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New method for HPLC separation and fluorescence detection of malonaldehyde in normal human plasma

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Abstract

A new method for the detection of free and total malonaldehyde (MDA) in human plasma samples based on the derivatization of MDA with 9-fluorenylmethoxycarbonyl hydrazine (FMOC-hydrazine) in an acidic medium was developed. Derivatization was achieved after 4 h at 50 °C. The derivatized samples were analyzed by HPLC using a reversed-phase C_{18} column with fluorescence detection (Ex = 270 nm, Em = 310 nm). The benefit of this direct injection of deproteinized plasma is to avoid the use of an internal standard. The detection limit was 0.1 pmol (4.0 nmol/L). The recovery of MDA spiked in different human plasma samples was 95.3% (n = 25; R.S.D. 5.1%) for the hydrolysation procedure. The total and free MDA in plasma of 15 healthy male volunteers are 426 ± 29.8 nmol/L and 153 ± 9.6 nmol/L, respectively.

Keywords: Malonaldehyde; 9-Fluorenylmethoxycarbonyl hydrazine; Plasma; Fluorescence detection

1. Introduction

Free radicals have been known for causing oxidative damages. It has been suggested that they might play a part in various pathological processes [1,2]. Free radicals could attack lipids to initiate lipid peroxidation [3,4]. Due to the difficulty of directly measuring the free radicals production, the measurement of lipid peroxidation has become a commonly used technique as to evaluate oxidative stress. The determination of malonaldehyde (MDA) is the most widely used method for the monitoring of lipid peroxidation [5]. The most common method for measuring MDA is based on its reaction with 2-thiobarbituric acid (TBA) in acidic media at a temperature of 100 °C and measuring absorbance at 533 nm [6]. However, TBA reacts not only with MDA but also with many other compounds that are present in biological samples [7,8]. Furthermore, the method exhibits limited sensitivity and selectivity. Although the selectivity and the sensitivity might be improved by using HPLC with UV or fluorescence detection [9,10], the drawback is that the harsh conditions used in sample preparation might generate artificial results [8]. Another common method for the determination of MDA is based on its reaction with 2,4-dinitrophenylhydrazine (DNPH) at low pH, with the formation of DNPH derivatives [11–13]. The benefit of this method is that the derivatization reaction does not require high temperature to proceed, but the assay process is not as quick as the TBA method. The DNPH method involves multiple liquid–liquid extractions [11–13], recrystallization and purification of DNPH reagent on a daily basis [14], and the samples can be very easily contaminated with atmospheric aldehydes or impurities present in the reagents [12]. In addition, an internal standard is required in this method. Recently, it was reported that MDA in plasma was successfully measured after its reaction with diaminonaphtalene (DAN) in an acidic medium at 37 °C. However, this method is not suitable for measuring MDA in urine, due to the presence of numerous interfering compounds [15].

A new HPLC method to measure MDA in plasma with fluorescence detection is described in this paper. The method is based on the derivatization of MDA with 9-fluorenylmethoxycarbonyl hydrazine (FMOC-hydrazine) to form a FMOC-hydrazone in an acidic medium.

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2. Experimental

2.1. Chemicals

1,1,3,3-Tetraethoxypropane (TEP, 97%), formaldehyde (37%), acetaldehyde (99%), propaldehyde (97%), were from Acros Organics (USA). FMOC-hydrazine (purity > 99%) was synthesized in our laboratory [16]. HPLC grade acetonitrile was purchased from Fisher Chemicals (New Jersey, USA). All other chemicals were of analytical grade and obtained from Beijing Chemical Reagent Factory. Triple distilled water was used throughout the study.

2.2. Sample collection

Peripheral blood samples were collected in Heparin Vacuettes (Greiner, Austria) from 15 healthy male volunteers (20–45 years old, nonsmokers). Blood samples were taken daily and plasma was separated within 1 h with centrifugation (4000 rpm for 10 min at 4 °C).

2.3. Preparation of reagents and calibration standards

FMOC-hydrazine was prepared at 1.0 mmol/L in acetonitrile, stored at 4 °C. NaOH solution (5.0 mol/L) was prepared and stored at 4 °C.

Standard stock solution of TEP (1.0 mmol/L) was prepared by dissolving 25 μL TEP in 100 mL of water. This stock solution was stored at 4 °C and freshly prepared on a weekly basis. MDA stock solution (final concentration ca. 20 μmol/L) was obtained daily by hydrolysis of 1 mL TEP stock solution in 50 mL of 1% sulfuric acid and incubation for 2 h at room temperature [17]. Standard solutions of MDA were prepared by further dilution of MDA stock solution with 1% sulfuric acid to get the standard curve and to spike plasma samples for determination of the recovery.

2.4. Derivatization of MDA

Solutions of 0.4 mL each of MDA standard and FMOC-hydrazine in various concentrations were placed into 1.0 mL PTFE vials with screen-on lid. The solutions were prepared in acetonitrile and the ratio of MDA to FMOC-hydrazine was maintained at 1:20. The solution was heated at 50 °C for 4 h in a water bath, then cooled to room temperature. The solution was neutralized with 5.0 mol/L NaOH, and 25 μL of solution was injected into the HPLC system for analysis.

2.5. HPLC analysis

The HPLC analysis was performed with a Shimadzu HPLC system including a LC-10ATvp pump, a 7725i Rheodyne injector, a FCV-10ALvp mixer, a DGU-14A degasser, a RF-10Axl fluorescence detector (excitation at 270 nm, emission at 310 nm) and a WDL-95 chromatography workstation (Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Liaoning, China). A Nucleodur® C_{18} column (5 μm, 250 mm × 4.6 mm, Macherey-Nagel, USA) with a guard cartridge kit (SecurityGuard™, Phenomenex®, USA) was used for separation. The mobile phase was acetonitrile/water (53/47, v/v). The flow-rate was 1.0 mL/min.

2.6. Sample preparation

2.6.1. Free MDA

In a 2.0 mL Eppendorf tube, 0.7 mL of acetonitrile was added to 0.7 mL of plasma sample. The tube was then vortexed to precipitate the protein in the sample. After centrifugation at 14 000 rpm for 10 min, 1 mL of the clear supernatant was transferred to a 1.5 mL Eppendorf tube. A 5.0 μL of sulfuric acid was then added to the supernatant to obtain a mixture containing 1% sulfuric acid. Subsequently, a 0.3 mL of the mixture and a 120 μL of 1.0 mmol/L FMOC-hydrazine solution were added into a 1.0 mL PTFE bottle with a screw-on lid. Then, the bottle was incubated for 4 h at 50 °C. The derivatized samples were neutralized with 5.0 mol/L NaOH prior to its analysis. The sample injection volume was 25 μL.

2.6.2. Total MDA

For hydrolysis of protein bound MDA, 10 μL of sulfuric acid was added to 1.0 mL of plasma in a 1.5 mL Eppendorf tube (final concentration of H_2SO_4 in the solution was 0.182 mol/L). The mixture was incubated for 60 min at room temperature. Then, 0.2 mL of the mixture, 0.2 mL of 1% sulfuric acid and 400 μL of 200 nmol/L FMOC-hydrazine solution were combined in a 1.0 mL PTFE bottle with a screw-on lid. The bottle was incubated for 4 h at 50 °C and the derivatized samples were centrifugated at 14 000 rpm for 10 min. The clear supernatant was adjusted to neutrality with 5.0 mol/L NaOH before injection with 25 μL of solution.

3. Results and discussion

3.1. Optimization of derivatization conditions

The reaction of FMOC-hydrazine with aldehyde groups is an addition reaction (shown in Fig. 1). The product of this addition reaction releases water to form a stable product, FMOC-hydrazone. Therefore, increasing the molar ratio of FMOC-hydrazine to MDA would be of benefit to improve the yield of MDA-FMOC-hydrazone. The derivative yield was investigated with the molar ratio in the range of 2:1 to 100:1 (see Fig. 2). The results showed in Fig. 2 indicated that the derivative yield reached a high level when the molar ratio is above 20:1, so the derivatization was completed at the molar ratio of 20:1.

The reaction temperature and time are always the important factors on the derivative yield (see Fig. 3). From Fig. 3, it can be concluded that, at low temperature (e.g., 40 °C), the reaction was slow, whereas at higher temperature (e.g., 60 °C), the reaction reached near completion in 3 h. However, at higher temperature, both the solvent and the MDA evaporated easily, so that it was difficult to maintain the concentration of the derivatives. At 50 °C, the reaction also reached near completion within 4 h. Therefore, the preferred reaction temperature and time is chosen to be at 50 °C and 4 h.

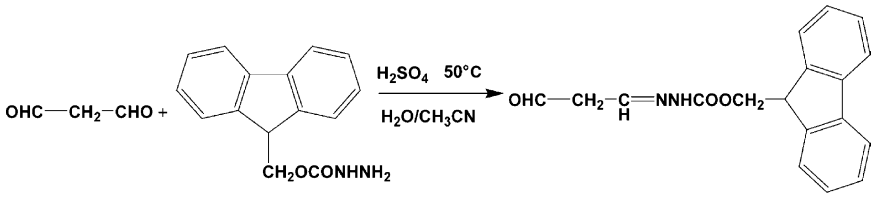

Fig. 1. The derivatization of malonaldehyde with FMOC-hydrazine.

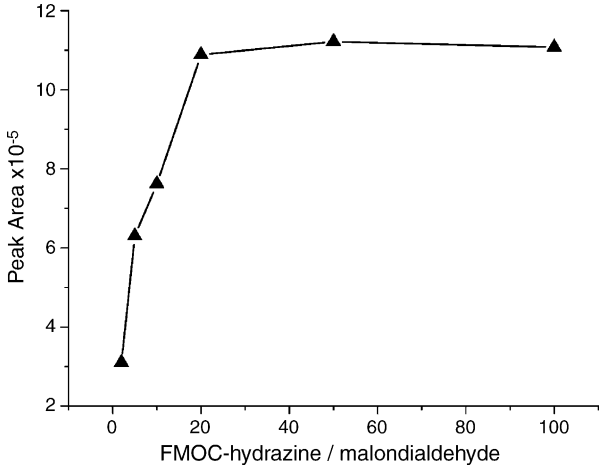

Fig. 2. Effect of molar ratio of FMOC-hydrazine to MDA on the derivatization yield.

3.2. Selectivity, recovery, reproducibility and stability of MDA-FMOC-hydrazone

In Fig. 4, the representative chromatograms of the FMOC-hydrazine derivatives are shown. The HPLC method we used resulted in excellent separation of the MDA-FMOC-hydrazone from the other short-chain water-soluble aldehydes derivatives, namely formaldehyde, acetaldehyde and propaldehyde.

In these experiments, different specimens of plasma ($n = 5$) obtained on five different days were divided in two aliquots and one of them was spiked with 4.0 nmol of MDA. Each specimen was analyzed in five replicated preparations for total MDA as described above. The recovery of MDA was $95.3 \pm 5.1\%$ ($n = 25$). Within the same run, the variations of two samples were 4.7% and 4.1%, while the between-run variations for two different samples were 5.8% and 4.5%.

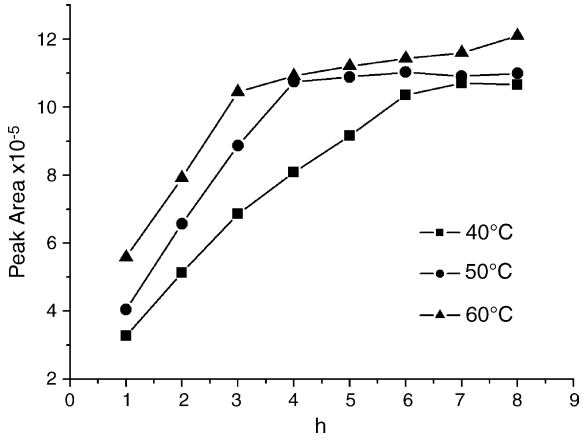

Fig. 3. Effects of time and temperature on the derivation of FMOC-hydrazine with MDA. The concentration of MDA was 4.82 nmol/mL.

The stability of the MDA-FMOC-hydrazone was tested by re-analysis of the same samples stored at room temperature (22–32 °C) for 72 h. The percent changes were less than 4.7%. This indicated that the derivatives are stable for at least 72 h when stored at room temperature.

The derivatization reagents, FMOC-hydrazine and DNPH possess identical hydrazine group, which are strong nucleophiles and react readily with aldehydes [18,19]. This characteristic facilitated the rapid derivatization reaction at mild acidic condition and lower temperature. These conditions suppress the formation of undesired artificial aldehyde, which are produced during the sample preparation. Unfortunately, the assay with DNPH involves multiple liquid–liquid extractions [11–13] due to the excess of unreacted DNPH, which was difficult to be separated from MDA-DNPH [20], and the DNPH requires purification on a daily basis because of its instability [14]. By adding additional extraction steps, the separation and sensitivity can be improved [19], but at the same time, factors like recovery, reproducibility and precision will suffer. The extraction process is not only time consuming but also difficult to keep the precision in the operation [21]. In order to minimize the shortcomings of extraction process, using an internal standard is a necessity for this kind of method. As for our method, on the contrary, a baseline separation of the MDA from the interfering peak was achieved directly as seen in Fig. 4. In other words, the excess of unreacted FMOC-hydrazine reagent did not affect the separation and the quantitation of MDA. The method benefited from this excellent separation character, so that the extraction procedure from sample preparation was completely eliminated. This helped to simplify the procedure and enhance the recovery, reproducibility and precision of the established method. Furthermore, the MDA-FMOC hydrazone was stable for at least 72 h at room temperature. This is also an important factor for developing a precise and accurate quantitative method.

3.3. Calibration curve and sensitivity

A typical calibration curve is shown in Fig. 5. It is linear up to 10 nmol/mL. The detection limit, measured on the real samples (mean baseline noise ± 3S.D.), is calculated to be 0.1 pmol (4.0 nmol/L). A comparison of sensitivity among the recently published methods is given in Table 1. The summarized results prove the high sensitivity of FMOC hydrazine method.

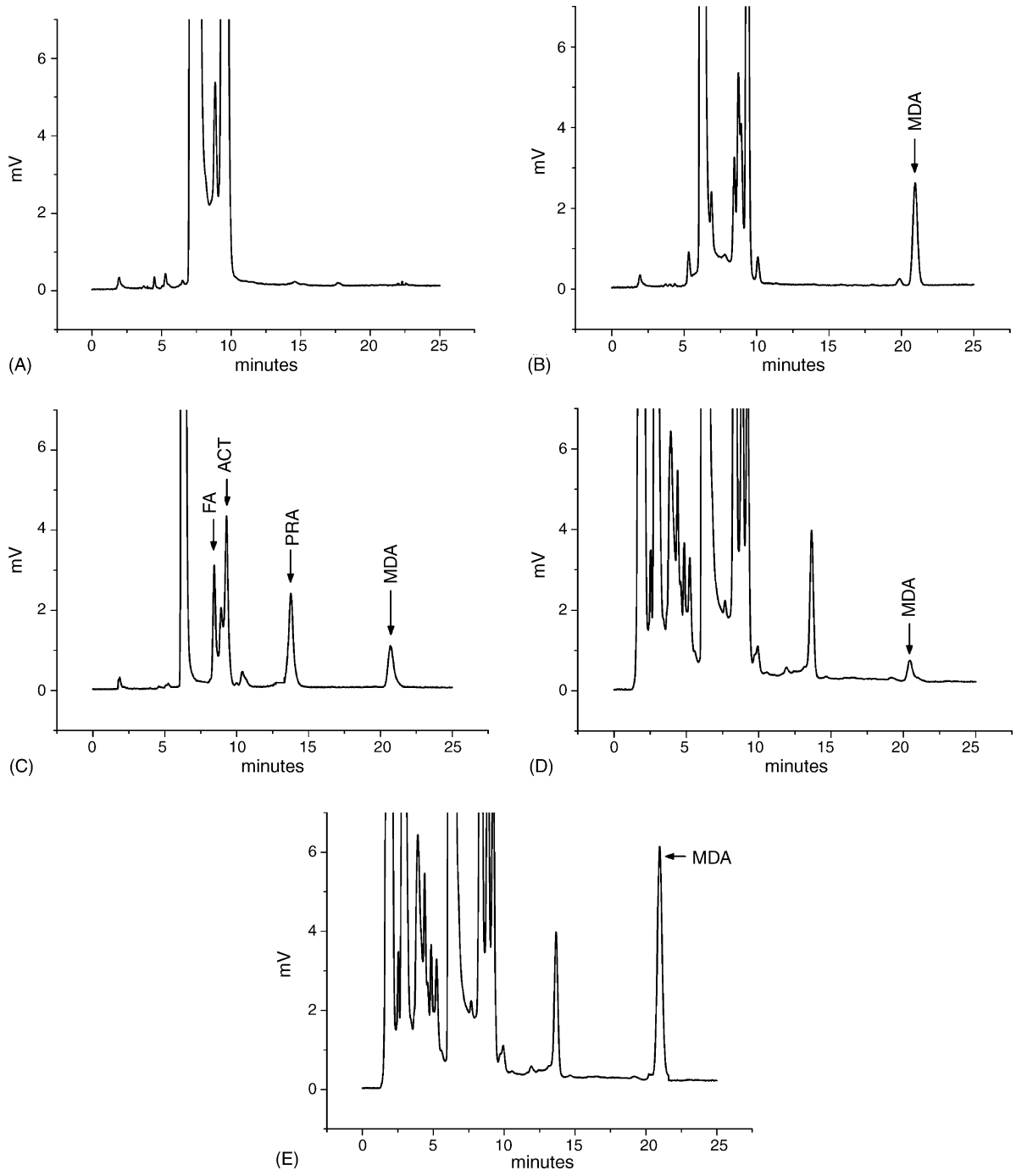

Fig. 4. Typical chromatograms of blank, standard MDA and plasma specimen after derivatization with FMOC-hydrazine. Conditions of derivatization are as described in Section 2. (A) Reagent blank; (B) standard MDA (2.41 nmol/mL); (C) chromatographic separation of MDA and the short-chain carbonyls. Abbreviations of FMOC-hydrazine derivatives with: formaldehyde, FA (0.5 nmol/mL); acetaldehyde, ACT (0.5 nmol/mL); propaldehyde, PRA (1.0 nmol/mL); malonaldehyde, MDA (1.0 nmol/mL). (D) Plasma specimen; (E) the same plasma specimen spiked with 4.0 nmol MDA.

3.4. Human studies

Concentrations of total and free MDA in plasma of healthy volunteers were measured. Plasma levels in 15 healthy male volunteers were 426 ± 29.8 nmol/L and 153 ± 9.6 nmol/L for total and free MDA, respectively. The concentrations of total MDA are generally lower than the values measured with recently published HPLC-UV method with DNPH derivatization [12,25]. But they are higher than the values measured with DAN techniques [15]. However, the total MDA concentrations we obtained are in agreement with those reported by Romero et al. [26], Suttnar et al. [27] and Carbonneau et al. [28] measured with TBA techniques. Bound MDA in plasma samples can only be measured after acid or alkaline hydrolysis of the samples. With our method, samples were measured after acid hydrolysis (final concentration of H_2SO_4 in the assay = 0.182 mol/L) for 1 h at room

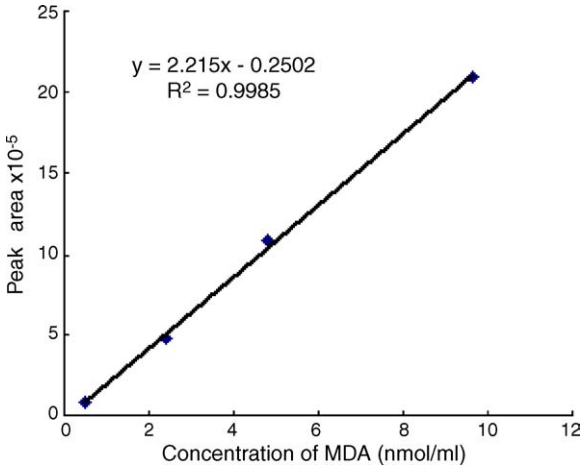

Fig. 5. Calibration curve.

temperature. The free MDA concentrations we measured are in agreement with those reported for MDA by Cighetti et al. [24,29] and Lepage et al. [30]. In addition, the results are also in agreement with the conclusion that values of free MDA in human plasma must be very low (<0.2 nmol/mL) [12]. Strong acidic conditions can lead to the release of bound MDA from its binding form so we have taken precautions by using only acetonitrile to precipitate proteins before the acidification.

Determination of MDA is commonly used for the monitoring of lipid peroxidation in biological samples. However, to estimate the MDA in plasma is difficult due to the complex matrix. The TBA method, although easy to use, is not specific and often gives results that are not reproducible and artificial [31]. The DNPH method, although does not require high temperature to proceed the derivatization reaction, involves multiple liquid–liquid extractions [11–13]. The DNPH method also cannot quantitate any free MDA in normal human plasma since the signals observed were below the lowest calibrator of the assay [12]. Even the use of sophisticated and complex methods like GC–MS does not guarantee reproducible results. Our goal was to develop a simply method for MDA determination with higher sensitivity and selectivity, and fits for routine analysis. Using the FMOC-hydrazine method, the derivatization of samples was relatively simple and rapid under mild acidic condition at relatively lower temperature and the resulted derivatives are unique for a given aldehyde. Furthermore, these derivatives are stable at room temperature and could be separated by HPLC to get a specific signal for MDA. Since centrifuged sample is injected directly, an internal standard is not required. These are important advantages for analyzing MDA content in samples with complex biological matrix. The advantages also enhance the certainty to measure true MDA as a main end-product of lipid peroxidation.

Table 1
Sensitivity of recently published methods for the determination of MDA

Derivative reagent	Method	Detection limit	Reference
DNPH	HPLC	1 pmol for free MDA	[12]
DNPH	HPLC	9 pmol for total MDA	[12]
TBA	HPLC	2.8 pmol for total MDA	[22]
DETBA	HPLC	5 pmol for total MDA	[23]
PH	GC–MS	5 pmol MDA injected	[24]
FMOC-hydrazine	HPLC	0.1 pmol MDA injected	Present paper

Abbreviations: PH, phenylhydrazine; DETBA, 1,3-diethyl-2-thiobarbituric acid.

In this paper, it is observed that the derivatization yield was lowered in the presence of urea based on the recovery result (<70%). The mechanism whereby urea influenced the derivatization was unknown but we found no evidence that it bound to FMOC-hydrazine. Similar results were also observed using the DNPH method [21]. Urea is the main organic component in urine, whose concentration may vary from one specimen to another due to differences in urinalysis. This new method is not convenient to measure MDA in human urine, but it is specific and advantageous to measure MDA in plasma.

4. Conclusions

A new method for MDA measurement in human plasma was developed. The method is based on the addition reaction of MDA with 9-fluorenylmethoxycarbonyl hydrazine (FMOC-hydrazine) to form a FMOC-hydrazone in an acidic medium at 50 °C. The derivatization conditions are mild and the derivatives are stable. After centrifugation, the derivatized solution could be analyzed using a C_{18} column with fluorescence detection. The detection limit, measured on the real samples (mean baseline noise ± 3S.D.), is 0.1 pmol (4.0 nmol/L). The advantages by using this method give great potential on the investigation of lipid peroxidation and the identification of the differences in MDA levels, which are not distinguishable by other assay methods.

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