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Free malondialdehyde determination in rat brain tissue by capillary zone electrophoresis: evaluation of two protein removal procedures

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Abstract

Two procedures for the determination of underivatised, free malondialdehyde in rat brain tissue have been evaluated. Both procedures are based on capillary zone electrophoresis (CZE) and UV detection at 267 nm and differ only with respect to the protein removal step, for which ultrafiltration or precipitation with acetonitrile have been employed. The total analytical processes include sample homogenisation, addition of antioxidant, protein removal, and separation and detection in the CZE system, and take less than 20 min. The CZE buffer consists of 10 m*M* borax and 0.5 m*M* CTAB at pH 9.3. The malondialdehyde peak reaches the detector about 3 min after injection as one of the very first peaks in the electropherogram. The limit of detection (3 S/N) is 0.2 μ*M*, corresponding to 4 fmol for an injection volume of 20 nl. The method is fast, reproducible and has a large linear range, spanning 0–200 μ*M*.

Keywords: Protein removal; Malondialdehyde

1. Introduction

There is great clinical interest in oxidative stress and lipid peroxidation, due to the suggestion that many significant disease states are associated with oxidative injury [1–4]. When various primary and secondary products of lipid peroxidation in biological systems are decomposed, aldehydes are formed [5,6]. One intensely studied aldehyde is malondialdehyde (MDA), and it is commonly used as a marker for the lipid peroxidation process [7,8].

The literature dealing with methods for the quantification of MDA is extensive [7,9]. These methods can be divided into two classes: direct methods where MDA itself is the analyte, and derivative methods dependent on the formation of a MDA reaction product. The most widely used techniques involve the reaction between MDA and thiobarbituric acid (TBA) generating a red, fluorescent complex [10]. This procedure, generally referred to as the TBA-test, is simple to perform but lacks specificity since various substances, besides MDA, also react with TBA [7]. Furthermore, MDA or MDA-like substances can be formed from sample components at the elevated temperatures and low pH values used in the procedure [7]. As MDA is known to bind to proteins [5,11], it is desirable to know whether or not a method is specific to either the free or bound MDA, or is sensitive to the total content.

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When a crude sample is exposed to relatively harsh assay conditions, such as boiling or addition of solutions with extreme pH values, a displacement of the equilibrium between free and bound forms of MDA can be expected. Consequently, the direct MDA analysis, performed with mild treatment conditions, is preferable since it limits the potential for artefacts. So far, the principal approach for the direct determination of MDA has been by high-performance liquid chromatography (HPLC) coupled to UV spectrophotometry [12–14].

Olsson et al. have recently described a method for determining the MDA content in rat brain homogenates using capillary zone electrophoresis (CZE) with UV detection [15]. The main advantage with CZE, for this application, is that small sample amounts are required. The present paper is an extension and refinement of the method by Olsson et al. The overall objective was to simplify the protein removal procedures and to study the outcome of this with respect to sensitivity, repeatability and recovery.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade and solutions were prepared with water from an Elgastat UHQII (Elga, High Wycombe, UK). Ferrous ammonium sulphate, sodium hydroxide, sulphuric acid, phosphoric acid, borax and cetyl trimethyl ammonium bromide (CTAB) were purchased from Merck (Darmstadt, Germany), acetonitrile (ACN) from KEBO (Spånga, Sweden) and butylated hydroxy toluene (BHT) and 1,1,3,3-tetramethoxypropane (TMP) from Sigma (St. Louis, MO, USA). The electrophoresis buffer, 10 m*M* borax, 0.5 m*M* CTAB, pH 9.3, was degassed and filtered through a 0.25-μm filter before use. The MDA standard solution, 10 m*M*, was prepared as described by Esterbauer and Cheeseman [16], i.e., by acid hydrolysis of TMP in 1% sulphuric acid at room temperature for 2 h. This solution was then further diluted with 5 m*M* phosphate buffer, pH 7.0, when preparing the working standard solutions.

2.2. Animals

Three adult, female Sprague–Dawley rats (B&K International, Sweden) were used in the study.

2.3. Capillary zone electrophoresis system

The capillary electrophoresis system ($HP^{3D}CE$, Hewlett-Packard, Palo Alto, CA, USA) was provided with UV detection at 267 nm. The diode array detector could also be operated in UV scanning mode. The running voltage was −25 kV. Untreated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA), 65 cm long (55 cm to the detection window)×50 μm I.D.×375 μm O.D. were employed. Before use the capillaries were preconditioned with 0.1 *M* NaOH for 10 min, distilled water for 10 min and running buffer for 15 min. The samples were injected by pressure, corresponding to an injection volume of 20 nl (1.6% of capillary length). To maintain capillary performance, conditioning cycles were applied comprising rinsing for 2 min with 0.1 *M* NaOH, 2 min with methanol and 2 min with running buffer.

2.4. Sample preparation procedures

The heads of the decapitated rats were stored at −70°C until further use. After thawing at room temperature the brains were quickly excised and put on ice. An ultraturrax set at 800 rpm was used to prepare the tissue homogenates (20%) in 5 m*M* phosphate buffer, pH 7.0. Directly after homogenisation each of the three homogenates was divided into four aliquots (I–IV), which were treated as follows.

I. 0.075% BHT was added to one aliquot from each set, to prevent oxidation and thereby provide a control brain sample.

II. Lipid peroxidation was stimulated in a second aliquot by the addition of ferrous ammonium sulphate to a final concentration of 100 μ*M* and continuous shaking for an hour at room temperature, after which the oxidation process was terminated by the addition of 0.075% BHT.

III. The third brain portion was spiked with MDA

standard to a final concentration of 5 μ*M* and, to prevent oxidation, 0.075% BHT was added.

IV. The fourth aliquot of each set was left untreated.

All samples were stored at −80°C until further use. Before injection into the CZE system the sample homogenates were centrifuged at 14 000 *g* for 3 min in order to remove particles, and one of the following two protein-removing techniques was applied. In the first, the supernatant was mixed 1:1 (by volume) with ice-cold acetonitrile, to precipitate the proteins. The mixture was then put on ice for 5 min prior to centrifugation for 3 min at 14 000 *g*. In the second, protein was removed by ultrafiltration using Microcon centrifugal filtration devices (Millipore, Bedford, MA, USA) with a molecular mass cut-off at 30 000.

3. Results and discussion

3.1. The buffer system

When a 10 m*M* borax buffer, pH 9.3, with 0.5 m*M* CTAB and a running voltage of −25 kV was employed, an electroosmotic flow from the injector side to the detector side of the capillary system was generated. Since MDA is a small molecule, and negatively charged at this pH, it migrated ahead of the electroosmotic flow and the peak appeared within 3 min after injection. With the selected buffer composition, fairly large injections, up to 1.6% of the capillary length, could be made without affecting peak width or migration time of the MDA peak. It was possible to use a larger injection volume to further decrease the limit of detection (LOD), but the peak widths then began to increase.

3.2. Peak identification

Fig. 1 shows an electropherogram of an ultrafiltered rat brain homogenate, spiked with MDA standard. The MDA peak was easily identified since it migrated as the first peak in the electropherogram. The migration time was consistent between days, the relative standard deviation (RSD_{time}) being 2.4% (5 days, 75 runs). Additional confirmation of the identity and purity of the MDA peak was obtained by comparing the sample peak spectra with MDA standard spectra, which overlapped, and by spiking the sample with MDA standard.

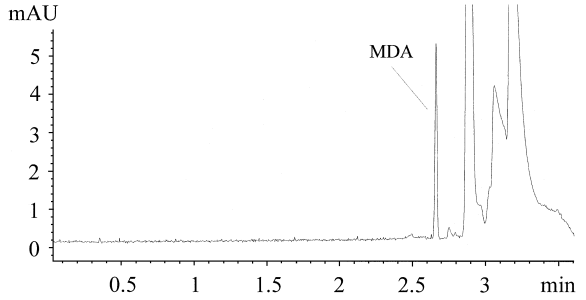

Fig. 1. Electropherogram obtained for ultra-filtered rat brain homogenate. The sample was treated according to procedure III, i.e., BHT was added and the sample was spiked with MDA standard to a 5 μ*M* concentration.

3.3. Calibration data

The sample matrix can affect the area and migration rate of MDA when real samples are analysed in the CZE system, causing deviations from those of pure standards. Therefore, the quantification of MDA was performed by applying a standard addition technique for both ultra-filtered and ACN-treated samples. The linearity and the operational ranges of the standard addition curves for ultra-filtered and ACN-treated samples, respectively, were tested in separate experiments. The linear ranges were found to span from the LOD to at least 200 μ*M*. The LOD for MDA was found to be approximately 0.2 μ*M*, corresponding to 4 fmol (20 nl injection) for both the ultra-filtered and the ACN-treated samples. However, according to our experience, the conventional quantification based on a calibration curve created by prepared standard solutions can be applied as well. Figures of merit for such a calibration curve; x range 0–200 μ*M*; y range 0–40 area units; equation $y=0.1819x-0.01$; confidence limits (95%) for the slope ±0.0017 and for the intercept ±0.13; $r^2=0.9998$; $n=13$.

3.4. Evaluation of sample pre-treatment

3.4.1. Protein removal procedures

The investigated sample matrix contained a relatively large amount of proteins. Adsorption of proteins on the inner surface of the uncoated fused-silica capillaries could lead to loss of efficiency, peak distortion or capillary clogging. It was thus important to remove the main part of these proteins from the samples. A further advantage is that elimination of proteins also reduces the protein bound forms of MDA, since we are interested in measuring exclusively the free fraction of MDA.

Cold acetonitrile instantly precipitated the proteins in the samples. For ultrafiltration, molecular mass cut-offs at 3000, 10 000 and 30 000 were tested (results not shown) and 30 000 was found to be sufficient. Ultrafiltration is most commonly used in concentrating or desalting protein solutions, but in this case we instead removed the proteins and used the filtrate.

MDA is believed to covalently bind to proteins forming a Schiff base type of adduct [11,17]. Such an adduct will probably be kept intact when applying either of the two protein removal procedures. Our results seem to support this view since the two procedures were found to yield almost identical results.

3.4.2. Evaluation of recovery

The lack of a suitable internal standard necessitates finding alternative ways to evaluate the recovery of MDA during the sample pre-treatment procedure. We chose to divide homogenates into aliquots and then add a known amount of MDA standard, both before and after the protein removal operations. Fig. 2 shows a description of the procedure. No significant difference (*t*-test, $n=4$, 95% confidence level) was found, in terms of peak area, between the samples where MDA was added before or after the protein removal step. The average recovery was 99% for ultrafiltration (Fig. 2a) and 101% for ACN treatment (Fig. 2b). These results imply that no free MDA is lost during either of the two sample pre-treatment procedures.

3.4.3. Repeatability

The between run repeatability was studied for both protein removal methods, using samples from pre-treatment procedures I and III (described in Section 2.4), in which concentrations were approximately 1 and 5 μ*M*, respectively, see Table 1. The repeatabili-

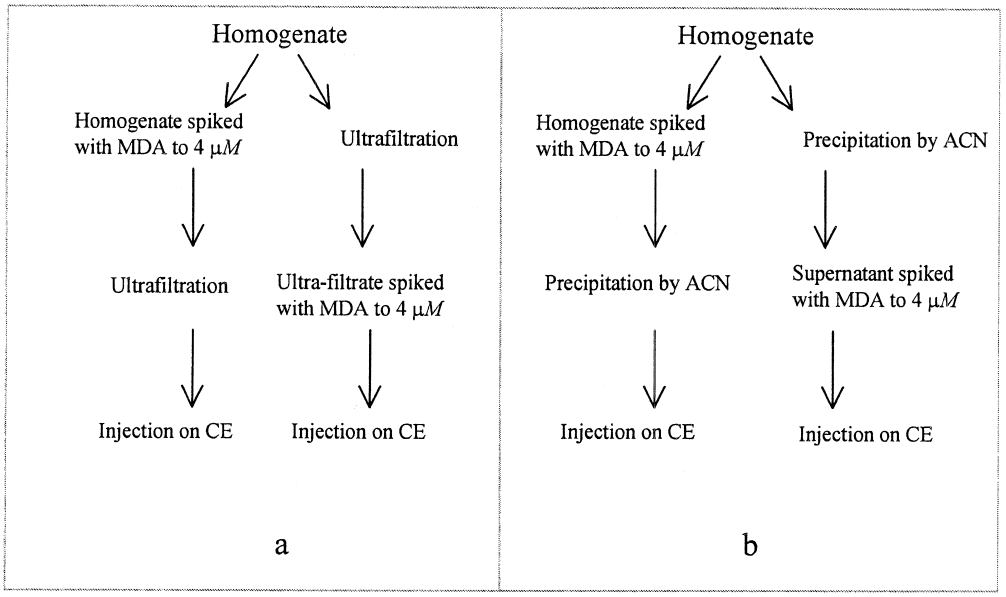

Fig. 2. Overview of procedures used for evaluation of recovery.

Table 1
RSD values for the relative peak areas obtained by repeated injections of samples subjected to various protein removal procedures

Protein removal procedure	*n*	RSD (%)
Ultrafiltration (~1 μ*M*)	8	5.6
Ultrafiltration (5 μ*M*)	7	2.5
ACN (~1 μ*M*)	7	6.1
ACN (5 μ*M*)	8	2.8

ty for the entire work-up protocol was also studied using samples treated according to procedure III, the RSD values obtained being 3.6% ($n=4$) for ultrafiltration and 4.8% ($n=4$) for precipitation with ACN.

3.4.4. Quantification

The control samples, treated according to procedure I, show the endogenous levels of free MDA found in the three rat brain homogenates. The amounts of MDA found in these samples (0.2–0.9 μg MDA/g brain tissue) are shown in Table 2. It can be seen that both methods give similar results, further supporting the conclusion that they both give a good estimate of the free MDA content in the samples. The addition of BHT is absolutely necessary, as is shown by data in Fig. 3, comparing results from procedures I and IV. The only difference between these procedures is the addition of BHT in I, and it can be seen that lipid peroxidation proceeded in vitro. Even though precautions were taken against undesired lipid peroxidation in the control samples, by the addition of BHT, we can not be completely certain whether the MDA amounts found in the rat brains are endogenous or are artefacts caused by the work-up procedure. The oxidative potential of the brain tissue is illustrated by results from the sample to which iron was added (procedure II), in Fig. 4. The MDA peak in this sample corresponded to 27 μg MDA/g brain tissue. Several studies have shown that the iron homeostasis of the brain is disturbed in some neurodegenerative diseases [18–21].

Table 2
Levels of free MDA found in three control rat brains using the two protein removal procedures[a]

	Acetonitrile	Ultrafiltration
Rat No. 1	0.9	0.9
Rat No. 2	0.3	0.2
Rat No. 3	0.5	0.5

[a] Results are given in μg MDA/g brain tissue.

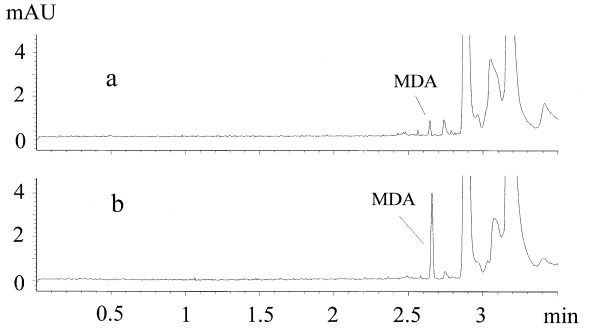

Fig. 3. Comparison of electropherograms obtained for (a) sample treated according to procedure I and (b) according to procedure IV. The proteins were removed by ultrafiltration.

3.5. Comparison of methods

In the previously published procedure, the samples were boiled in order to remove the proteins [15]. This boiling and subsequent chilling procedure takes about 60 min, in comparison with 15 min required for the ACN or ultrafiltration methods. Since boiling was tedious and these samples frequently caused capillary clogging due to insufficient protein removal, we decided to discontinue use of the boiling procedure. Furthermore, the LOD has been lowered from 1.2 to 0.2 μ*M* using the modified protocols presented here. Homogenising the tissues in pH 7

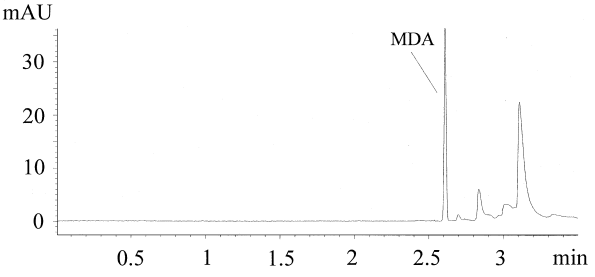

Fig. 4. Electropherogram obtained for sample treated according to procedure II, i.e., lipid peroxidation was induced by the addition of iron. The proteins were removed by ultrafiltration.

phosphate buffer rather than water probably contributed to the increased sensitivity, promoting sharper electrophoretic peaks and higher plate numbers.

4. Conclusions

The determination of free MDA in a complex biological sample is an analytical challenge. We have evaluated two procedures for removing proteins from samples: precipitation by ACN and ultrafiltration. Both procedures are suitable for this application since they are robust, have low LOD values and give similar results. The entire procedures, from homogenate to electropherogram, take less than 20 min. Other implications of the results include the following.

(1) Ultrafiltration is to be preferred for samples containing MDA at levels close to the LOD since no dilution is performed.

(2) Only minute amounts of sample are required to perform an analysis. About 10 mg of brain tissue is sufficient to analyse MDA via ultrafiltration, and even less via ACN.

(3) The LOD values are of the same order of magnitude as the lowest figures reported for HPLC methods for determining free, underivatised MDA.

(4) The simplicity of the improved method, and the fact that only free MDA is measured, without any derivatisation, makes it suitable for validation of other methods.

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