FABAD J. Pharm. Sci., 29, 15-19, 2004

RESEARCH ARTICLE

Malondialdehyde Quantification in Blood Plasma of Tobacco Smokers and Non-Smokers

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Summary

We adapted the thiobarbituric acid assay (TBAA) in our laboratory and validated it according to the study conditions in order to quantify plasma malondialdehyde (MDA) concentration. MDA-thiobarbituric acid (TBA) derivative was synthesized as a reference compound and characterized by UV visible and fluorescence spectra. Calibration curves of the spectrophotometric assay were linear ($r^2 = 0.979$-0.999) over a concentration range of 2.5-10 μM. The limit of detection was 1.1 μM. Within-day coefficient of variation (CV) for pooled human plasma samples was 8.2%, and between-day variation was 17.3%. The accuracy of the assay for the standard concentrations of 2.5, 5.0, 7.5 and 10.0 mM was calculated as 94.1%, 90.0%, 91.3%, and 94.7%, respectively.

The assay was further applied to the fresh plasma samples of male human smokers (n=10) and their age- and sex-matched counterparts (n=10). The plasma levels were found as 6.7±0.2 μM (mean± SEM), and 4.9±0.1 μM (mean±SEM), respectively. The difference was statistically significant ($p < 0.05$).

Key Words : *Malondialdehyde, validation, spectrophotometry, blood plasma, human.*

Received : 21.10.2004
Revised : 02.02.2005
Accepted : 03.02.2005

Tütün İçicileri ve İçici olmayan bireylerin kan plazmalarında malondialdehit miktar tayini

Özet

Plazmada malondialdehit (MDA) miktar tayini yapmak için tiyobarbitürik asit testini (TBAA) laboratuvarımıza adapte ettik ve çalışma koşullarında validasyonunu gerçekleştirdik. MDA-TBA türevi referans bileşik olarak kullanmak amacıyla kimyasal yoldan sentezlendi ve UV-görünür bölge spektrumu ve floresans spektrumu yardımı ile karakterize edildi. Spektrofotometrik yöntemin kalibrasyon doğruları 2.5-10 μM konsantrasyon aralığında doğrusal bulundu ($r^2 = 0.979$-0.999). Saptanabilirlik sınırı 1.1 μM olarak hesaplandı. Gün-içi varyasyon katsayısı (C.V.) birleştirilmiş insan plazma örnekleri için %8.2, günler-arası varyasyon katsayısı ise %17.3 olarak bulundu. Yöntemin doğruluk değeri 2.5, 5.0, 7.5 ve 10.0 μM konsantrasyonlarda hazırlanmış standart çözeltiler için sırasıyla %94.1, %90.0, %91.3, ve %94.7 olarak hesaplandı.

Yöntem daha sonra sigara içen erkek gönüllülerin (n=10) ve bunlarla yaş ve cinsiyet açısından eşleştirilmiş gönüllülerin (n=10) taze olarak hazırlanmış plazma örneklerine uygulandı. MDA plazma konsantrasyonları sırasıyla 6.7±0.2 μM (ortalama±S.E.M.) ve 4.9±0.1 μM (ortalama±S.E.M.) olarak ölçüldü. Bu fark istatistiksel olarak $p < 0.05$ düzeyinde önemli bulundu.

Anahtar Kelimeler : *Malondialdehit; validasyon; spektrofotometri; kan plazması; insan*

INTRODUCTION

Tobacco smoking has been claimed to cause a wide variety of health problems such as atherosclerosis, mutagenesis of exposed cells, and cancer in the upper respiratory system as well as lungs[1-5]. One of the putative mechanisms of the hazardous effect of tobacco smoking is oxidative stress, which is caused by the numerous reactive chemicals both in tar and gas phases. Oxidative stress is defined as the disrupted balance between oxidants and antioxidants in the body in favor of oxidants. As a consequence, cellular critical macromolecules such as lipids, proteins, carbohydrates and DNA are oxidized and degraded.

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When oxidizing compounds attack lipids, peroxidation of lipids initiates by abstraction of a proton from fatty acid side-chains and this process results in several degradation products: small molecule alkanes, alkenes, and aldehydes[6,7]. Malondialdehyde (MDA) is an aldehydic product of this process and its determination via the thiobarbituric acid assay (TBAA) is commonly used as a test for evaluating oxidative stress in the body as well as in *in vitro* antioxidant investigation studies. Despite criticism of the assay's low specificity[8], it is capable of reflecting oxidative lipid damage in the body and has been used in many studies.[9-13]

We adapted this assay according to our laboratory conditions in the present study. For this purpose, the assay was analytically re-validated. Subsequently, the validated assay was applied to the plasma samples of a group of tobacco smokers and their non-smoker counterparts, in order to investigate whether smoking increases MDA in blood plasma and whether the assay is capable of showing this elevation, if it exist.

MATERIALS and METHODS

All chemicals used in this study were of analytical grade. TBA, tetraethoxypropane (TEP), perchloric acid, *n*-butanol, and butylated hydroxytoluene (BHT) were purchased from SIGMA Co. (St. Louis, MO, USA). Dipotassium hydrogen phosphate and potassium dihydrogen phosphate were obtained from MERCK Co. (Darmstadt, Germany). All other chemicals were purchased from common commercial sources.

Preparation of MDA

MDA was obtained by hydrolysis of TEP according to the method of Csallany *et al.*[14] TEP (1 mmol) was dissolved in 10 ml of 0.01 M hydrochloric acid and left at 50°C in a water bath for 1 h. At the end of hydrolysis, pH of the solution was adjusted to 7.40. The MDA stock solution was kept in the dark until used. Standard working solutions were prepared by diluting the stock solution in water.

Synthesis and characterization of MDA-TBA derivative

The MDA-TBA derivative was synthesized chemically. 200 µl of TEP was hydrolyzed in 50 ml HCl (0.01 M) as described above. After checking the UV-visible spectrum of MDA, 250 ml of TBA in 125 ml perchloric acid (7%) was added. After vigorous stirring, the solution was kept at 95˚C in a water bath for 1 h. At the end of derivatization, one aliquot of MDA-TBA derivative was extracted twice with two volumes of n-BuOH. The butanol phases were combined and evaporated in vacuo and yielded a pinkish solid. The other aliquot was evaporated directly without extraction with n-BuOH. The product was characterized by UV-visible spectrum of λ = 532 nm, and by fluorescence properties (ex. 532, em. 553).

Subjects

Ten male smokers and 10 age- and sex-matched non-smokers were studied. None of the subjects was taking any medication. All subjects provided written informed consent. The ethical standards described by the Helsinki Declaration were followed in the course of the study.

5 ml of blood samples were drawn by venopucture into the tubes containing heparin. Each sample was centrifuged for 10 min. at 2500 *xg*. Plasma samples were separated for the analysis.

For the validation of the assay, plasma samples were obtained from three healthy individuals as described above. The samples were pooled and stored at -80˚C until studied.

Analysis of MDA by UV-visible spectrophotometer

The TBAA test for the plasma MDA concentrations was performed using the method described by Richard *et al.*[9] BHT (100 µM) was added before the reaction took place at high temperature in order to prevent further/artifactual MDA formation.

Linearity, reproducibility and sensitivity of the thiobarbituric acid assay

The sensitivity and linearity of the method was tested for MDA in spiked plasma samples (concentrations: 2.5, 5.0, 7.5 and 10.0 µM). For sensitivity, limit of detection (LOD) value was calculated as three fold of the standard deviation of the lowest concentration, 2.5 µM, among four different assays. Informati-

on on reproducibility was obtained from pooled plasma samples. For within-day variation, five individual samples were analyzed on the same day. For between-day variation, these samples were measured on four different days.

Statistics

Statistical analyses of the data were performed by Student's t-test. A probability value of $p<0.05$ was considered to denote a statistically significant difference. Data are presented as mean values ± SEM (standard error of the mean).

RESULTS

Preparation of MDA

The absorbance of MDA at 267 nm ($\varepsilon = 3.18 \times 10^4$ $M^{-1}cm^{-1}$) was used to determine the MDA concentration of the stock solution. The concentration was diluted to 10 mM and used for further studies. A typical UV-visible spectrum of MDA is represented in Figure 1.

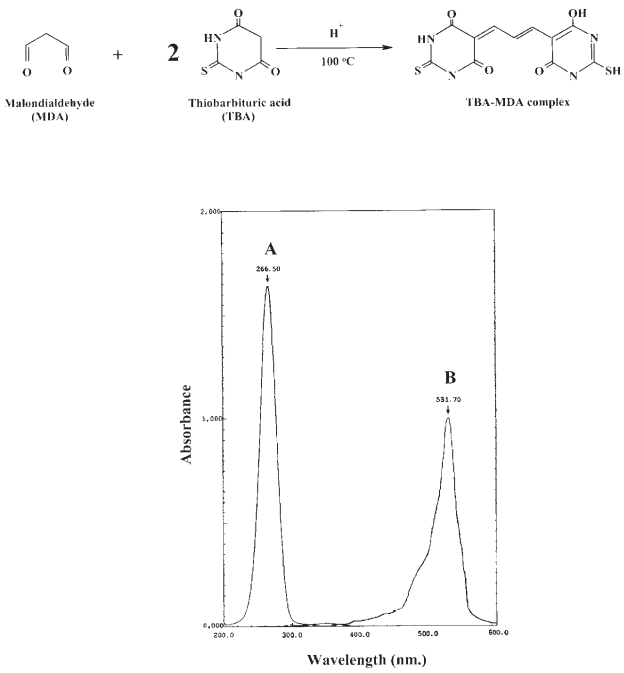

Figure 1. The reaction of TBA with MDA (upper panel), and overlaid UV-visible spectra of malondialdehyde (A) and MDA-TBA complex (B) (lower panel).

* The maximum absorbance wavelengths (nm) of both compounds have been shown on the top of each peak.

Synthesis and characterization of MDA-TBA derivative

The derivatization reaction and UV-visible spectrum of MDA-TBA derivative are shown in Figure 1. The compound was further characterized by fluorescence spectrophotometry (532 nm ex. / 553 nm em.).

Linearity, reproducibility and sensitivity of the spectrophotometric assay

Calibration curves of the spectrophotometric method were linear ($r^2 = 0.979$-0.999) over a concentration range of 2.5 µM to 10 µM. The analytical specifications of the assay are summarized in Table 1.

Table 1. Validation data of plasma MDA analysis by spectrophotometry

LOD (µM)	Regression analysis	Precision (%) within-day	Precision (%) between-day	Accuracy (%) 2.5µM	5µM	7.5µM	10µM
1.1	y = 0.0073 (±0.00036)x + 0.0036 (±0.0014) r2 = 0.988	8.2	17.3	94.1	90.0	91.3	94.7

For the determination of reproducibility, two samples were included in each assay, one at the beginning and one at the end of the assay row, and analyzed with the samples. The data derived from these samples were analyzed by a Shewhart chart, which is represented in Figure 2.

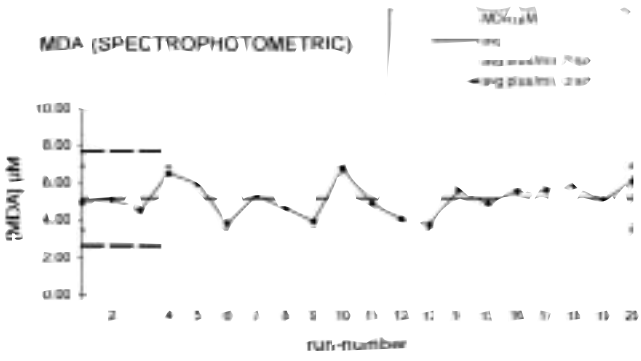

Figure 2. Shewhart chart for plasma malondialdeyhde analysis in MDA-spiked human plasma sample (5 µM) by spectrophotometry.
avg: average; sd: standard deviation

Analysis of plasma samples from smokers and non-smokers

Plasma MDA concentrations of smokers and non-smokers are presented in Table 2. The mean value smokers was higher compared to that of non-smokers. The difference was statistically significant ($p<0.05$).

Table 2. Plasma malondialdehyde concentration of smokers and non-smokers

		MDA concentration (μmol/L)*
Non-smokers	(n = 10)	4.9 ± 0.1
Smokers	(n = 10)	6.7 ± 0.2**

* Data are represented as mean ± SEM

** Statistically significant compared to non-smokers ($p<0.05$).

DISCUSSION

Our aim in the present study was to re-validate the TBAA for the quantitative determination of plasma malondialdehyde according to our laboratory conditions, and to apply the method to smokers' samples. Analysis of MDA by TBAA offers advantages, since it does not require pre-purification of samples before analysis, and the only necessary instrument is a spectrophotometer. With this simple and rapid method, a large number of samples can be analyzed in a short time.

The present assay reached a LOD of 1.1 μM. This is approximately three times lower than the usual levels of 3 μM observed in plasma samples of healthy individuals[15-17]. This simple assay method for plasma MDA has been found reproducible (Within-day coefficient of variation, CV, 8.2%). Between-day variation was calculated as 17.3%. Shewhart chart of the assay proved that the quality of the method is assured in a safe quantitation range (Figure 2; 95% confidence interval).

It has been previously reported that tobacco smoking causes increases in plasma MDA concentrations[18-20]. However, there have also been reports indicating no significant differences[21,22]. We observed a statistically significantly increased MDA concentration in plasma of the smokers, which is in accordance with the former reports. As mentioned in the Introduction, cigarette smoke (CS) may be expected to induce peroxidation of cellular membrane lipids. CS contains numerous precursors in the tar and gas phases, which were converted to electrophilic compounds during burning, and/or during biotransformation in the body. These reactive electrophiles cause lipid peroxidation by abstracting a proton from the methylene bridge adjacent to double bonds of fatty acids. After a series of reactions, MDA is formed as a reactive aldehyde among other degradation products.[23]

The TBAA is associated with two drawbacks: the high temperature used during derivatization is a source of artifactual reactions, and several other endogenous and exogenous compounds in plasma react with TBA and yield products that absorb the light at the same wavelength (532 nm).[8] Despite this lower sensitivity, however, TBAA has been applied successfully in many studies to reflect changes in MDA levels in biological media. In addition, the ease of the method makes it popular world-wide.[9-13]

In conclusion, TBAA is a simple, rapid and reliable method for the quantitative analysis of plasma MDA. A small group of smokers and non-smokers (n = 10) is sufficient to prove statistically significant differences in MDA concentrations.

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