ANALYTICAL SCIENCES APRIL 2003, VOL. 19



Notes

# Determination of Thiobarbituric Acid Adduct of Malondialdehyde Using On-line Microdialysis Coupled with High-Performance Liquid Chromatography

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An on-line analytical system for the continuous monitoring of malondialdehyde (MDA) was developed. This method involves the use of microdialysis perfusion, on-line derivatization and on-line HPLC analysis. This method gave a linear response for MDA concentrations and HPLC peak areas in the range from 0.051 μM to 2.43 μM. The intra-day (RSD = 1.6 – 10.5%) and inter-day (RSD = 1.1 – 9.3%) precisions were acceptable. The average *in vitro* probe recovery of MDA standard was 18.4 ± 1.0%. The detection limit was 0.03 μM, corresponding to 0.6 pmol for an injection volume of 20 μl. This method was used for *in vitro* peroxidation investigations, which provided evidence for elevated MDA levels following the incubation of metal ions to a linoleic acid solution.

**(Received September 25, 2002; Accepted December 9, 2002)**

Lipid peroxidation, a general mechanism whereby free radicals induce tissue damage, has been implicated under diverse pathological conditions, including aging, atherosclerosis, brain disorders, cancer, cardiac myopathy, lung disorders, rheumatoid arthritis and various liver disorders.[1,2] Malondialdehyde (MDA), an end product of polyunsaturated fatty acid oxygenation, is a reliable and commonly used biomarker for assessing lipid peroxidation. In recent years, using MDA as a marker of lipid peroxidation, there has been growing interest in studying the role played by lipid peroxidation in various kinds of disease.[3]

The measurement of MDA is based on its reaction with thiobarbituric acid (TBA) to form a colored MDA-TBA adduct. Owing to the nanomolar sensitivity, a spectrophotometric or spectrofluorometric measurement of the MDA-TBA adduct is widely mentioned in the literature.[4,5] However, the major shortcoming of this method is a lack of specificity.[6] To overcome this defect, HPLC was used as a separation tool to determine the MDA level in biological samples. In spite of this, the determination of lipid peroxidation in a complicate biological matrix is still a sophisticated task, which would suffer from a tedious and time-consuming pretreatment by HPLC.

Recently, microdialysis has become an important technique for the *in vivo* sampling of the extracellular fluid in discrete compartments of living systems. The advantage of microdialysis is that it achieves *in situ* sampling and sample clean-up and, consequently, results in a clean dialysate that may be directly injected into a chromatographic system without any further pretreatment. Although microdialysis has been widely applied for continuous monitoring of the concentrations of unbound drugs and neurotransmitters *in vivo*,[7-9] few researchers have used the technique to evaluate lipid peroxidation which occurs in the biological matrix. On-line microdialysis coupled with HPLC provides many advantages, such as a reduction in the exposure of dialysate to air, which is a major advantage of the on-line method, because MDA is not a stable substance. In addition, it also provides simplified sample preparation and automated analysis. To obtain the real-time and *in-situ* MDA concentrations in *in vitro* or *in vivo* tests, on-line microdialysis coupled with HPLC would be a suitable hyphenated method.

In general, sample pretreatment still remains a bottleneck for a large number of analytical procedures, and often one which seriously hinders both automation and miniaturization. The development and applications of miniaturized sample preparation and separation methods is a trend which is important in analytical science.[10] In this study, we established an on-line microdialysis–HPLC detection system to measure the MDA-TBA adduct. Furthermore, the effect of metal ions on the levels of the MDA-TBA adduct was investigated after incubation with hydrogen peroxide and linoleic acid.

## Experimental

*Reagents*

All reagents were of analytical grade, unless stated otherwise. Tetraethoxypropane (TEP), $MnCl_2 \cdot 4H_2O$ and linoleic acid were purchased from Sigma (St. Louis, MO, USA). Methanol (HPLC grade), $FeSO_4 \cdot 7H_2O$, $H_2O_2$, $MgSO_4 \cdot 7H_2O$, $Na_2HPO_4$, and thiobarbituric acid were purchased from Merck (Darmstadt, Germany). Phosphoric acid was purchased from Riedel-de Haën (Seelze, Germany).

*On-line microdialysis sampling and HPLC analysis*

A schematic diagram of the on-line derivatization and heating system for dialysate and automatic injection onto an HPLC system for the determination of MDA is shown in Fig. 1. The

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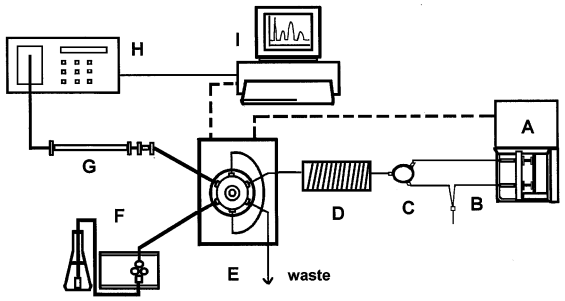


Fig. 1 Schematic diagram of the on-line microdialysis–HPLC system for the analysis of the MDA-TBA adduct. A, microinjection pump; B, microdialysis probe; C, Micro T; D, dry bath; E, on-line injector with a 20 μL sample loop; F, HPLC delivery pump; G, separation column; H, spectrophotometric detector; I, computer-integrated system.

microdialysis sampling system was purchased from Carnegie Medicine Associate (CMA, Stockholm, Sweden). The sampling system consists of a microinjection pump (CMA/100), a microdialysis probe (CMA/20) with a 4 mm length and a 0.5 mm diameter polycarbonate membrane, and an on-line injector (CMA/160) with a 20 μl sample loop. The probe was perfused (1 μl/min) with a CMA/100 perfusion pump. The dialysates flowed into a Y-shaped mixing tee, and were mixed with TBA (1.25 mM TBA in 0.33 mM phosphoric acid), which flowed into the mixing tee from another syringe pump. The mixtures then flowed into an internal volume approximate 60 μl FEP tubing that was incubated in a dry bath (Thermolyne, USA) kept at 85˚C. The dialysates were collected every 10-min with an on-line injector, and directly connected to the HPLC system.

The HPLC system used consisted of a solvent delivery pump (JASCO 980-PU, Tokyo, Japan), a reversed-phase column (Luna C18, 250 × 4.6 mm, Phenomenex, CA, USA), and a UV-visible detector (JASCO, UV-975, Tokyo, Japan) operated at 532 nm. The mobile phase consisted of a 45:55 (v/v) 0.05 M methanol–potassium phosphate buffer (pH 6.8); the flow rate was 1.2 ml/min with an average retention time of 4.47 min for the MDA-TBA adduct. Typical chromatograms of the MDA-TBA adduct are shown in Fig. 2.

*Recovery of the microdialysis probe*

For a probe-recovery test, the microdialysis probes were put into the MDA standard solution, and the perfusate was perfused through the probes at a constant flow-rate (1 μl/min) using a microdialysis pump. The probe recovery upon the TBA reaction and HPLC analysis was determined using the formula: Recovery = $(A_1 - A_2)/A_1$, where $A_1$ represents the area of the peak obtained from the same amount of MDA standard and $A_2$ represents the area of a peak obtained from a microdialysis sample with a known amount of MDA standard.

*Metal-catalyzed peroxidation of linoleic acid*

To monitor the temporal changes of metal-induced lipid peroxidation using the proposed method, a mixture of a linoleic acid solution with metal ions was used. Linoleic acid (5 μl) was dissolved in 2 ml of 95 percent ethanol, and then mixed with 3 ml of a phosphate–saline solution (0.024 M phosphate, 0.15 M NaCl).[11] In a final volume of 5.5 ml, the mixture contained the following reagents at the final concentrations stated: $FeSO_4$ (0.89 mM), $H_2O_2$ (49 μM), $MnCl_2$ (100 μM), $MgSO_4$ (100 μM). The mixture was incubated in a water bath at 37˚C for 150 min.

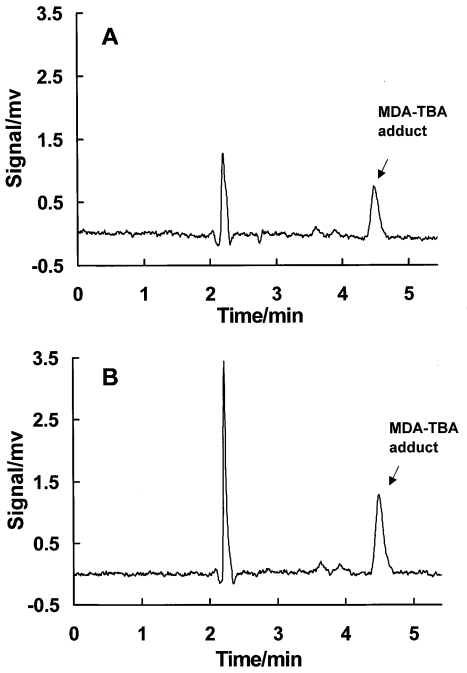


Fig. 2 Chromatograms obtained by the on-line microdialysis–HPLC system. Shown are typical chromatograms of the MDA-TBA adduct from dialysate sample (A) and standard (0.61 μM MDA) (B). The dialysate sample was obtained through the perfusion of a mixture containing linoleic acid (0.1%), $FeSO_4$ (100 μM) and $H_2O_2$ (100 μM).

## Results and Discussion

Although HPLC with various kinds of detection has been commonly used in determining biological MDA levels,[4-6] there have been few literature reports concerning microdialysis sampling for MDA determination. Although Yang *et al.*[12] used microdialysis and HPLC to monitor brain extracellular MDA levels, they used gradient elution and fluorescent detection. In this study, simple isocratic elution and more popular UV/Vis detection were employed and the on-line system was improved to quicken the analysis. In our previous study, an automated and continuous atomic spectrometric method using microdialysis coupled on-line with isocratic HPLC was successfully developed for urinary arsenic speciation.[35]

*Optimization of the chromatographic separation of the MDA-TBA adduct by on-line derivatization*

The determination of the MDA-TBA adduct by the isocratic HPLC method was modified from that of Wong *et al.*[14] In the present study, the optimum parameters of each ingredient of the reaction mixture and the reaction temperature for the on-line measurement of the MDA-TBA adduct were determined. The effect of the temperature on the formation of the MDA-TBA adduct in on-line derivatization was studied over the range from 75˚C to 90˚C. It was found that the maximum MDA-TBA adduct level was obtained when using a temperature of 85˚C. Therefore, a reaction temperature of 85˚C was used throughout in further experiments. The effect of the TBA concentration on the peak area of the MDA-TBA adduct was examined over the range from 8.5 to 13.8 mM. It was found that the maximum MDA-TBA adduct was detected at a TBA concentration of 12.5 mM. Hence, 12.5 mM TBA was selected in this study. The $H_3PO_4$ concentration in the reaction mixture was tested over the

Table 1 Intra-day and inter-day precision (% RSD) of the on-line microdialysis–HPLC system for the determination of malondialdehyde

| Nominal conc./μM | Observed conc./μM[a] | RSD, % | Bias, % |
|---|---|---|---|
| Intra-assay ($n$ = 6) | | | |
| 0.051 | 0.057±0.006 | 10.5 | 11.8 |
| 0.106 | 0.095±0.007 | 7.4 | –10.4 |
| 0.304 | 0.316±0.005 | 1.6 | 4.0 |
| 0.608 | 0.603±0.031 | 5.1 | –0.8 |
| Inter-assay ($n$ = 6) | | | |
| 0.051 | 0.054±0.005 | 9.3 | 5.8 |
| 0.106 | 0.101±0.009 | 8.9 | –4.7 |
| 0.304 | 0.302±0.012 | 4.0 | –0.7 |
| 0.608 | 0.608±0.007 | 1.1 | 0.3 |

a. Data are expressed as mean ± standard deviation.

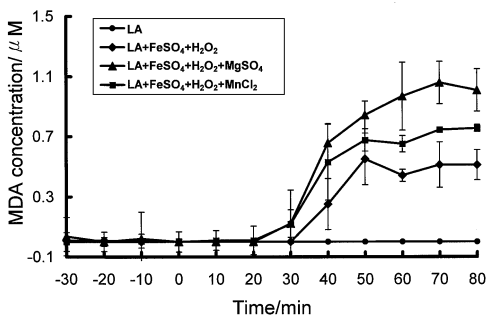


Fig. 3 Temporal effect of $MgSO_4$ and $MnCl_2$ on the induction of malondialdehyde by the peroxidation of linoleic acid (LA) incubated with $FeSO_4$ and $H_2O_2$ at 37˚C. The reaction mixtures contained the following reagents at the final concentrations stated: $FeSO_4$ (0.81 mM), $H_2O_2$ (49 μM), $MgSO_4$ (100 μM) and $MnCl_2$ (100 μM).

range from 0.26 to 0.41 M. The $H_3PO_4$ concentration of 0.33 M was found to be optimal for the analysis.

*Method validation*

A calibration curve was prepared by plotting the peak areas of the blank and TEP standard samples, and was made prior to the experiments. The standard curve gave a linear response for the MDA concentrations and the HPLC peak areas in the range from 0.051 μM to 2.43 μM ($y$ = 186755$x$ + 23.169; $r$ = 0.9999, for $y$ = peak area, $x$ = MDA concentration). The intra- and inter-day variations of the MDA-TBA adduct were assayed (six replicates) at concentrations of 0.051, 0.108, 0.304 and 0.608 μM on the same day, and on six sequential days, respectively. The intra-day and inter-day precision (% RSD) values of the MDA-TBA adduct within ± 15% covering the range of the actual experimental concentrations were considered to be acceptable (Table 1). The average *in vitro* probe recovery of MDA standards was 18.4 ± 1.0%. Based on a signal-to-noise ratio of 3, the limit of detection in our assay was found to be 0.03 μM, corresponding to 0.6 pmol for an injection volume of 20 μl.

*Temporal changes of MDA levels in metal-treated oxidative linoleic acid solution*

Linoleic acid is the most abundant polyunsaturated fatty acid in mammals, and is often applied to assess the induction of lipid peroxidation in *in vitro* studies. Free radicals can be generated by mixing $Fe^{2+}$ and $H_2O_2$ with linoleic acid. The free radicals generated attack the linoleic acid, and set off a series of reactions that eventually result in the formation of MDA. A time-course study, as shown in Fig. 3, demonstrates the reliability of the on-line microdialysis-HPLC system, which allows continuous MDA monitoring.

Manganese is necessary for the development and function of biological systems.[15] The presence of $Mn^{2+}$ has suggested that manganese can enhance the generation of reactive oxygen species.[16] Prime targets of reactive oxygen species are the polyunsaturated fatty acid (such as linoleic acid) in the cell membranes, causing lipid peroxidation, which may lead to damage of the cell structure and function. Magnesium catalyzes or activates more than 300 enzymes in the body, and is pivotal in the transfer, storage, and utilization of energy. The $Mg^{2+}$ levels have been shown to decrease rapidly after central nervous system injuries; also, an enhancement of lipid peroxidation was observed owing to a decrease in extracellular magnesium.[17] Based on the developed method, as shown in Fig. 3, the temporal effect on MDA formation by the peroxidation of linoleic acid during incubation at 37˚C in the presence of $Mn^{2+}$ or $Mg^{2+}$ indicated that both $Mn^{2+}$ and $Mg^{2+}$ enhanced lipid peroxidation under the *in vitro* condition.

In conclusion, a method involving microdialysis sampling, fully automated on-line injection and HPLC analysis for the rapid and continuous monitoring of MDA has been developed. This method has been used in *in vitro* peroxidation investigations, which provided evidence for elevated MDA levels following the incubation of metal ions to a linoleic acid solution.

## Acknowledgements

Financial support from the National Science Council of Republic of China (NSC 90-2113-M-037-019) is gratefully acknowledged.

## References

1. J. A. Knight, *Ann. Clin. Lab. Sci*., **1995**, *25*, 111.
2. B. A. Wagner, G. R. Buettner, and C. P. Burns, *Biochemistry*, **1994**, *33*, 4449.
3. K. Moore and L. J. Roberts, *Free Radical Res*., **1998**, *28*, 659.
4. J. Lykkesfeldt, *Clin. Chem*., **2001**, *47*, 1725.
5. J. Tsaknis, S. Lalas, M. Hole, G. Smith, and V. Tychopoulos, *Analyst*, **1998**, *123*, 325.
6. A. M. Jentzsch, H. Bachmann, P. Furst, and H. K. Biesalski, *Free Radical Biol. Med*., **1996**, *20*, 251.
7. M. I. Davies, J. D. Cooper, S. S. Desmond, C. E. Lunte, and S. M. Lunte, *Adv. Drug Delivery Rev*., **2000**, *45*, 169.
8. F. C. Cheng, J. Wang, and D. Y. Yang, *Neurochem. Res*., **2000**, *25*, 1089.
9. D. Liu, L. Li, and L. Augustus, *J. Neurochem*., **2001**, *77*, 1036.
10. Y. Saito, M. Kawazoe, M. Imaizumi, Y. Morishima, Y. Nakao, K. Hatano, M. Hayashida, and K. Jinno, *Anal. Sci*., **2002**, *18*, 7.
11. B. Halliwell, J. M. C. Gutteridge, and O. I. Aruoma, *Anal. Biochem*., **1987**, *165*, 215.
12. C. S. Yang, P. J. Tsai, W. Y. Chen, and J. S. Kuo, *J. Chromatogr. B*, **2001**, *752*, 33.
13. W. C. Tseng, M. H. Yang, T. P. Chen, and Y. L. Huang, *Analyst*, **2002**, *127*, 560.

14. S. H. Y. Wong, J. A. Knight, S. M. Hopfer, O. Zaharia, C. N. Leach Jr., and F. W. Sunderman, *Clin. Chem*., **1987**, *33*, 214.
15. T. Miyake and T. Shibamoto, *Food Chem. Toxicol*., **1996**, *34*, 1009.
16. H. Roels, G. Meiers, M. Delos, I. Ortega, R. Lauwerys, J. P. Buchet, and D. Lison, *Arch. Toxicol*., **1997**, *71*, 223.
17. M. E. Üstiin, M. Gürbilek, A. Ak, H. Vatansev, and A. Duman, *Int. Care Med*., **2001**, *27*, 264.