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A Rapid Method for Estimating the Levels of Urinary Thiobarbituric Acid Reactive Substances for Environmental Epidemiologic Survey

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Malondialdehyde (MDA), used as an oxidative stress marker, is commonly assayed by measuring the thiobarbituric acid reactive substances (TBARS) using HPLC, as an indicator of the MDA concentration. Since the HPLC method, though highly specific, is time-consuming and expensive, usually it is not suitable for the rapid test in large-scale environmental epidemiologic surveys. The purpose of this study is to develop a simple and rapid method for estimating TBARS levels by using a multiple regression equation that includes TBARS levels measured with a microplate reader as an independent variable. Twelve hour urine samples were obtained from 715 subjects. The concentration of TBARS was measured at three different wavelengths (fluorescence: λ-$_{ex}$ 530 nm and λ-$_{em}$ 550 nm; λ-$_{ex}$ 515 nm and λ-$_{em}$ 553 nm; and absorbance: 532 nm) using microplate reader as well as HPLC. 500 samples were used to develop a regression equation, and the remaining 215 samples were used to evaluate the validity of the regression analysis. The induced multiple regression equation is as follows: TBARS level (μM) = –0.282 + 1.830 × (TBARS level measured with a microplate reader at the fluorescence wavelengths λ-$_{ex}$ 530 nm and λ-$_{em}$ 550 nm, μM) –0.685 × (TBARS level measured with a microplate reader at the fluorescence wavelengths λ-$_{ex}$ 515 nm and λ-$_{em}$ 553 nm, μM) + 0.035 × (TBARS level measured with a microplate reader at the absorbance wavelength 532 nm, μM). The estimated TBARS levels showed a better correlation with, and are closer to, the corresponding TBARS levels measured by HPLC compared to the values obtained by the microplate method. The TBARS estimation method reported here is simple and rapid, and that is generally in concordance with HPLC measurements. This method might be a useful tool for monitoring of urinary TBARS level in environmental epidemiologic surveys with large sample sizes.

Key words: Malondialdehyde, Thiobarbituric acid reactive substances, HPLC, Microplate, Regression equation

INTRODUCTION

Aging and disease, have been linked to oxidative stress damage of DNA and protein that results in various deleterious effects on cells, such as lipid peroxidation (1,2). As a marker for oxidative stress, lipid peroxidation can be indirectly evaluated by assay of malondialdehyde (MDA) produced during the process of lipid peroxidation (3).

The most common method of measuring MDA is to determine the amount of TBARS that reacts with MDA and TBA, using HPLC. Although direct determination of TBARS by HPLC may increase the specificity of the assay, the use of HPLC is attendant with the issues of expensive columns. There is also a limitation on the number of samples that can be measured in a day because it takes approximately 10 min to analyze each sample (4,5). In addition, given that the HPLC method requires frequent instrument cleanup, the rate of sampling decreases accordingly (6). Therefore, it is

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inconvenient to use this method for large-scale environmental epidemiologic studies. On the other hand, measurement using a microplate facilitates high throughput screening, because it allows the simultaneous analysis of tens of samples and does not require cleaning.

The microplate methods for TBARS measurement are less expensive, less time consuming, and less laborious than the HPLC methods. However, based on the experience of the authors, microplate readers generally yield higher values for TBARS levels than HPLC for the same samples. This discrepancy could be explained by the fact that the MDA-TBA adduct can be distinguished from other TBA-bound substances in case of HPLC-based methods, but not in case of microplate methods.

The purpose of this study is to develop a valid method for estimating TBARS levels by applying the microplate method while maintaining simplicity and time-based efficiency.

MATERIALS AND METHODS

Subject. The subject includes 303 adults, 148 primary and middle school students, and 264 infants and toddlers, who participated in a nationwide epidemiologic survey conducted in 2011 by the Korea Food and Drug Administration to study the extent of exposure to toxic metals and the related effects in general Korean population. We collected 12 hr urine samples from every subject. All adult subjects and parents of all students, infants, and toddlers provided written consent, which included approval for the measurement of urinary MDA levels. Of the 715 samples, 500 randomly selected samples were used to develop a new method, and the remaining 215 samples were used to evaluate the validity of the method. The study protocol was approved by the Chung-Ang University Ethical Committee for Medical Research (IRB approval 2011-02-02).

TBARS measurement. Standard solutions were prepared by dissolving the required amounts of 1,1,3,3-tetramethoxypropane (TMP) in 40% ethanol to yield 2, 2.5, 5, and 10 μM solutions. Fifty microliters, each of the urine samples and the standard solutions, was mixed with 50 μL of 0.05% butylated hydroxytoluene, 150 μL of 0.1125 N HNO_3, and 150 μL of 42 mM TBA. After 1-h incubation at 95°C, the mixture was cooled with ice for 5 min. To the cooled mixture, 300 μL of *n*-butanol was added, and then, the solution was centrifuged for 5 min at 1,500 rpm and 4°C. The supernatant was used for analyses.

Two hundred microliters of the pretreated samples was dispensed into a 96-well microplate (F96 Microwell Plate; Nunc, Denmark), and fluorescence was measured by the top method at λ_{ex} = 530 nm and λ_{em} = 550 nm; λ_{ex} = 515 nm and λ_{em} = 553 nm, and the absorbance was measured at 532 nm. Both fluorescence and absorbance measurements were carried out in using a SPECTRAmax PLUS microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Using an autosampler (SIL-10ADvp; Shimadzu, Japan), 10 μL of the supernatants was injected into a HPLC system fitted with a fluorescence detector (RF-10AxL; Shimadzu) and a reverse-phase column (TSK-gel; TOSOH, Japan). The fluorescence wavelengths were set at λ_{ex} = 515 nm and λ_{em} = 553 nm. The mobile phase consisted of a mixture of 50 mM KH_2PO_4 (pH 6.8), methanol, and acetonitrile (65 : 20 : 15, v/v/v), and the flow rate was 1.0 mL/min. The external standard method was applied to the TBARS quantification methods.

Statistical analysis. A multiple regression analysis was performed for TBARS levels measured by the HPLC method. The statistically analyzed regression model is as follows:

$$H = \alpha_1 + \beta_1 \times A + \beta_2 \times (B - H) + \beta_3 \times (C - H),$$

where H: TBARS level measured by the HPLC method at the fluorescence wavelengths λ_{ex} = 515 nm and λ_{em} = 553 nm (μM),

A: TBARS level measured by the microplate method at the fluorescence wavelengths λ_{ex} = 530 nm and λ_{em} = 550 nm (μM),

B: TBARS level measured by the microplate method at the fluorescence wavelengths λ_{ex} = 515 nm and λ_{em} = 553 nm (μM), and

C: TBARS level measured by the microplate method at the absorbance wavelength of 532 nm (μM).

The statistically induced model was converted to the following equation:

$$H = \alpha_2 + \beta_4 \times A + \beta_5 \times B + \beta_6 \times C$$

All independent variables were tested for their collinearity.

RESULTS

Table 1 shows the distribution of urinary TBARS levels measured using the HPLC and microplate method in 500 subjects (211 adults, 97 primary and middle school students, and 192 infants and toddlers). The average TBARS level measured using the HPLC method was 2.15 μM, whereas the average TBARS level measured by the microplate method was 2.79 μM (fluorescence wavelengths: λ_{ex} = 530 nm and λ_{em} = 550 nm), 4.42 μM (fluorescence wavelengths: λ_{ex} = 515 nm and λ_{em} = 553 nm), and 10.21 μM (absorbance wavelength: 532 nm), respectively, all of which were higher than that determined by the HPLC method. The value obtained by the microplate method at λ_{ex} = 530 nm and λ_{em} = 550 nm showed the highest R-square value, i.e., 0.788, with that obtained by HPLC.

The results of a multiple regression model for TBARS level measured by HPLC are presented in Table 2. This can be summarized as follows:

Table 1. Distributions of measured TBARS levels and R-squares between the HPLC method concentration and the other 3 measured values

Device/method	Wavelength	N	TBARS (μM)		R-square
			Mean ± S.D.	Range	
HPLC/fluorescence	λ_{ex} = 515 nm, λ_{em} = 553 nm	500	2.15 ± 1.60	0.01~11.86	
Microplate/fluorescence	λ_{ex} = 530 nm, λ_{em} = 550 nm	500	2.79 ± 1.55	0.01~10.86	0.788
Microplate/fluorescence	λ_{ex} = 515 nm, λ_{em} = 553 nm	500	4.42 ± 2.29	0.30~12.09	0.495
Microplate/absorbance	λ = 532 nm	500	10.21 ± 5.51	0.94~29.94	0.251

Table 2. Multiple regression analysis result for TBARS concentration measured with an HPLC

Variables	β	Std. Error	t	p-value
Constant	−0.171	0.035	−4.910	< 0.0001
A	1.109	0.013	86.366	< 0.0001
B - H	−0.415	0.016	−25.237	< 0.0001
C - H	0.021	0.005	3.390	< 0.0001

A: TBARS concentration measured with microplate at fluorescence wavelength λ_{ex} = 530 nm and λ_{em} = 550 nm, B: TBARS concentration measured with microplate at fluorescence wavelength λ_{ex} = 515 nm and λ_{em} = 553 nm, C: TBARS concentration measured with microplate at absorbance wavelength 532 nm, H: Dependent variable, TBARS concentration measured with HPLC, at λ_{ex} = 515 nm and λ_{em} = 553 nm.

Table 3. Collinearity statistics: tolerances and variance inflation factors

	Collinearity statistics	
	Tolerance	VIF
A	0.564	1.772
B-H	0.522	1.915
C-H	0.410	2.440

A: TBARS concentration measured with microplate at fluorescence wavelength λ_{ex} = 530 nm and λ_{em} = 550 nm, B: TBARS concentration measured with microplate at fluorescence wavelength λ_{ex} = 515 nm and λ_{em} = 553 nm, C: TBARS concentration measured with microplate at absorbance wavelength 532 nm, H: Dependent variable, TBARS concentration measured with HPLC, at λ_{ex} = 515 nm and λ_{em} = 553 nm.

$$H = 0.282 + 1.830 \times A - 0.685 \times B + 0.035 \times C$$

where H is the TBARS level measured using the HPLC method (μM), A is the TBARS level measured by the microplate method at λ_{ex} = 530 nm and λ_{em} = 550 nm (μM), B is the TBARS level measured by the microplate method at λ_{ex} = 515 nm and λ_{em} = 553 nm (μM), and C is the TBARS level measured by the microplate method at the absorbance wavelength 532 nm (μM).

Tables 3 and 4 show the results of the collinearity test for the independent variables of multiple regression analysis. The tolerances for each independent variable listed in Table 3 were 0.564, 0.522, and 0.410, and the variance inflation factors (VIFs) were 1.772, 1.915, and 2.440, respectively, indicating no collinearity. None of the condition indices suggested collinearity, with all being less than 30 (Table 4).

Compared to the average value obtained by the HPLC method, i.e., 2.21 μM, all the average values obtained by the three different microplate conditions (fluorescence wavelengths: λ_{ex} = 530 nm and λ_{em} = 550 nm, and λ_{ex} = 515 nm and λ_{em} = 553 nm; and absorbance wavelength: 532 nm), i.e., 2.82 μM, 4.40 μM, and 10.28 μM, respectively, were higher (Table 5). However, the average TBARS level estimated from the regression equation was 2.22 μM, which is almost identical to the average TBARS level measured using the HPLC method. The R-square value between the value obtained by the HPLC method and the estimated values obtained using the regression model was 0.933, which was highest among the four R-square values.

Fig. 1 illustrates scattergrams between the levels determined by the HPLC method and those determined by three different microplate methods and those estimated from the regression equation. In the scattergrams for the microplate methods, most of the data points lie above the y = x regres-

Table 4. Collinearity diagnostic results

Model	Dimension	Eigenvalue	Condition index	Variance Proportions			
				Constant	A	B - H	C - H
1	1	3.676	1.000	0.01	0.01	0.01	0.01
	2	0.145	5.042	0.96	0.04	0.07	0.09
	3	0.114	5.679	0.01	0.67	0.46	0.00
	4	0.065	7.518	0.02	0.29	0.47	0.90

A: TBARS concentration measured with microplate at fluorescence wavelength λ_{ex} = 530 nm and λ_{em} = 550 nm, B: TBARS concentration measured with microplate at fluorescence wavelength λ_{ex} = 515 nm and λ_{em} = 553 nm, C: TBARS concentration measured with microplate at absorbance wavelength 532 nm, H: Dependent variable, TBARS concentration measured with HPLC, at λ_{ex} = 515 nm and λ_{em} = 553 nm.

Table 5. Distributions of TBARS levels measured and estimated with the regression model and R-squares between the HPLC method concentration and the other measured or estimated values

Device/method	Wavelength	N	TBARS (μM)		R-square
			Mean ± S.D.	Range	
HPLC/fluorescence	λ_{ex} = 515 nm, λ_{em} = 553 nm	215	2.21 ± 1.95	0.04~14.20	
Microplate/fluorescence	λ_{ex} = 530 nm, λ_{em} = 550 nm	215	2.82 ± 1.90	0.41~12.17	0.876
Microplate/fluorescence	λ_{ex} = 515 nm, λ_{em} = 553 nm	215	4.40 ± 2.56	0.75~14.48	0.591
Microplate/absorbance	λ = 532 nm	215	10.28 ± 5.80	2.17~27.23	0.170
Regression model		215	2.22 ± 2.01	0.01~13.02	0.933

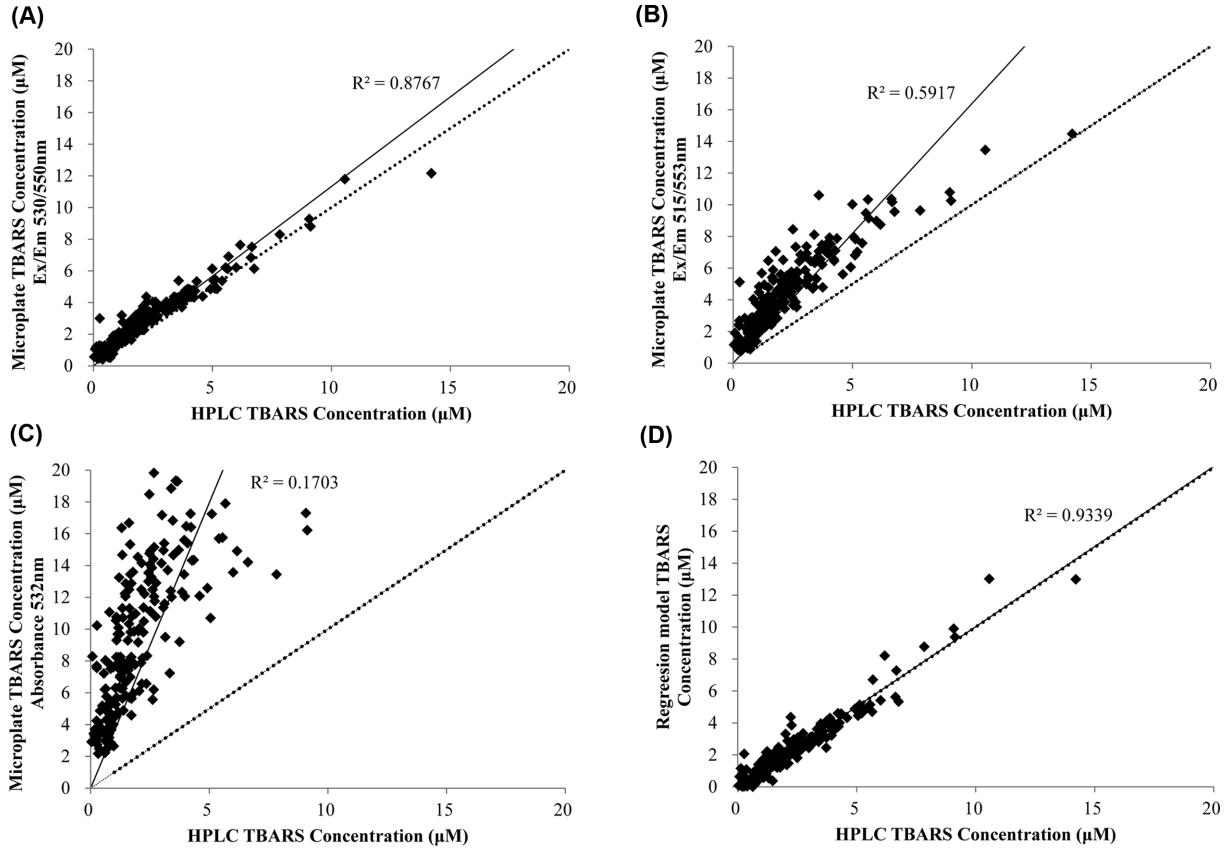

Fig. 1. Scattergrams between TBARS concentrations determined by the HPLC method, and those by 3 different microplate reader methods ((A) fluorescence at λ_{ex} = 530 nm/λ_{em} = 550 nm, (B) fluorescence at λ_{ex} = 515 nm/λ_{em} = 553 nm, (C) absorbance at λ = 532 nm) and those estimated from the regression equation (D). The dotted line in each scatter plot represents the identity line.

sion line, indicating that the TBARS levels measured by the three different microplate methods are higher than the corresponding TBARS level measured by the HPLC method. In case of the value estimated from the regression equation, however, the data points are in close proximity with the y = x regression line and evenly scattered above and below the line.

DISCUSSION

Since a lot of diseases have been linked to oxidative damages of DNA and proteins that result in various deleterious effects on cells, evaluation of TBARS, a marker for oxidative stress, have been widely used in environmental epidemiologic studies. The HPLC method, though highly specific, is time-consuming, expensive and labor-intensive, therefore, rapid and inexpensive methods evaluating urinary TBARS level need to be developed, especially for large-scale environmental epidemiologic surveys.

In the present study, the TBARS levels estimated from the regression equation showed a higher correlation with those measured by the HPLC method than any TBARS levels measured with the three different microplate methods. In comparison of TBARS levels measured by HPLC method

and the microplate method, the TBARS levels measured by all of the three different microplate methods were higher than that by the HPLC method. This overestimated TBARS levels measured by single absorbance or fluorescence wavelength are due to the interfering chromogens (5). The interfering chromogens signals can be removed effectively in the HPLC-based assay. In case of the value estimated from the regression equation, however, the data points are in close proximity with the y = x regression line and evenly scattered above and below the line. This indicates that the TBARS level estimated from the regression equation rather than microplate method is more consistent with that measured using the HPLC method.

With the objective of developing a simple and rapid estimation method of urinary TBARS level, we adopted a multiple regression analysis approach. The regression model is not valid if there is collinearity among the independent variables. To avoid the issue of collinearity, the values obtained by the microplate methods corrected by subtracting the corresponding value obtained by the HPLC method were used as independent variables. In the collinearity test, all the tolerances were greater than 0.1, and all the VIFs were smaller than 10. In addition, every condition index was below the cut-off value of 30. These facts suggest that collinearity was not an issue.

This study has one limitation; since the TBARS level was estimated from a regression equation, the values for around 2% of the total samples were negative. In this study, we substituted the negative TBARS levels with the value of the detection limit divided by 2. In addition, the regression equation derived does not reflect individual urine characteristics such as levels of bile pigment, hemoglobin, and bilirubin, which can interfere with the measurement of TBARS level (7,8).

In summary, we proposed a simple, rapid and reliable method for TBARS estimation which might be a convenient tool for estimation of urinary TBARS level in large-scale environmental epidemiologic studies.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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