ELSEVIER

Journal of Chromatography B, 752 (2001) 33–38

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

On-line, continuous and automatic monitoring of extracellular malondialdehyde concentration in anesthetized rat brain cortex

Chung-Shi Yang*, Pi-Ju Tsai, Wen-Ying Chen, Jon-Son Kuo

Department of Education and Research, Taichung Veterans General Hospital, Taichung 407, Taiwan

Received 6 June 2000; received in revised form 12 September 2000; accepted 13 September 2000

Abstract

An assay for in vivo, continuous and automatic monitoring of extracellular malondialdehyde concentrations in anesthetized rat brain cortex was developed. This method involved the use of microdialysis perfusion, on-line derivatization and on-line high-performance liquid chromatographic analysis. Microdialysate from an implanted microdialysis probe was on-line reacted with thiobarbituric acid at 80°C for 10 min prior to on-line collection and automatic injection into a HPLC system equipped with a fluorescence detector. This method gave a linear response between the concentrations of the malondialdehyde in the microdialysates and the TEP solution where the microdialysis probe was placed. This method was used to observe the increased extracellular malondialdehyde production following elevated extracellular glutamate levels, which were achieved by perfusion of L-*trans*-pyrrolidine-2,4-dicarboxylate, a competitive inhibitor of glutamate uptake transporter.

Keywords: On-line microdialysis; Malondialdehyde

1. Introduction

Oxygen-derived free radicals and their metabolites have been implicated in pathophysiological progression of various brain disorders such as cerebral ischemia and Parkinson's disease [1–3]. Following their production, oxygen-derived free radicals could attack lipids to initiate lipid peroxidation [4–6]. Thus, the extent of lipid peroxidation is commonly used to evaluate oxidative stress in brain, since brain tissue is rich in phospholipid (approximately 40% dry mass) and in vivo free radicals production is difficult to measure directly.

Various methods can be used to assess biological lipid peroxidation [7–10]. Direct and indirect measurements of malondialdehyde (MDA), a side product of enzymic polyunsaturated fatty acid oxygenation and a secondary end product of autooxidative peroxide formation and decomposition [11,12], are simple, convenient and commonly used methods. Direct measurement is usually achieved by high-performance liquid chromatography (HPLC) with UV detection [13]. For indirect measurement, MDA can be reacted with thiobarbituric acid (TBA) prior to analysis by HPLC with fluorescence detection [14–16].

Recently, microdialysis perfusion, which provides a minimally invasive method for in vivo sampling of organ interstitial fluids from awake or anesthetized animals, can be used in combination with HPLC to

*Corresponding author. Tel.: +886-4-3592-525; fax: +886-4-3592-705.

E-mail address: csymdr@vghtc.vghtc.gov.tw (C.-S. Yang).

0378-4347/01/$ – see front matter
PII: S0378-4347(00)00510-7

evaluate in vivo MDA production in rat brain [17–21]. Both UV–Vis and fluorescence detection can be used in these methods, however, fluorescence detection appears to be a better choice due to the low malondialdehyde concentrations in tissue extracellular fluids. When fluorescence detection was used, those assays were performed exclusively by off-line analyses probably because the microdialysates have to be reacted with TBA for approximately 20 min at high temperature (usually at 80°C).

On-line HPLC with microdialysis perfusion provides many advantages such as simplified sample preparation and automated analyses. The reduction in exposure of microdialysate to air, which is a major advantage of on-line method, is important since MDA is not a stable substance. Thus, in the present study, we report an assay system for on-line monitoring of MDA production in anesthetized rat brain cortex. Furthermore, the effect of glutamate uptake inhibition on the MDA production was investigated in anesthetized rat brain cortex.

2. Materials and methods

2.1. Chemicals

The following chemicals were purchased from Sigma (St. Louis, MO, USA): 1,1′3,3′-Tetraethoxypropane (TEP), urethane, 2′-thiobarbituric acid, sodium phosphate, butylated hydroxytoluene and sodium hydrogenphosphate. Monochloroacetic acid and tetrahydrofuran (THF) were obtained from Merck (Darmstadt, Germany). Reagent-grade acetonitrile and methanol were obtained from J.T. Baker (Phillipsburg, NJ, USA). L-*trans*-Pyrrolidine-2,4-dicarboxylate (PDC) was purchased from RBI. All other chemicals were of reagent-grade and deionized distilled water was used.

2.2. General procedure for microdialysis

The microdialysis system was obtained from Camegie Medicine Associates (Stockholm, Sweden). Microdialysis probes (CMA/20) were purchased from CMA. The probe length was 24 mm. The membrane for the probes was made of polycarbonate, with dimensions of 4 mm×0.5 mm. the molecular mass cut-off for the membrane was 20 000. The probe was perfused (2 μl/min) with a CMA-100 perfusion pump. The microdialysates flowed into a Y-shaped mixing tee and were mixed with 2′-thiobarbituric acid (0.4% in 0.2 *M* HCl), which flowed into the mixing tee from another syringe pump. The mixtures then flowed into an approx. 100-cm long polyethylene tubing that was incubated in a water bath kept at 80°C. The microdialysates were collected every 10-min with a CMA-160 on-line collector and injector, which had a loading loop of 19.6 μl and was directly connected to the HPLC system.

2.3. Animal preparations

Male Sprague–Dawley rats (280–330 g) were used. The animals were anesthetized with pentobarbital (50 mg/kg, i.p.). Body temperature was maintained at 37°C with a heating pad. Polyethylene catheters were inserted into the femoral artery for monitoring of systemic arterial blood pressure (SAP) via a Gould pressure processor. The rat's head was mounted on a stereotaxic apparatus (Davis Kopf Instruments, Tujunga, CA, USA) with the nose bar positioned 3.3 mm below the horizontal. Following a midline incision, the skull was exposed and one burr hole was drilled on the skull for insertion of a dialysis probe. The microdialysis probe was implanted into the cortex (0 mm anterior, 6 mm lateral to the bregma and 4 mm from the brain surface). The microdialysates were flowing into the mixing tubing as described above.

2.4. Analysis of MDA

Two TEP stock solutions were prepared. The original TEP stock consisted of the addition of 0.05 ml of standard TEP to 24.95 ml of 40% aqueous ethanol. The intermediate stock solution was prepared by the addition of 0.25 ml original standard TEP solution to 49.75 ml of 40% ethanol solution. Final concentration for the intermediate standard TEP stock solution was 41.8 μ*M*. The stock solution was diluted with 0.01 *M* HC1 to yield experimental concentrations.

The HPLC system consisted of a type 1050 series quaternary pump, a 1050 series autosampler, a 1050

series on-line degasser, and a 1046A HPLC fluorescence detector, all obtained from Hewlett-Packard (Hewlett-Packard Taiwan Branch, manufactured in Waldbronn, Germany). Optimum responses were observed when excitation and emission wavelengths were set at 515 nm and 550 nm, respectively. Peak areas and concentrations were determined using the Hewlett-Packard Chemstation Chromatographic Management System.

Separations were achieved using a Merck LiChrospher 100, LiChroCART (5 μm) 125 mm×4 mm RP-18 cartridge column. Binary gradient elution was used. Mobile phase A consisted of methanol–50 m*M* sodium phosphate buffer (35:65) (final pH 7). Mobile phase B consisted of methanol–50 m*M* sodium phosphate buffer (90:10). In all cases, the mobile phases were filtered (filter pore size: 0.45 μm). Binary gradient elution was performed at a flow-rate of 1.0 ml/min. The elution profile was: 0 to 7 min isocratic with mobile phase A, followed by a column wash with 90% mobile phase B for 5 min. Then, the column was reequilibrated with mobile phase A for 5 min before the next injection.

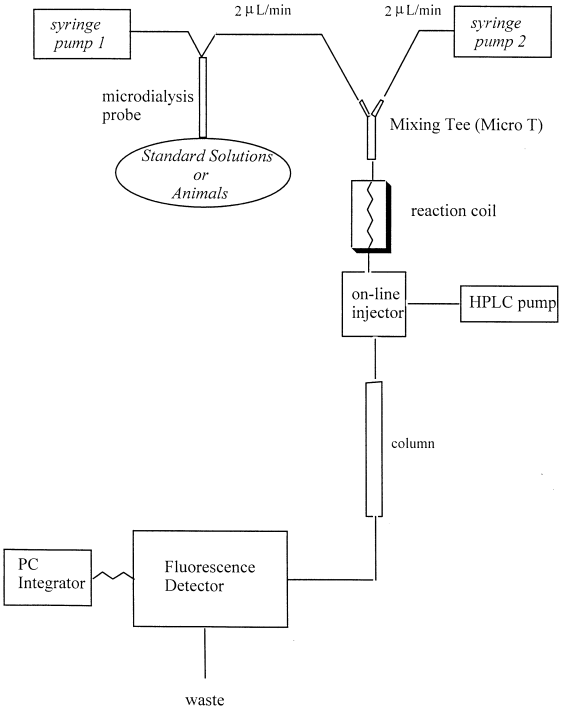

Fig. 1. Schematic representation of on-line derivatization and heating system for microdialysate and the automatic injection on to a HPLC system equipped with a fluorescence detector.

3. Results and discussion

Microdialysis perfusion is a minimally invasive method for in vivo sampling, and it can used with HPLC–fluorescence detection to monitor brain extracellular MDA production in anesthetized rat brain cortex. We examined the possibility for on-line reaction of MDA with the aim of developing an automatic and continuous MDA monitoring system.

A schematic representation of the microdialysis–on-line HPLC system is summarized in Fig. 1. The microdialysate perfused from an implanted microdialysis probe flowed (2 μl/min) into a Y-shaped mixing tee to mix with TBA (0.4% in 0.2 *M* HCl), which was perfused with another syringe pump (2 μl/min). The mixture then flowed into a coiled PTFE tube that was placed in a water bath maintained at either 25°C or 80°C. The coiled tube was directly connected to a loading loop of an on-line injector for a HPLC system equipped with a fluorescence detector.

We examined the effects of reaction time and temperature on the MDA reaction with TEA. TEP was used as the standard compound for MDA since MDA is unstable and TEP hydrolyzes to yield MDA at high temperature (80°C). The hydrolysis of TEP at 80°C to react with TBA to form conjugate has been widely reported in the literature, and TEP has been commonly used as the standard compound for MDA. The results are summarized in Fig. 2. From our earlier experiments, we understand that the brain extracellular MDA is usually present at a concentration below 1 μ*M*, thus TEP at a concentration of 0.75 μ*M* was chosen to react with TBA at either 25°C and 80°C. When the reaction temperature was set at 25°C, the reaction did not appear to go to completion after 60 min. When the temperature was set at 80°C, which is the reaction temperature that has been widely reported for the MDA–TBA reaction, the reaction for MDA and TBA appeared to complete after 10 min. Based on these observations, the volume inside the coiled PTFE tube was set to 40 μl. Thus, it would take 10 min for the MDA–TBA mixture (2 μl/min for microdialysis perfusion pump

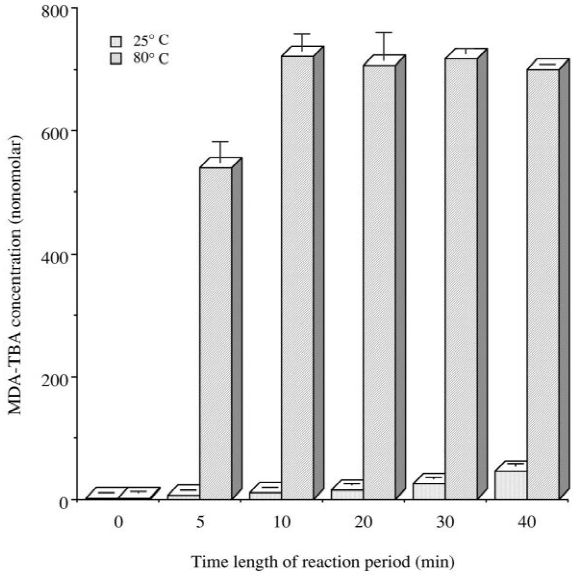

Fig. 2. Effect of temperature and reaction time length for MDA and TBA on the conjugation of MDA–TBA. The MDA–TBA conjugate concentrations were determined using the off-line HPLC method as described in the Materials and Methods section of this manuscript.

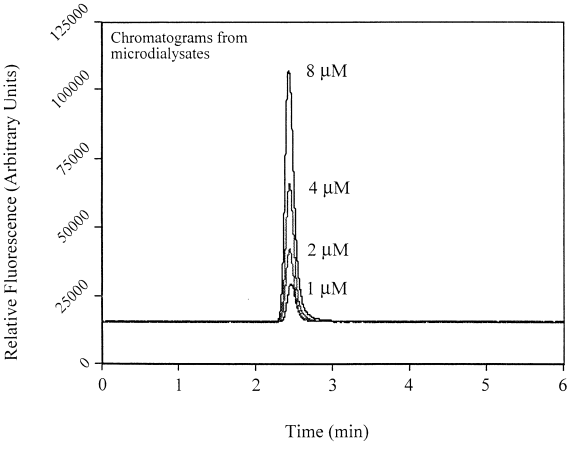

Fig. 3. Effect of various tetraethoxypropane (TEP) concentrations on the microdialysis probe recovery. (A) Chromatograms from injections of microdialysates perfused through TEP concentrations of 1, 2, 4, 8 μ*M*. Original TEP concentrations (1, 2, 4, 8 μ*M*) are on *x*-axis. The MDA–TBA reaction mixture was automatically injected at a 10-min interval. There were six runs (60 min) for each concentration. Data shown were obtained from microdialysates collected at the 20 to 30 min interval.

and 2 μl/min for TBA flow) to flow through the coiled PTFE tube when conversely, they flowed quickly into the sampling loop located in the automatic injector.

TEP was also used as the standard solution for in vitro recovery in microdialysis perfusion. Ringer solution was continuously perfused through the microdialysis probes placed in TEP solutions of four different concentrations (1 μ*M*, 2 μ*M*, 8 μ*M*, 16 μ*M*). The microdialysates were hydrolyzed and on-line reacted with TBA, and the MDA levels were analyzed in a continuous and automatic manner. Shown in Fig. 3 are the chromatograms obtained from the injections of microdialysates perfused through four different TEP concentrations. A very distinct MDA–TBA peak was observed, while no other peak appeared in the same region. Additionally, the peak identification was also performed by the observation of corresponding increase in the peak areas from injections of TEP–TBA mixtures with various TEP concentrations (Fig. 3). Furthermore, the TEP concentrations in which the probes were places were analyzed separately, and the results were compared with those obtained from the microdialysates. A linear relation can be observed between the standard TEP solutions before and after the microdialysis perfusion. The linear response demonstrated that the operational system for MDA–TBA was adequate. Additionally, the slope can be used to represent the in vitro recovery of TEP.

Shown in Fig. 4A are the typical chromatograms obtained from injections of microdialysates collected at the time period from 110 to 120 min after the probe implantation into anesthetized rat brain cortex, and from the first 10 to 20 min after PDC perfusion. MDA levels in the microdialysates before PDC perfusion were very low (from 0.02 μ*M* to 0.16 μ*M*, mean±SD, 0.07±0.05 μ*M* from the average of 10 rats). PDC is a competitive inhibitor for sodium-dependent glutamate uptake transporter, and the perfusion of PDC would result in accumulation of glutamate in the brain extracellular space. Prolonged presence of glutamate in brain extracellular space would overactivate its receptors and induce excitotoxicity, whose damage mechanisms involve the production of oxygen radicals. Thus, we used the on-

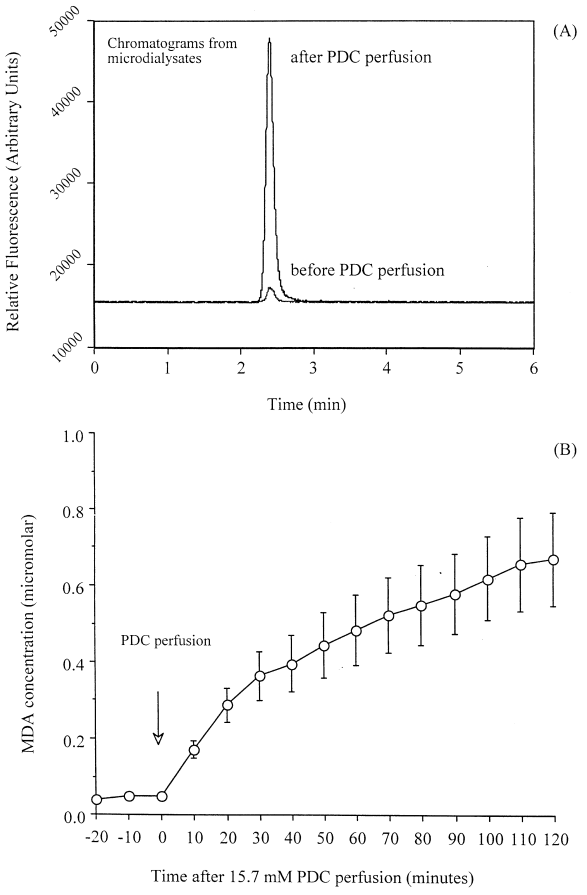

Fig. 4. Effect of PDC perfusion (15.7 m*M*) on brain cortex extracellular MDA contents in anesthetized rats. Data are represented as fold of increase relative to basal status.

line HPLC–microdialysis perfusion system to monitor the production of MDA, an end product of lipid peroxidation initiated by oxygen radicals. Extracellular MDA production is very evident at the PDC perfusion (15.7 m*M*) (Fig. 4A). The concentration profile for extracellular MDA after PDC perfusion is shown in Fig. 4B. The MDA concentrations remained elevated while the PDC was perfused through the probe. These results are in good correspondence with earlier published results [21].

Oxidative stress is an important factor in the damage mechanism for various central nervous system diseases. Production of MDA is a convenient index for oxidative stress in the central nervous system [22]. In vivo data of organ extracellular MDA levels from anesthetized or awake animals could provide valuable information for evaluation of oxygen radical-induced oxidative stress. However, the data are usually difficult to obtain due to the difficulties in the in vivo sampling, which can be overcome by the microdialysis perfusion. Waterfall et al. used microdialysis perfusion and a HPLC system equipped with a UV detector to directly monitor MDA content in anesthetized rat brain [17,18]. Qian and Liu used microdialysis perfusion with HPLC to monitor the increased production of malondialdehyde in rat spinal cord extracellular space following a contusive injury [20]. Although HPLC–UV detection can be used for direct measurement of MDA, several limitations exist [23,24]. For example, the UV detector may not have adequate sensitivity when used to monitor the low basal levels of MDA in brain extracellular space. Furthermore, MDA is not a stable substance, immediate injection is needed to avoid possible variations. Fluorimetric detection provides good sensitivity, which is important since MDA levels are usually low in the extracellular space. However, artifacts may be formed during the derivatization procedure. In Qian and Lu's report, butylated hydroxytoluene was used to minimize the artifact for MDA derivatization [20]. Additionally, the MDA–TBA conjugate compound is much more stable, and the immediate conjugation of MDA with TBA following microdialysate collection might reduce possible variations in MDA levels. Therefore, the on-line method reported in the present investigation may provide adequate sensitivity, and minimize the artifact formation in that the method shorten the sample preparation and minimize its exposure to air.

Several other important and more selective assays exist for assessing lipid peroxidation such as the measurement of lipid peroxide or hydroperoxide [25,26]. Besides, an intermediate in lipid peroxidation such as 4-hydroxynonenal [27,28] rather than an end product (such as MDA) is also a more selective marker for biological lipid peroxidation. However, the in vivo assay of these metabolites using microdialysis perfusion would face difficulties since the hydrophilicity of these metabolites are low, which dramatically reduce sampling efficiency since the perfusion solution is aqueous. The addition of

biocompatible amphipathic molecules into the perfusion solution may help increasing the sampling efficiency, and could facilitate the on-line assay for these hydrophobic metabolites in the future.

In conclusion, we developed an on-line assay, involving microdialysis perfusion and a HPLC system equipped with a fluorescence detector, to monitor extracellular MDA content in anesthetized rats. This assay has been applied to observe a dose-dependent increase in MDA production following inhibition of glutamate uptake transport by PDC in anesthetized rat brain cortex. This on-line microdialysis–HPLC system can be applied to investigations focusing on the oxidative stress in central nervous system disorders, such as cerebral ischemia and spinal cord injury.

Acknowledgements

This research was supported by a grant from National Science Council (NSC88-2113-M-075A-001), and a joint grant from Taichung Veterans General Hospital, National Tsinghua University and National Yang-Ming University (VTY-88-P5-51), Taiwan.

References

[1] C.E. Cross, B. Halliwell, E.T. Borish, W.A. Pryor, B.N. Ames, R.L. Saul, J.M. McCord, D. Harman, Ann. Intern. Med. 107 (1987) 526.
[2] B. Halliwell, J.M.C. Gutteridge, in: Free Radicals in Biology and Medicine, Clarendon Press, Oxford, 1993, p. 416.
[3] D.C. Borg, in: M. Tarr, F. Samson (Eds.), Oxygen Free Radicals in Tissue Damage, Birkhauser, Boston, MA, 1993, p. 12.
[4] B.A. Wagner, G.R. Buettner, C.P. Bums, Biochemistry 33 (1994) 4449.
[5] A. Sakamoto, S.T. Ohnishi, T. Ohnishi, R. Ogawa, Brain Res. 554 (1991) 186.
[6] T.H. Risby, W. Maley, R.P. Scott, G.B. Buildey, M. Kazui, S.S. Sehnert, K.B. Schwarz, J. Potter, E. Mezey, A.S. Klein et al., Surgery 115 (1994) 94.
[7] H.H. Draper, M. Hadley, Methods Enzymol. 186 (1990) 421.
[8] M. Tomita, T. Okuyama, S. Kawai, J. Chromatogr. 515 (1990) 391.
[9] J.M.C. Gutteridge, G.J. Quilan, J. Appl. Biochem. 5 (1983) 293.
[10] H. Kosugi, T. Kojima, K. Kikugawa, Lipids 24 (1989) 873.
[11] E.N. Frankel, Free Radic. Res. Commun. 3 (1987) 213.
[12] D.R. Janero, B. Burghardt, Lipids 24 (1989) 125.
[13] R.P. Bird, S.S. Hung, M. Hadley, H.H. Draper, Anal. Biochem. 128 (1983) 240.
[14] H.H. Draper, E.J. Squires, H. Mahmoodi, J. Wu, S. Agarwal, M. Hadley, Free Radic. Biol. Med. 15 (1993) 353.
[15] B. Halliwell, J.M.C. Gutteridge, in: Free Radicals in Biology and Medicine, Clarendon Press, Oxford, 1993, p. 218.
[16] R.P. Bird, H.H. Draper, Methods Enzymol. 105 (1984) 299.
[17] A.H. Waterfall, G. Singh, J.R. Fry, C.A. Marsden, Neurosci. Lett. 200 (1996) 69.
[18] A.H. Waterfall, G. Singh, J.R. Fry, C.A. Marsden, Brain Res. Brain Res. Proc. 2 (1997) 17.
[19] C.S. Yang, P.J. Tsai, J.P. Wu, N.N. Lin, S.T. Chou, J.S. Kuo, J. Chromatogr. B 693 (1997) 257.
[20] H. Qian, D. Liu, Neurochem. Res. 22 (1997) 1231.
[21] C.S. Yang, P.J. Tsai, N.N. Lin, J.S. Kuo, Neurosci. Lett. 243 (1998) 33.
[22] C. Rice-Evans, R. Burdon, Prog. Lipids Res. 32 (1993) 71.
[23] D.R. Janero, Free Radic. Biol. Med. 9 (1990) 515.
[24] C.H. Cheeseman, T.F. Slater (Eds.), Oxygen Free Radicals in Medicine, Churchill Livingston, London, 1993, p. 494.
[25] J.R. Zhang, H.M. Scherch, E.D. Hall, J. Neurochem. 66 (1996) 355.
[26] T. Miyazawa, Free Radic. Biol. Med. 7 (1989) 209.
[27] H. Esterbauer, H. Zollner, Free Radic. Biol. Med. 7 (1989) 187.
[28] F.J. Van Kuijik, L.L. Holte, E.A. Dratz, Biochim. Biophys. Acta 1043 (1990) 116.