobarbitirük asit yöntemi malondialdehit miktarı sonuçlarının HPLC yönteminden elde edilen sonuçlara kıyasla önemli derecede yüksek olduğu gözlenmiştir. Doku ve plazma örneklerinde tiyobarbitirük asit yöntemiyle malondialdehit miktarının fazla ölçülmesi lipit olmayan malondialdehide benzer ve tiyobarbitirük asit reaktif maddelerin oluşumu ve interferans vermesiyle açıklanabilmektedir. Buna karşılık direk ve derivatize edilme işleminden sonra uygulanan HPLC yöntemiyle elde edilen MDA sonuçları önemli derecede düşük bulunmuştur.

Sonuç: Sonuçlarımız tiyobarbitirük asit testinin, HPLC yöntemine kıyasla gerçek bir lipit peroksidasyon olayının değerlendirilmesinde spesifik ve geçerli olmadığını ortaya koymuştur. *©2006, Fırat Üniversitesi, Tıp Fakültesi*

Anahtar kelimeler: *Malondialdehit; Yüksek basınçlı sıvı kromatografisi; Plazma; Doku.*

Reactive oxygen species and particularly free radical induced lipid peroxidative tissue damage have been implicated in the pathogenesis of various diseases. Lipid peroxidation is assessed indirectly by the measurement of the secondary products, such as malondialdehyde (MDA) (1). MDA is a three-carbon low molecular weight aldehyde and spontaneous breakdown product of peroxides that can be produced from free radical attack on poly unsaturated fatty acids (2-5).

The analysis of MDA by the thiobarbituric acid (TBA) assay has been widely employed over the many years in biological systems for the assessment of lipid peroxidation. It is a spectrophotometric assay, based upon heating of the sample under acidic conditions to form the adduct of MDA-TBA (3, 6, 7). This reaction, although simple and reproducible, is unfortunately rather non-specific as TBA reacts with many other carbonyl-containing compounds. Plasma fatty acids can also be oxidized during the 95°C heating step with TBA, generating artificially high results (7, 8). In an attempt to overcome these difficulties, in a direct chromatographic assay suitable to be applied for accurate and specific quantification of MDA have been developed (3, 8, 9). However, TBA reacts not only with MDA but also with many other compounds. Therefore, derivatization of MDA with 2,4-dinitrophenylhydrazine (DNPH) and conversion into pyrazol and hydrazine derivatives has since been found to allow a more specific estimation of these compounds, especially if combined with their separation using high pressure liquid chromatography (HPLC) in the biological samples (2, 5). To date these are few data on HPLC analysis after derivatization of MDA with DNPH and comparison with TBA method in biological samples.

In this study, our aim was to measure the level of total MDA with TBA test and HPLC-UV detector after derivatisation of MDA with DNPH in plasma obtained from

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healthy volunteers and various control tissues from guinea pigs including liver, renal cortex and brain in order to compare the results.

MATERIAL AND METHODS

All the reagents and chemicals used in these experiments 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) were obtained from Sigma-Aldrich Com Ltd. Acetonitrile was purchased from Labscan (Dublin, Ireland).

Experimental animals:

Guinea pigs weighing 200–400 g (n: 10) were anesthetized with pentobarbital. Kidney, liver and brain were removed and washed with cold NaCl 0.9% and immediately placed in liquid nitrogen. Then tissues were frozen at -70°C until use.

Blood:

Blood was collected by venipuncture into EDTA from 10 healthy male and female volunteers (18-30 years, non smokers). After centrifugation (400°g 10 min at 4°C) plasma was immediately frozen on dry ice and stored at -70°C.

Sample preparation for total MDA in HPLC:

Tissue preparation:

500 mg tissue was homogenized by Vetra-Turrax in a volume of 1.15% KCl, 24000 rpm/min (7). For alkaline hydrolysis of protein bound MDA, 200 µL 6 M NaOH was added to 1 ml homogenenate in an eppendorf cup and the sample was incubated in a 600C water bath for 45 min. An aliquot of 1 ml was diluted with an equal volume of acetonitrile to precipate proteins. The resulting suspension was then vortex mixed for 30 s and centrifuged at 15000g for 10 min. The upper clear supernatant (0.25 ml) was transferred to a 2 ml eppendorf cup, mixed with 25 µL DNPH solution (5mM in 2 M HCl, pH = 0.09) and incubated for 10 min at room temperature. After derivatisation, the samples were filtered through a 0.2 µm filter. Aliquots of 20 µL were injected into HPLC system (5, 7, 8, 10- 11).

Protein levels of tissues were measured by Lowry method (12).

Plasma preparation:

The same procedure for the tissues was applied to plasma. 50 µL of 6 M NaOH was added to 0.250 ml plasma and incubated in a 600C water bath for 45 min. The hydrolyzed sample was acidified with 0.125 ml of 35% (v/v) perchloric acid. After centrifugation, 0.250 ml supernatant was mixed with 25 µL DNPH solution and incubated for 10 min. A 20 µL volume of the reaction mixture was directly injected onto HPLC system (5).

Preparation of the standard curve:

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 hydrolysis of 1 ml TEP stock solution in 50 ml 1% sulfuric acid and incubation for 2 h at room temperature. The resulting MDA standard of 20 nmol/ml was further diluted with 1% sulfuric acid to yield the final concentration of 10.5, 2.5, 1.25 and 0.625 nmol/ml to get the standard curve for the estimation of total MDA (5). 0.250 ml of standards were mixed with 25 µL DNPH solution and incubated for 10 min. A 20 µL volume of the reaction mixture was directly injected onto HPLC system by filtered through a 0.2 µm filter or unfiltered (5). Plasma samples were not filtered therefore their results were evaluated with unfiltered curve.

HPLC analysis:

The samples were analyzed on an Agilent HP 1100 series HPLC apparatus (USA). The analytical column ODS 2 C18 (5 µm particle size, 125 µ4 mm). In our experiment, analytical column was 5 µm pore size spherisorb ODS-2 C18 reserve phase column (125µ4 mm) the retention time of the MDA-hydra zone averaged 6.0 min for daily runs.

The mobile phase was acetonitrile-distilled water (38: 62, v/v) containing 0.2 % (v/v) acetic acid. HPLC apparatus was isocratic a condition at a flow rate 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 1,1,3,3 tetraethoxypropane and expressed as nmol/ml for the plasma and nmol/mg protein for the tissues (2, 5).

Thiobarbituric acid method:

Plasma TBA:

MDA level of the plasma was measured by the following procedure according to Tomotsu et al. 0.5 plasma was shaken with 2.5 ml of 20% trichloroacetic acid (TCA) in a 10 ml centrifuge tube. 1ml of 0.6 % TBA was added to the mixture, shaken, and warmed for 30 min in a boiling water bath followed by rapid cooling. Then it was shaken into a 4 ml of n-butyl-alcohol layer in a separation tube and MDA content in the plasma was determined from the absorbance at 535 and 520 nm by spectrophotometer against butanol. The standards of 5, 10, 20 nmol/ml TEP were used. The results were expressed as nmol/ml plasma (13).

Tissue TBA:

The level of MDA was determined in the tissue samples homogenized in a ratio of 1/10 in 1.15 % (w/v) cold KCl solution, by the aid of TBA method reported by Uchiyama and Mihara (14). The results were evaluated from the standard curve (2.5, 5, 10, 20 nmol/ml) and expressed as nmol/mg protein.

Statistical analysis:

Statistical analyses were performed using a software program (SPSS 11.5 for windows, Chicago, IL, USA). Plasma and tissue MDA levels of HPLC and TBA methods were compared by Mann-Whitney U test. For tests of significance a p value of less than 0.05 was considered to be significant. The results were presented as mean ±SD.

RESULTS

The standard curve gave a linear response for MDA concentrations and HPLC peak areas in the range 0.078 nmol/ml to 5 nmol/ml for total MDA of plasma (y= 0,972+12,2A; r=0,9990 y: peak area 5 point) (Fig. 1).

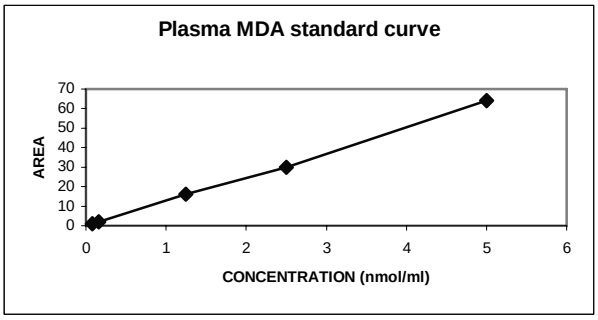

Figure 1. Plasma MDA standard curve, with regression line, prepared in the same condition with the sample (unfiltered before HPLC)

The tissue MDA levels were evaluated from another standard curve, because after the preparation of the tissue samples, the filtered samples were injected onto HPLC colon. For this reason, standards (0.078 nmol-5nmol) were also filtered (y=0.671 + 9.27A; r= 0.9975 y: peak area 5 point) except for 20 nmol (Fig. 2).

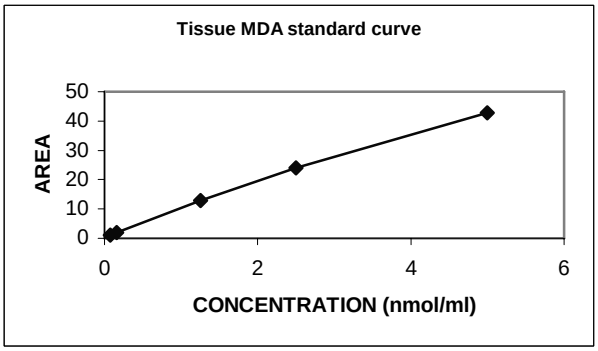

Figure 2. Tissue MDA standard curve, with regression line, prepared in the same condition with sample (filtered before HPLC)

A standard curve (2.5, 5, 10, 20 nmol/ml) was prepared for TBA method for the calculation of the tissue and plasma MDA by using TEP (y=0.693 + 55.741x) (Fig. 3).

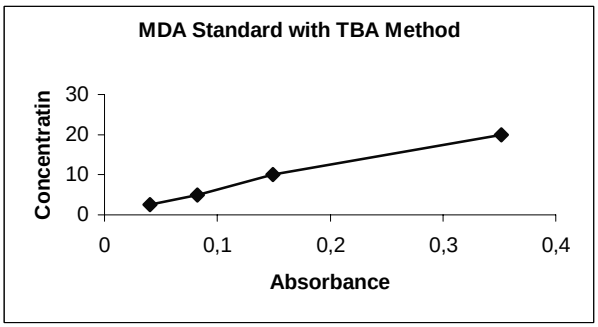

Figure 3. MDA standard curve used for TBA method, prepared in the same condition with the sample

When they were measured as triplicate in the TBA method, an important difference between MDA levels were observed (Figs. 4,5,6,7). As indicated in Table 1, the mean±SD values of plasma MDA levels with HPLC and TBA method were 0.066±0.021 nmol/ml and 14.46±0.712 nmol/ml respectively, at a significance of $p<0.001$. Brain MDA level with HPLC was 0.0323±0.011 nmol/mg protein whereas with TBA method it was 5.30±1.578 nmol/mg protein (at a significance of $p<0.05$). The MDA level of kidney cortex with HPLC was 0.028±0.006 nmol/mg protein, while with TBA it was 0.321±0.029 nmol/mg protein (at a significance of $p<0.05$). In the same way, liver MDA level when measured with HPLC was 0.028±0.004 nmol/mg protein, whereas with TBA this value was 0.196±0.051.nmol/mg protein (at a significance of $p<0.05$).

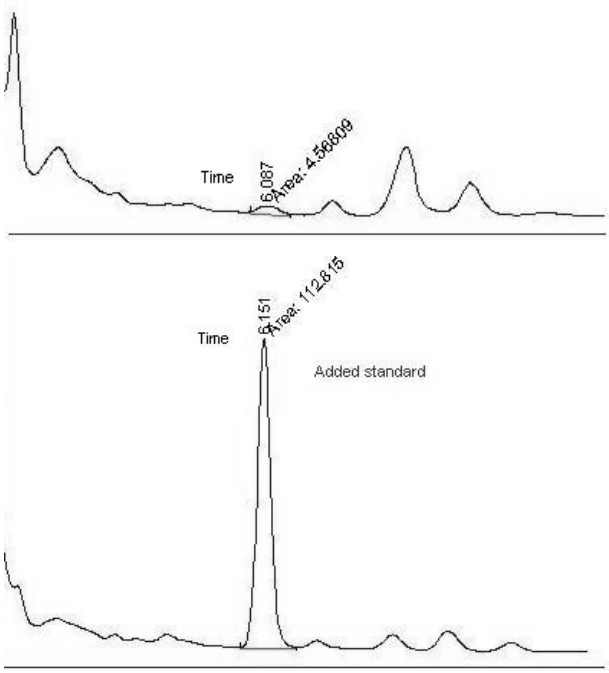

Figure 4. HPLC chromatograms of plasma total MDA after DNPH derivatization with and without added standard (20 µl injected) column ODS2 C18 reverse phase (125x4.6mm) mobile phase: acetonitrile-distilled water. Sample preparation is as described in the text.
a) Control plasma, b) Control plasma + added standard

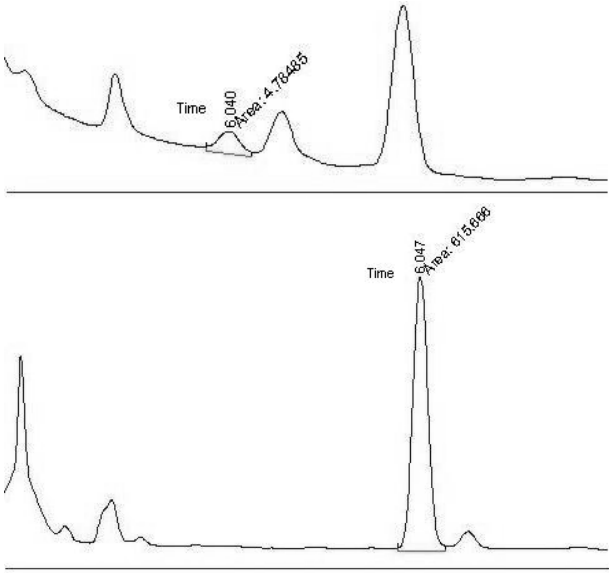

Figure 5. HPLC chromatograms of liver total MDA deproteinized with acetonitrile after DNPH derivatization with and without added standard (20 µl injected) column ODS2 C18 reverse phase (125x4.6mm) mobile phase: acetonitrile-distilled water. Sample preparation is as described in the text. a) Control liver, b) Control liver + added standard

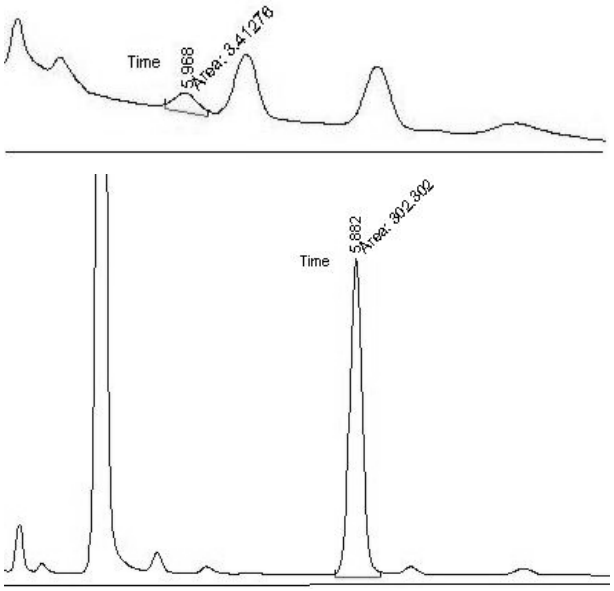

Figure 6. HPLC chromatograms of kidney total MDA deproteinized with acetonitrile after DNPH derivatization with and without added standard (20 µl injected) column ODS2 C18 reverse phase (125x4.6mm) mobile phase: acetonitrile-distilled water. Sample preparation is as described in the text.
a) Control renal cortex
b) Control renal cortex + added standard

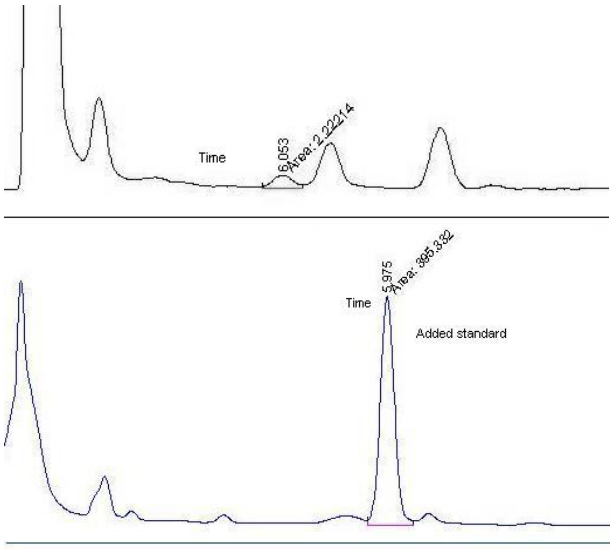

Figure 7. HPLC chromatograms of brain total MDA deproteinized with acetonitrile after DNPH derivatization with and without added standard (20 µl injected) column ODS2 C18 reverse phase (125x4.6mm) mobile phase: acetonitrile-distilled water. Sample preparation is as described in the text.
a) Control brain
b) Control brain + added standard

Table 1. The mean±SD values of plasma, brain, liver, renal cortex MDA levels with HPLC and TBA methods.

	Plasma (n:10)	Brain (n:10)	Kidney (n:10)	Liver (n:10)
HPLC Method	0.066 ± 0.021 nmol/ml	0.0323 ± 0.011 nmol/mg protein	0.028 ± 0.006 nmol/mg protein	0.028 ± 0.004 nmolmg protein
TBA Method	14.46 ± 0.712 nmol/ml	5.30 ± 1.578 nmol/mg protein	0.321 ± 0.029 nmol/mg protein	0.196 ± 0.051 nmol/mg protein
p	p< 0.001	p< 0.001	p< 0.001	p< 0.001

Results were presented as means± SD. A p value of less than 0.05 was considered to be significant.

DISCUSSION

The determination of MDA is one of the most commonly used methods for monitoring lipid peroxidation (1, 2-3). Several methods are available for the quantification of MDA in biological samples. Although simple and reproducible, the frequently used TBA method is fairly sensitive but not specific (2, 3). Recently, various HPLC methods have been applied to improve the specificity of the MDA method (3-6, 8-9).

In this study, MDA was determined in the plasma obtained from healthy volunteers and control guinea pig tissues by means of both HPLC UV detector and TBA method and the obtained results were compared. Our objective was to develop a method for MDA measurement simple enough for routine determination. The samples were derivatised with DNPH since this procedure proceeds rapidly under mild acid pH and temperature conditions and resulting derivatives were unique for a given aldehyde. In the 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 MDA content in samples with complex biological matrix in relation to the commonly used TBA assay (2, 5).

Materials of animal origin and plasma samples contain proteins to which MDA may be bound. Bound MDA in samples can only be measured after acid or alkaline hydrolysis with 6 M NaOH for 30 min under heating to 600C which was found to be optimal. This is in good agreement with the findings of Pilz et al (5).

We found 0.066 nmol/ml of total MDA in plasma with our method. This value is approximately 40% below then those reported by Pilz et al (5) despite the fact that the sample preparation is quite similar. In the other hand Yeo et al (15) found only 0.03 nmol/ml. This difference may be related to the factors such as age, tobacco smoking, and nutrition of healthy volunteers.

In addition to plasma, we also measured the MDA amount of liver, brain and renal cortex of control guinea pig with HPLC and TBA method. In tissue preparation for HPLC, we applied the same procedure used for plasma except protein deproteination with acetonitril and these samples were filtered through a 0.2 µm filter for column life. As shown in Table 1, our results of tissue MDA were high compared with those reported by Lazzorion et al (16) and Stevens et al (10).

However they measured free MDA by HPLC after DNPH-derivatization.

When our findings were compared to the other published studies, our results were low. The reason may be that our samples were derivatised with DNPH, but in the other studies the samples were treated with TBA to produce thiobarbituric acid-malondialdehyde complex. This complex was then isolated using HPLC procedure. In this reaction TBA may react with many other carbonyl-containing compounds and results in high MDA levels (2, 16-17). Our results of brain MDA levels were consistent with the findings reported by Cini and Moretti (11).

In this study, MDA also was measured with TBA method. The same homogenate prepared for HPLC was utilized for TBA. As previously reported, we also have not been able to find any relationship between the TBA-reactive substance production and total MDA level of the plasma and tissues when it was determined directly and selectively by HPLC. These conflicting results of the two assays raise several important considerations methodologically.

As our data demonstrated, the concentration of TBA reactive material is higher than the real concentration of MDA. Our results are consistent with Ceconi et al (7) who found high MDA with TBA and low MDA level by HPLC in the heart tissue. Pilz et al (5) also found 100 fold difference of MDA level between the two methods. This can be due to the lack of the specificity of the reaction of TBA with MDA or the artificial formation of MDA-like material during the heating stage. As seen in Table 1, the brain MDA levels with TBA method were higher than the other tissues MDA. As it is well known that the brain tissue contains more phospholipids and fatty acids than the other tissues.

In our experiments, although we could not find any change in MDA measurement with HPLC within day and between days, there was an important difference between MDA levels when measured as triplicate with TBA method.

In conclusion, the HPLC method after derivatization with DNPH provided a more accurate and sensitive assessment of lipid peroxidation than the conventional spectrophotometric method (TBA). The procedures for the tissue samples and plasma can be modified depend ing on the material being analyzed. The short retention time in MDA also reduced the total analysis time.

Acknowledgements: This work has been supported in part by the Research Funds of Gazi University.

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Kabul Tarihi: 18.04.2006