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Sensitive determination of malondialdehyde in exhaled breath condensate and biological fluids by capillary electrophoresis with laser induced fluorescence detection

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ABSTRACT

In this work, a sensitive capillary electrophoresis method with laser induced fluorescence detection for determination of malondialdehyde in various biological fluids was developed. Malondialdehyde reacts with thiobarbituric acid under optimized conditions of pH=2, reaction time of 60 min and temperature of 90 °C, yielding an adduct that can be separated in a 50 mM sodium borate background electrolyte at pH 9. The separation of the formed adduct was accomplished in less than 6 min with limit of detection of 1.1 nM due to the use of 532 nm laser module, exactly matching the maximum excitation wavelength of the formed adduct. The developed method offers unprecedented sensitivity and was for the first time used for analysis of malondialdehyde in exhaled breath condensate. The method proved to be also applicable to other samples of biological fluids, such as blood plasma and saliva.

1. Introduction

Lipid peroxidation is a process under which oxidants, such as free radicals as well as non-radical species, attack lipids containing double bonds, especially polyunsaturated fatty acids. Oxidative attack of essential cell components by reactive oxygen species is a mechanism generally recognized as relevant in the pathogenesis of several human diseases such as atherosclerosis [1], Alzheimer's disease [2] or cancer [3]. During lipid peroxidation, lipid peroxyl radicals, hydroxyperoxides and number of secondary products including malondialdehyde (MDA), propanal, hexanal, dienals and 4-hydroxynonenal (4-HNE), are formed [4]. MDA appears to be the most mutagenic product of lipid peroxidation (reacts with DNA to form adducts), whereas other significant aldehyde, 4-HNE, is the most toxic product. Under physiological condition, MDA exist in its enolate form (pKa=4.5) [5], which is relatively unreactive and has been therefore used as a biomarker for lipid peroxidation [6]. The simplest and most frequently used, non-specific assay for assessment of lipid peroxidation is the thiobarbituric acid reactive substances (TBARS) assay [7,8]. Under strong acidic condition and heating, MDA is converted to its reactive enol form that reacts with thiobarbituric acid (TBA) and forms a $MDA(TBA)_2$ pink-colored adduct that has an absorption maximum at 530–535 nm (for reaction scheme, excitation and emission spectra, see Supporting information Figs. S-1 and S-2). The formed adduct can be determined spectrophotometrically or spectrofluorimetrically [9], making TBARS assay a valuable screening tool in the assessment of the oxidative damage to lipids [10,11]. However, the TBARS assay is often mistakenly interpreted as MDA-specific, whereas other species, such as nucleic acids, amino acids, proteins, phospholipids, pyridines and low molecular weight aldehydes also react with TBA. Therefore, when using spectrophotometry or spectrofluorimetry, TBARS assay often leads to the overestimation of the MDA content in the samples.

Improved selectivity and sensitivity can be achieved by using a separation technique such as high performance liquid chromatography (HPLC) or capillary electrophoresis (CE). A number of HPLC methods were developed to selectively determine MDA either based on fluorescence detection of the $MDA(TBA)_2$ adduct [7,12–15], or based on mass spectrometry (MS) detection after derivatization with 2,4-dinitrophenyl hydrazine solution [16,17]. CE is another, highly efficient technique amenable for analysis of low sample volumes (advantageous for most biological samples) and is generally less prone to matrix effects. Most CE methods for MDA determination in different biological samples (human plasma, tears, rat brain tissue) rely on UV detection, because MDA absorbs at wavelengths between 260 and 267 nm [18–24]. The sensitivity of direct MDA analysis by CE with UV detection is however too poor and is applicable only for biological samples with high MDA

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content (blood plasma, saliva). This is especially true for the free, unbound MDA form. The concentration range of MDA in human blood plasma samples was reported to be between 0.1 and 3 μM [25–30], but the higher values may stem from formation of other oxidation products during sample treatment or partial release of the bound MDA. Lykkesfeldt et al. [13] established that even infinitesimal hemolysis in a plasma sample raises the MDA concentrations from 0.5 to about 2–5 μM. Samples need to be therefore processed immediately after collection. Korizis et al. [31] suggested the derivatization with tetrabutylammonium hydrogen sulphate using the product's UV maximum at 267 nm, improving the separation of MDA from other plasma components, and reaching somewhat lower limit of detection (LOD) of 0.2 μM. Unless field amplified sample stacking is used in CE-UV methods, as suggested by Zinellu et al. [23,24], the sensitivity is not sufficient. However, sample stacking is flawed by the requirement of low conductance of the sample and is often not achievable with biological samples. CE with laser induced fluorescence (LIF) is a viable option to decrease the limits of detection to the nM range and extend the applicability of CE to other biological samples, for instance exhaled breath condensate (EBC). The reported levels of MDA in EBC vary typically between 0 and 50 nM [16,32–36], which is about 1–2 orders lower concentration than in plasma or saliva [37]. Surprisingly, there are only 2 articles on the CE-LIF determination of MDA. Banos and Silva [38] have developed a method for analysis of MDA and other aldehydes in urine using derivatization with fluorescein 5-thiosemicarbazide, a fluorophore that matches well the 488 nm Ar-ion laser line, but the sensitivity was not significantly higher than using the CE with UV detection (0.12 μM). Cooley and Lunte [39] have analyzed MDA in in vivo microdialyzates of rat brain using derivatization with TBA with LODs reaching 25 nM. In this case the sensitivity was much higher, but still would not allow the detection of MDA in EBC samples where the concentrations of MDA are often below 20 nM [33,34,36]. Arguably, use of Ar-ion laser at 488 nm for the determination of the $MDA(TBA)_2$ adduct with absorption maxima wavelength of 530 nm is not optimal. In this work, a 532 nm laser module, exactly matching the maximum of excitation spectra of the formed $MDA(TBA)_2$ adduct was used for sensitive MDA determination in a CE-LIF system. As EBC was the primary sample of our interest, the reaction conditions, such as reaction time, reaction temperature, ratio of reagent to analyte and pH of the reaction solution, were optimized mainly for EBC samples, in which the concentrations of MDA are below the LODs of the previously published CE-LIF methods [38,39]. The developed CE-LIF method provides so far unprecedented sensitivity (LOD of 1.1 nM for MDA). MDA in EBC was analyzed by CE-LIF for the first time. The method proved to be also applicable to other samples of interest, such as blood plasma and saliva.

2. Material and methods

2.1. Chemicals and reagents

All chemicals were of reagent grade and deionized (DI) water (Purite, Neptune, Watrex, Prague, Czech Republic) was used for stock solution preparation and dilutions. MDA was purchased from Sigma-Aldrich (Steinheim, Germany) as malondialdehyde tetrabutylammonium salt (MDA, ≥96% purity). MDA stock solution was prepared fresh each day by dissolving MDA in DI water purged with nitrogen. The working solutions of MDA were prepared by diluting the fresh stock solution with DI water to the required concentration, typically 100 μL aliquots of standard solutions were prepared. TBA (≥98%) was also purchased from Sigma-Aldrich (Steinheim, Germany). The TBA derivatization solution was prepared as 0.4% TBA (w/v) in 0.4% H_2SO_4 (≥96%, PENTA, Czech Republic). The background electrolyte (BGE) for CE measurements was prepared weekly and consisted of 50 mM sodium borate buffer, prepared from 50 mM boric acid (≥99%, Sigma-Aldrich, Germany) that was adjusted by 1 M NaOH (JT Baker, USA) to pH 9. The derivatization solutions were prepared immediately prior to use.

2.2. Instrumentation

2.2.1. Electrophoretic system

A compact CE system with LIF that allows easy exchange of different laser modules was constructed. Its schematic is in Fig. 1. The whole system including the high voltage supply, capillary, LIF detector and electronic controls was accommodated in a black box made from polyvinyl chloride sheets with anodized Al-bottom, constructed in house (30×30×25 cm) to shield the detection part from ambient light. The CE separation part consisted of a high voltage (HV) unit (EMCO DX250, EMCO High Voltages, USA) providing +15 kV to a fused-silica (FS) capillary (50 μm id, 375 μm od, 60 cm total length, Polymicro Technologies, Phoenix, AZ, USA).

Two tubular platinum electrodes (0.7 mm od, 0.4 mm id, 3 cm length, Goodfellow Cambridge Limited, Huntingdon, UK) were incorporated in the electrolyte vial and the poly(methyl methacrylate) (PMMA) interface (see below).

2.2.2. Detection system

An in-house built LIF detector was assembled from parts machined from an Al-alloy that was anodized to give it a non-reflective black finish. A green laser module operating in continuous wave mode with nominal wavelength of 532 nm and power ~4 mW, (GLM-02–532-05-P-D, GM Electronic, Brno, Czech Republic) was used. The module was inserted in a 50 mm OD, 12 mm ID Al-cylinder that served to align the module and as a passive cooling element. The laser beam was focused onto the separation capillary using a plano-convex lens (diameter: 5 mm, focal length: 20 mm, PCX, 532 nm V-coat, Edmund Scientific, NJ, USA). Fluorescence was collected orthogonally by a 60x microscope objective with working distance 0.15 mm (Edmund Scientific, NJ, USA) and focused on a photomultiplier tube (model H10722-20,

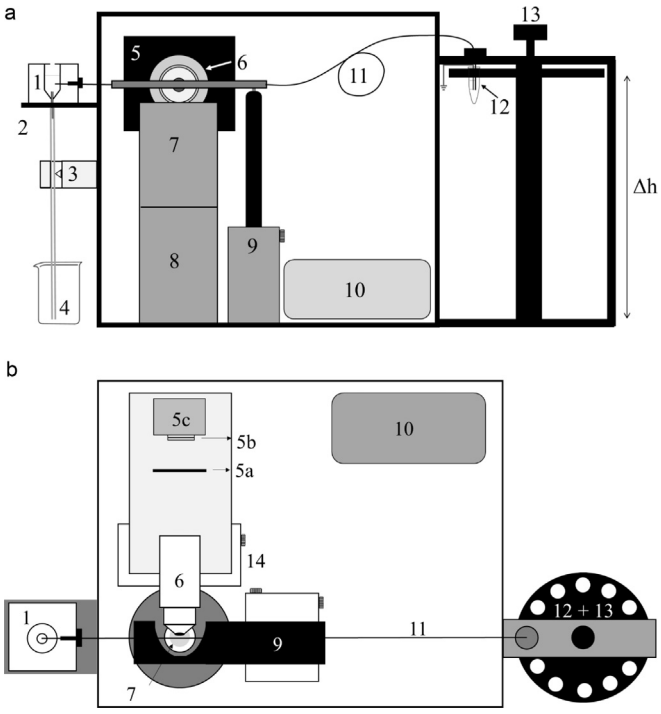

Fig. 1. A schematic diagram of the in-house built CE-LIF instrument: A-front view, B-top view, 1 – vial with BGE, 2 – Pt tubular electrode, 3 – pinch valve, 4 – waste, 5 - shield with slit (5a), filters (5b) and PMT (5c), 6 – microscope objective, 7 – lens, 8 - laser with passive cooling, 9 - dual-axis goniometer with capillary holder, 10 - HV supply + electronic board, 11 - separation capillary, 12 - injection vial + ground, 13 - carousel, 14 - one-axis goniometer with microscope objective (6).