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Detection of Malondialdehyde *in vivo* Using Microdialysis Sampling with CE-Fluorescence

Justin Carl Cooley and

Department of Chemistry, University of Kansas, 1251 Wescoe Hall Dr, Lawrence, KS 66045

Craig Edward Lunte, Ph.D.

University of Kansas, MRB 2030 Becker Dr, Lawrence, KS 66047

Abstract

Oxidative damage is a naturally occurring process where reactive oxygen species (ROS) attack and disrupt normal cellular function, however, these effects become elevated during a stress event, such as ischemia/reperfusion or seizure. One result of oxidative stress is lipid peroxidation, where ROS attack free unsaturated fatty acids forming lipid hydroperoxides, which then breakdown to form secondary products acrolein, 4-hydroxynonenal, and malondialdehyde (MDA) resulting in irreversible membrane damage and ultimately cell death. Described here is a CE – fluorescence method for the determination of MDA in conjunction with *in vivo* microdialysis sampling. MDA was derivatized with thiobarbituric acid under acidic conditions for 20 minutes and injected directly onto the capillary without any pretreatment. This method provided a limit of detection of 25 nM (S/N = 3) and a linear range of 25–2400 nM (1.8–174 ng/ml). This method was used to quantify MDA in rat heart, muscle, liver, and brain dialysate.

Keywords

Capillary Electrophoresis; Malondialdehyde; Microdialysis; Thiobarbituric acid; Interferences

1. Introduction

Malondialdehyde (MDA) has been a molecule of interest in the scientific community since the late 1950's when it was first used as a measure of food rancidity [1] and is currently viewed as a biomarker for lipid peroxidation. During oxidative stress, polyunsaturated fatty acids can be attacked by ROS to form lipid hydroperoxides which readily decompose to form secondary products such as MDA, 4-hydroxynonenal, and dienals [2, 3]. Because of the stability of MDA, it has been used as a reliable biomarker to determine the extent of lipid peroxidation. MDA has been studied in such matrixes as plasma [4, 5], breath condensate [6] and tissue homogenate [7, 8]; in conjunction with separation techniques such as HPLC-UV/fluorescence [8–12] GC-MS [13], and CE-UV/fluorescence [4, 5, 12]. Although MDA absorbs at 245 nm and 267 nm in acidic and basic solutions respectively, the most common method for detection is by derivatization to produce a fluorophore, in particular through the thiobarbituric acid (TBA) assay (for review see Janero [14] and Grotto [15]). Briefly, TBA reacts with MDA in a 2:1 ratio in acidic media and at high temperatures to form a pink product with strong absorbance band at 530 nm and emission at 550 nm. It is important to note, however, that the TBA assay is not specific for MDA and will react with dialdehydes and pyrimidines producing similar absorbance spectra [16]. However,

comparatively MDA is more reactive and has greater fluorescence intensity than other TBA products per molar ratio [17]. This is why use of fluorescence detection as well as a separation technique must be employed in order to increase selectivity and more accurately quantify MDA.

Microdialysis was chosen to couple this MDA method for *in vivo* use because of its ability to sample at localized areas and on short time scales. It would be advantageous to evaluate the degree of lipid peroxidation and oxidative stress on smaller time scales than tissue harvesting would allow in order to help determine the efficacy of preventative measures and treatments. Microdialysis involves the implantation of a semipermeable membrane (probe) into the tissue of interest, perfusing isotonic solution through it and collecting dialysate samples. Analytes in the extracellular fluid will diffuse across the membrane and be collected in the dialysate. Microdialysis provides clean, protein free samples that are tissue specific with sampling on the minute time scale and no further sample preparation needed for analysis. Tissue harvesting requires homogenization, deproteinization, and usually an additional extraction step, either liquid-liquid or SPE. These types of measures are not required with microdialysis sampling; however, the samples are of low volume (a few μL) and have a high salt concentration which can be problematic for conventional HPLC separation techniques to obtain adequate sensitivity. Although MDA has been monitored previously in microdialysis samples using HPLC methods [18, 19], the sample volume required limits temporal resolution during animal experiment. Here we describe the development of a CE-fluorescence method for the detection of MDA in microdialysis samples through derivatization with the TBA assay.

2. Methods and Materials

2.1 Chemicals

MDA (malondialdehyde tetrabutylammonium salt), boric acid 3-mercaptopropionic acid (3-MPA) and TBA were purchased from Sigma-Aldrich (St. Louis, MO). TBA was also purchased from Cayman Chemical (Ann Arbor, MI). Brij 35 was purchased from MP Biomedicals (Solon, OH). Sulfuric acid and salts for Ringer's solution were purchased from Fisher (Fair Lawn, NJ). Ringer's solution composition was: 147 mM NaCl, 3 mM KCl, 1 mM MgCl_2 , and 1.2 mM CaCl_2 . All solutions were made using nanopure water obtained from a Labconco Water Pro Plus purification system (Kansas City, KS).

2.2 MDA Derivatization

MDA was derivatized using the thiobarbituric acid method. Briefly, 8 μL of standard/sample in Ringer's solution was added to a 200 μL microcentrifuge tube. To that 3 μL of a 2:1 mixture of 0.4% (w/v) thiobarbituric acid and 0.4% (v/v) sulfuric acid (both in nanopure water) was added just before heating. The TBA solution was made fresh daily. The mixture was vortexed for about 5 seconds and then heated in a heating block at 95 °C for 20 minutes. The microcentrifuge tubes were then flash frozen in liquid nitrogen and stored at -20 °C.

2.3 CE Method

MDA was separated by capillary electrophoresis with fluorescence detection. A 75 μm ID capillary was cut to 55 cm and a window was burned at 45 cm. The background electrolyte (BGE) was a 200 mM boric acid solution with 4.5 mM brij 35 adjusted to pH 8.4 with NaOH. The capillary was flushed between runs with methanol (2 min), NaOH (2 min) and BGE (3 min) at 20 psi each. Prior to injection, the derivatized standard/sample was removed from the freezer and allowed to warm to room temperature and then diluted in half with nanopure water. The diluted mixture was injected with 0.5 psi for 5 seconds and the separation voltage was set at 10 kV. The separation was performed on a Beckman Coulter P/

ACE MDQ CE system with a 488 nm Laser Module as the excitation source and a 560 nm band pass emission filter. The data was collected and analyzed using 32 Karat software.

2.4 Mass Spectrometry

Mass spectrometry data was collected using an LTQ XL ion trap mass analyzer (Thermo Scientific) with flow injection analysis. Analytes were ionized using electrospray ionization in negative ion mode and collision-induced dissociation was attained using helium gas. A TBA blank and MDA standard solutions were reacted as stated above but up-scaled by 50 times. Butanol was used for liquid-liquid extraction to remove the salts and sulfuric acid. The butanol layer was transferred into another centrifuge tube, blown dry under argon and reconstituted in 500 μ L of 50% methanol/water with 0.1% acetic acid. The reconstituted solutions were then injected at a flow rate of 2 μ L/min. Spectral data was observed using LTQ Tune Thermo Tune software.

2.5 Probe Fabrication

Microdialysis probes were implanted into multiple tissue sites to help validate the application of the MDA method. For liver, heart, and muscle implantation, polyacrylonitrile (PAN) membrane (Hospal, Lakewood, CO) of dimensions 350 μ m OD and 260 μ m ID with a molecular weight cutoff of 20 kDa was used as the microdialysis window. The PAN membrane was cut and attached using UV glue to two strips of polyimide tubing (MicroLumen, Tampa, FL) of dimensions 160 μ m OD and 120 μ m ID. A 2 cm piece of Tygon tubing was attached to the inlet tube in order to affix the probe to a syringe. The probe window was 10 mm for liver and muscle and 2 mm for heart. For brain implantation, a CMA 12 Elite (CMA, N. Chelmsford, MA) 2 mm probe with a cutoff of 20 kDa was implanted into the hippocampus at coordinates – 3.3 A/P, +1.7 L/M, –3.7 D/V reference from bregma line.

2.6 Animal Procedures

All animal experiments were performed using Sprague-Dawley rats (Charles River) in weight range of 300-500 g. Rats were preanesthetized with isoflurane inhalation to effect and then fully anesthetized using a subcutaneous cocktail (ketamine (67.5 mg/kg), xylazine (3.4mg/kg), acepromazine (0.67 mg/kg)). During the experiment animals were maintained under anesthesia with intramuscular booster doses of ketamine equal to one fourth the original dose. Animal body temperature was maintained at 37 °C using an electronically controlled heating pad. Ringer's solution was used as the perfusate for all animal experiments with a flow rate of 1 μ L/min and collection every 10 min. Samples were stored at –20 °C until derivatization. All animal experiments were performed in accordance with the local Institutional Animal Care and Use Committee.

3. Results and Discussion

3.1 Derivatization

At neutral or basic pH, MDA exists as an enolate anion, however, in acidic medium (pH <4.5) MDA will predominantly be in the undissociated enol form. The enol will readily react with nucleophiles forming condensation products [14, 20] as is the case for the TBA reaction (Figure 1). Here MDA was reacted with TBA in sulfuric acid at 95 °C in order to produce the condensation product. The MDA-TBA adduct has a strong absorption at 530 nm with an emission at 560 nm [6, 12, 21, 22]. A fluorescence scan obtained from an MDA standard after derivatization had an excitation maximum at 532 nm with emission maximum at 560 nm signifying a successfully production of the fluorophore (Figure 1).

3.2 Separation and Identification

An initial separation was attempted using only boric acid. This was sufficient when water was the matrix; however, there were substantial destacking effects when Ringer's solution was the matrix due to its high salt concentration. One possible remedy is to add a surfactant to the BGE at a concentration above its critical micelle concentration (CMC). When potential is applied, the micelles will stack at the detector side of the sample plug which can help prevent the destacking of the analyte. Neither sodium dodecylsulfate (SDS) nor tetradodecylammonium bromide (TTAB) provided any advantage, above or below their respective CMC, over straight boric acid. Another approach the dilemma is to use a neutral surfactant to increase the viscosity of the BGE in order to slow the adduct based on its hydrophobicity as it crosses the sample plug boundary [23, 24]. Brij 35 was used at concentrations ranging from below the CMC to well above (0.5, 4.5, and 15 mM) and a large increase in sensitivity at 4.5 and 15 mM was observed. A concentration of 4.5 mM was chosen because there appeared to be no advantage to using a higher concentration. Unfortunately there is a contaminant in the TBA that is unresolvable from the MDA-TBA adduct. It is postulated that this contaminate could be a barbituric-thiobarbituric adduct formed during the heating. The barbituric acid contaminate has been previously observed and characterized using LC/MS [25].

To confirm this theory, butanol extracted constituents from both a blank and MDA standard were analyzed by MS/MS using ESI in negative ion mode. The MDA standard had an expected peak at m/z 323 representing the MDA-TBA adduct which has been previously characterized [25]. The blank had a TBA peak at m/z 143 but also had peaks at m/z 269, 285, and 301. These peaks were identified to be barbituric-thiobarbituric, thiobarbituric-thiobarbituric and a methylene bridged TBA adduct, respectively. All three contaminate peaks can also be seen in MDA standards at similar abundance to the blank (Figure 2). It is felt that the interfering peak in the electropherogram is resulting from the methylene-thiobarbituric acid product because it is more structurally similar to the MDA-TBA product. These contaminate peaks were also observed when just the TBA reagent was injected. TBA was purchased from two vendors (Sigma and Cayman) both with purity >98% to examine if there would be a difference in the formulation. Unfortunately, there was no difference in either mass spectra or electropherograms from the two vendors. However, this interfering peak was observed to be consistent from every fresh TBA solution made but was less than 25% of the smallest basal MDA detected. Because MDA levels are expected to only increase from oxidative stress, the blank can simply be subtracted.

To identify the MDA peak an MDA-TBA standard was run first with a UV/Vis photodiode array detector connected to the system (figure 3A). The peak at 10.1 minutes was easily identified as the adduct based on the UV/Vis spectrum (figure 3C) which matched well with literature [16, 25, 26]. The PDA detector was then replaced with the fluorescence module and another MDA-TBA standard was run on the same capillary producing a similar electropherogram (figure 3B). The UV data along with varying standard concentrations helped further identify the MDA-TBA formation as well as its migration time.

3.3 Reaction Optimization

There were no observable changes in sensitivity from varying concentrations of sulfuric acid; however, there was a noticeable difference in sensitivity at lower concentrations of TBA (Figure 4). The majority of TBA methods call for 0.4-0.5 % TBA [14, 20], however, in an attempt to reduce the TBA contaminate its concentration was decreased. There were interesting results between 0.1 and 0.4% TBA. With a decrease in the TBA concentration of four times, the sensitivity only decreased by a factor of two and y-intercept by two and a half. Even with a greater decrease in the TBA contaminate than sensitivity, it was felt that it

would be more advantageous to use 0.4 % TBA and have the added sensitivity; this is based off triplicate injections. The limit of detection was determined to be 25 nM (S/N=3). This method has a range of linearity from 2400 to 25 nM with an R^2 of 0.998. The inter-day RSD observed for standards ranging from 800 – 25 nM were between 7-19% (n=5) over a 3 month period. The high variance for low standards can be attributed to them being at the limits of detection for the method.

3.4 Microdialysis Validation

In order to demonstrate this method's applicability, it has been used to monitor MDA levels in a variety of rat organs (heart, muscle, and liver). *In vitro* studies using a microdialysis probe of 10 mm showed MDA had a relative recovery of 49 ± 6 % (n=6) at a flow rate of 1 μ L/min. This is a reasonable recovery considering MDA's low molecular weight, and there is no reason to believe MDA will cause any probe failure from sticking to the membrane. MDA was detected in dialysate collected from probes implanted in liver, heart and leg muscle (Figure 5). The samples were derivatized as previously stated and compared to standards. The dialysate concentrations were calculated by a calibration curve with linear regression to be: 84 ± 18 , 80 ± 8 , and 48 ± 9 nM averaged over 5 basal samples for the liver, muscle and heart respectively. An unreacted dialysate sample was also run and showed no interferences with the MDA peak.

Because MDA is used as a biomarker to evaluate oxidative stress it would seem prudent to test this method during such an event. A chemically induced seizure dosing 3-MPA through a microdialysis brain probe was chosen as the model of oxidative stress to demonstrate this method. Using 3-MPA to induce seizures via femoral vein infusion [27] and microdialysis probe perfusion (data not published) have previously employed in this lab. In the experiment, 10 mM 3-MPA was dosed through the probe into the hippocampus of an anesthetized rat for 50 minutes. Samples were collected every 10 minutes with a perfusion flow rate of 1 μ L/min. Figure 6 shows the time course of the recovered MDA throughout the experiment where data is normalized to basal samples collected one hour prior to seizure induction. There is an observable increase in MDA immediately following the start of 3-MPA perfusion, peaking shortly after perfusion stops and slowly decreasing over the subsequent 4 hours. This data would indicate that there is a massive amount of necrosis occurring from the 3-MPA induced seizure; however more data is needed to verify this conclusion.

4 Concluding Remarks

Here we describe a method that is applicable for monitoring MDA *in vivo* through microdialysis. The use of microdialysis sampling allows for site specific sample acquisition through the course of an animal experiment. The ability to use this method for the detection of MDA in microdialysis samples provides a time course for cellular lipid peroxidation that tissue harvesting or blood sampling cannot.

Acknowledgments

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Glossary

MDA	Malondialdehyde
TBA	Thiobarbituric acid

ROS Reactive Oxygen Species
3-MPA 3-Mercaptopropionic acid

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Figure 1. Reaction diagram of MDA and TBA along with a respective fluorescence scan of the obtained product.

Figure 2. Mass Spectra from flow injection analysis of A) blank and B) 10 μ M MDA standard. Y-axes have been zoomed in to show contaminate peaks. The base peak at m/z 143 (TBA) has an intensity of 4.8E4 and 4.4E4 for spectrum A and B respectively.

Figure 3.

A) Electropherogram of 25 μ M MDA-TBA with PDA detection. (B) Electropherogram of 100 nM MDA-TBA with LIF detection. (C) UV/Vis spectrum from peak at 10.1 min in A.

Figure 4.
Differing concentrations of TBA (◆ 0.4%, ■ 0.1% and ▲ 0.05%)

Figure 5. Electropherograms of dialysate from rat: liver, muscle, and heart along with TBA blank and an underivatized sample.

Figure 6. Electropherogram and subsequent MDA data from 3-MPA chemically induced seizure. Red arrows indicate start and stop of 3-MPA perfusion.