Pathology and Laboratory Medicine International

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ORIGINAL RESEARCH

Evaluation of a simple colorimetric analysis for urinary malondialdehyde determination

This article was published in the following Dove Press journal:
Pathology and Laboratory Medicine International
6 January 2010
Number of times this article has been viewed

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Abstract: Oxidative stress results when the amount of free radical formation exceeds the capacity of the antioxidant defense system. It is related to a number of pathological conditions including: cardiovascular disease, neurodegenerative disease, cancer, diabetes mellitus, gastrointestinal system disorders, inflammation and aging. It is difficult to quantify free radicals directly due to their reactive nature and short half-life. For this reason, most people prefer to measure antioxidant levels and/or the end products of free radical attack on macromolecules such as lipids, proteins and nucleic acids. In this study we have established a short and easy method to determine urinary levels of malondialdehyde, an end product of lipid peroxidation. The method depends on the spectroscopic measurement of color intensity of the product formed by the reaction of the aldehyde groups with the Schiff reagent. The method is linear at a concentration range of 1–10 μM and correlates with the widely used thiobarbituric acid method.

Keywords: urinary malondialdehyde, oxidative stress, Schiff reagent

Introduction

Free radicals and reactive oxygen species are continuously produced during cellular metabolism.[1,2] These are highly reactive electrophilic oxidant species which attack surrounding molecules. Overproduction of these reactive species results in oxidative stress, a condition in which the accumulation of free radicals exceeds the antioxidant capacity. Oxidative stress is related to a number of pathological conditions including cardiovascular disease, neurodegenerative disease, cancer, diabetes mellitus, gastrointestinal system disorders, inflammation and aging.[3]

It is difficult to quantify free radicals directly due to their short half-life and high reactivity. Instead, it is preferred to determine their "footprint" after attacking cellular components such as lipids, proteins and DNA. Lipid peroxidation is a free radical mediated chain of reactions that results in the oxidative breakdown of polyunsaturated fatty acids.[4] The initial products formed are lipid peroxides which tend to degrade rapidly to form a variety of secondary products.[5] Many different methods have been developed to assess the level of lipid peroxidation by measuring a variety of sub-products including: conjugated dienes, volatile gases such as ethane and pentane, isoprostanes, and aldehydes such as 4-hydroxynonenal and malondialdehyde.[5,6] Other methods measuring oxidative damage to protein and DNA are also available.[7,8]

Measurement of malondialdehyde (MDA) is by far the most popular indicator of oxidative damage to cells and tissue.[5] MDA, $CH_2(CHO)_2$ is produced from the breakdown of polyunsaturated fatty acids. In the past 20 to 25 years, MDA has been

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recognized as an important indicator of lipid peroxidation and oxidative stress-related disease states.[9,10]

The Schiff test, devised by Hugo Schiff, is a chemical test for the detection of aldehydes[11]. In this study, we have established a simple colorimetric analysis based on the Schiff reagent for the quantification of urinary malondialdehyde.

Materials and methods

Subjects, chemicals and standards

Spot urine samples were collected in the morning from apparently healthy individuals (n = 218, 114 male, 104 female) aged 1 to 80 years. Basic fuchsin and methanol (HPLC Grade) was obtained from Merck. Malondialdehyde bis(diethyl acetal), sodium metabisulfite, phosphoric (V) acid and thiobarbituric acid was obtained from Sigma-Aldrich. All other chemicals used were of the purest grade commercially available. Malondialdehyde (MDA) standard solution (5 mM) was prepared and diluted with 0.1 M HCl. The solution was hydrolyzed for 3 hours at room temperature. Serial dilutions (1–10 µM) were made and used as standards. The concentration of MDA was verified by spectrophotometry at 245 nm ($\varepsilon = 13{,}700\ M^{-1}\,cm^{-1}$).

Colorimetric assay

Schiff reagent was prepared by dissolving 10 mL phosphoric (V) acid, 10 g sodium metabisulfite and 5 g basic fuchsin in 1 L of ultra pure water. The mixture was left for 2 hours in the dark before 30 g of activated carbon was added. The solution was set aside for 24 hours and filtered before use. The colorimetric assay was carried out by adding 20 µL of urine to 970 µL of ultra-pure water. After thorough mixing 10 µL of the Schiff reagent was added and the absorbance at 545 nm was read within 2 to 5 minutes.

HPLC method

For the quantification of urinary MDA by HPLC,[12,13] to 3.0 mL (1%) phosphoric (V) acid and 0.4 ml ultra pure water 0.6 mL of sample or standard (1–10 µM MDA) was added to a screw capped test tube and mixed thoroughly. Then, 1.0 mL of 0.67% thiobarbituric acid was added to all tubes and kept in a boiling water bath for 1 hour. After chilling in crushed ice, the tubes were centrifuged at 14,000 × g for 15 minutes. The supernatant was filtered before being applied to a C-18 column. Measurements were made with a UV-Vis detector at 532 nm. Mobile phase was 0.05 M potassium phosphate buffer (pH 6.8) with methanol (50:50, v/v). The flow-rate was 0.5 mL/min.

Statistical analysis

Statistical analysis was carried out using the Student's t-test and the differences were considered to be significant at $P < 0.05$.

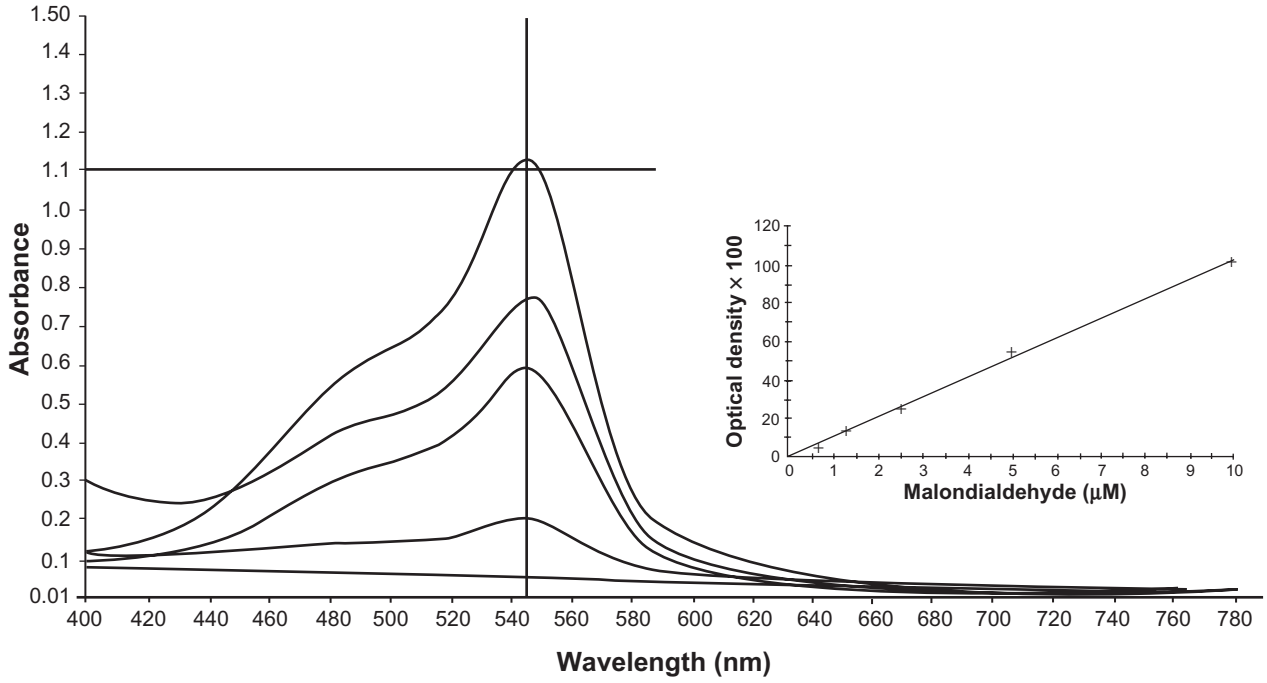

Figure 1 Absorption curve obtained by the Schiff method at different malondialdehyde concentrations.

Table 1 Mean, standard deviation and variances of within-day measurements (n = 10) of low and high absorbance samples

	Mean	Standard deviation	Variance
Low absorbance			
Sample 1	0.030	0.006	0.00007
Sample 2	0.025	0.005	0.00005
Sample 3	0.028	0.009	0.00009
High absorbance			
Sample 1	0.251	0.084	0.00798
Sample 2	0.382	0.132	0.01935
Sample 3	0.296	0.101	0.01137

Results and discussion

It is well known that thiobarbituric acid is a useful general reagent for the determination of MDA as an index of lipid peroxidation.[5,10] In recent years, methods based on HPLC have been developed for measuring MDA in urine and other body fluids.[12–15] The use of HPLC with UV-Vis detection has improved the selectivity and sensitivity of the method. However, the method is not practical enough for a large number of samples and requires expensive equipment together with skillful personnel.

Figure 1 shows the absorption curve obtained by the colorimetric Schiff method. The absorbance at 545 nm was a linear function of the MDA concentration over the range from 0.5 µM to 10 µM. Table 1 summarizes the precision of the method determined by measuring low and high absorbance samples. Within day values are presented with their average, standard deviation and variances. It was observed that the standard deviation and variances of high absorbance samples was much higher than low absorbance samples indicating that at high concentrations of MDA the reliability of the test is low. Figure 2 shows the correlation between the colorimetric Schiff method and the HPLC method.

We have tested the colorimetric Schiff assay in 218 individuals, from both sexes aged between 1 to 80 years. There was no significant difference between values obtained from males and females as well as different age groups. But the distribution of the Schiff assay values showed that more than 70% of the individuals had an absorbance value that was lower than 0.200 corresponding to <4 mM MDA (Figure 3). This value is in agreement with a previous report that studied the stability and intra-individual variation of urinary malondialdehyde.[15] Thus, we suggest this as a cut-off value for using this simple method as a screening test that may be adapted by small scale laboratories for routine monitoring of oxidative stress. The ability to simultaneously process many samples, ease of use and the rapidity of the method

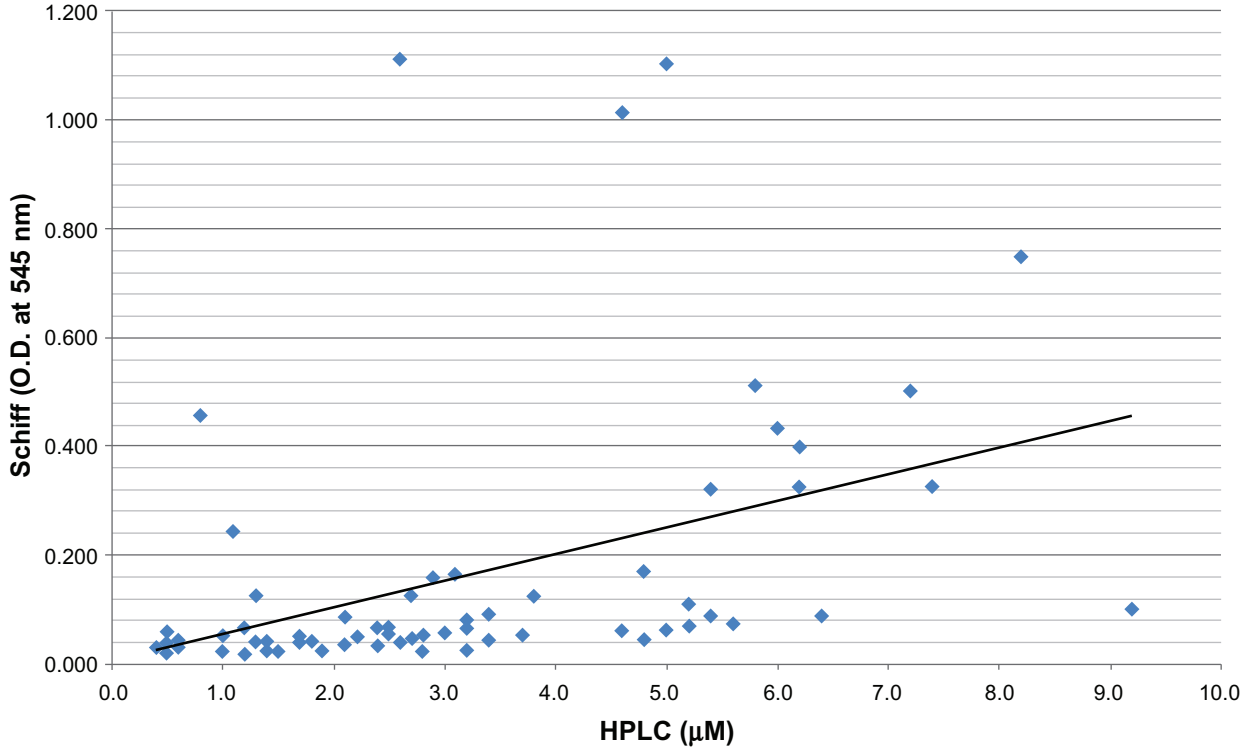

Figure 2 Comparison of the colorimetric Schiff assay results with the HPLC method.

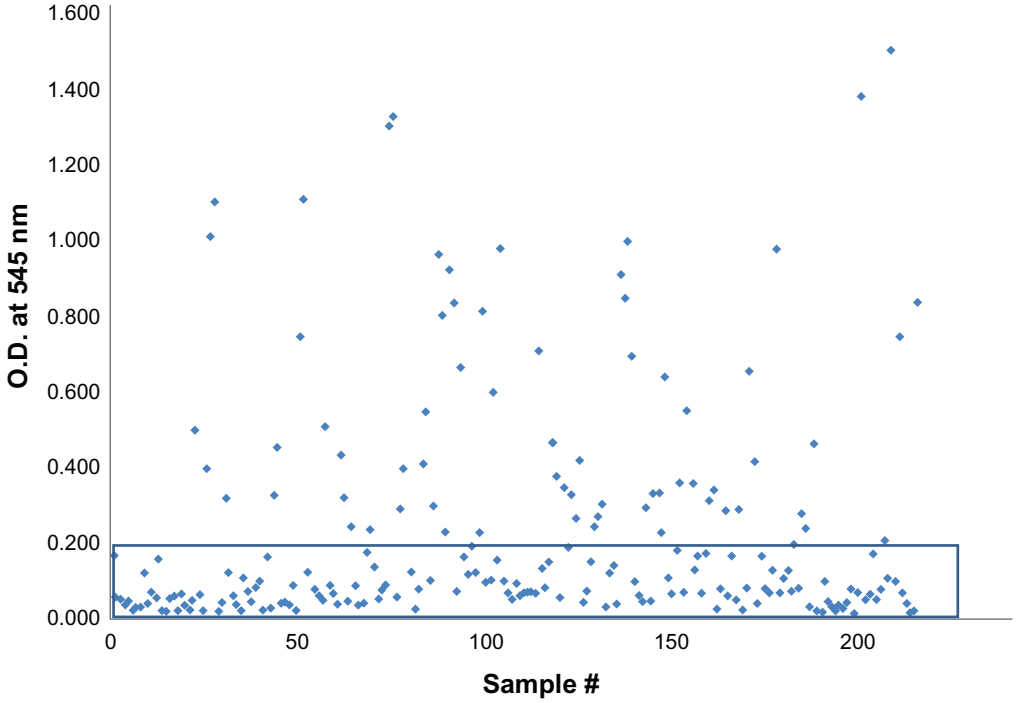

Figure 3 Distribution of colorimetric Schiff assay values in 218 individuals aged 1 to 80 years.

are practical reasons for supporting its use in the screening of free radical related human disorders.

Acknowledgment

Supported in part by the Turkish Scientific and Technological Research Council (TUBITAK-TEYDEB, Project no. 7070041). This work was first presented at the 20th National Biochemistry Congress, 29 October–1 November 2008, Kapadokya-Nevşehir, Turkey.

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