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Micellar electrokinetic chromatography separation and laser-induced fluorescence detection of the lipid peroxidation product 4-hydroxynonenal

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Abstract

4-Hydroxnonenal (HNE) is a product of lipid peroxidation in biological systems that causes a variety of harmful biological effects. A method for identifying HNE based on derivatization with the fluorescent reagent dansylhydrazine (5-(dimethylamino)naphthalene-1-sulphonehydrazine (DNSH) followed by micellar electrokinetic chromatography separation laser-induced fluorescence detection has been developed. The derivatization reaction has also been investigated for significant experimental parameters and rat brain homogenates with induced lipid peroxidation have been analysed for HNE contents. The limit of detection (3 S/N) was 30 nM or 0.3 fmol in the injected sample. © 2001 Published by Elsevier Science B.V.

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1. Introduction

An increase in the amount of reactive oxygen species in living cells beyond physiological limits, or a corresponding decrease in their ability to eliminate them, leads to oxidative stress. A consequence of oxidative stress is the degradation of cell membrane polyunsaturated fatty acids (PUFAs), i.e. lipid peroxidation. A great number of chemical substances are formed during lipid peroxidation [1]. Following peroxidation of ω-6 and ω-3 PUFAs, relatively unstable fatty acid hydroperoxides are converted to more stable aldehydes. A group of aldehydes formed by degradation of ω-6 PUFAs is the hydroxyalkenals, the most abundant of which is 4-hydroxynonenal (HNE). HNE has been claimed to be one of the most cytotoxic substances produced during lipid peroxidation [2].

Various effects have been found in cells treated with HNE, including: rapid depletion of glutathione, reductions in protein thiol contents, induction of lipid peroxidation, disturbance of calcium homeostatis, inhibition of DNA, RNA and protein synthesis, inhibition of respiration and glycolysis, lactate release, and morphological changes [3]. A number of reviews on the topic of aldehydes originating from lipid peroxidation have been published [2,4–6].

HNE is difficult to determine in biological samples

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since there are always questions as to whether metabolism and reactions generated by the sample itself, or by the sample handling, give rise to erroneous results. One way to increase the understanding of these problems is to develop new methodology.

Several methods for the determination of HNE have already been presented. At higher concentrations, HNE can be measured directly using HPLC with UV detection [6]. Derivatization of HNE with 2,4,-dinitrophenylhydrazine or dabsylhydrazine to the corresponding hydrazones has been used to increase the sensitivity for both HPLC–UV techniques [6,7] and HPLC with electrochemical detection [8]. Trimethylsiloxyethers of the dinitrophenylhydrazones have also been analysed by GC–MS [9]. An alternative GC–MS procedure is to react the aldehyde group with pentafluorobenzyl (PFB) oxime [10]. The reagent 4-(2-carbazoylpyrrolidine-1-yl)-7-nitro-2,1,3-benzoxadiazole (NBD-ProCZ) has been used to derivatize HNE prior to HPLC separation and laser-induced fluorescence (LIF) detection [11]. A method based on electrospray mass spectrometry has also been presented [12]. All the above methods use microlitre injection volumes and have LODs in the order of femtomoles to picomoles.

The further development of accurate and sensitive methods to measure 4-hydroxyalkenals, especially HNE, is desirable for the future progress of research into oxidative injury. Recently, interest has been given to brain damage caused by HNE, where it has been associated with serious disease states [13,14].

The minute (usually nanolitre) sample volume requirements and high separation efficiencies of techniques such as capillary electrophoresis (CE) and micellar electrokinetic chromatography (MEKC) are useful features when working with biological samples like brain tissue. An advantage with MEKC as compared to CE is that problems with adsorption of hydrophobic sample components and proteins to the capillary wall are reduced. This is mainly due to the ability of the micelles to solubilize these components, thereby allowing separations of samples that are relatively unpurified [15]. Laser-induced fluorescence detection is the most sensitive detection technique for CE and MEKC to date [16,17]. In this paper a MEKC–LIF method for the identification of HNE after derivatization with the fluorescent reagent dansylhydrazine (5-(dimethylamino)naphthalene-1-sulphonehydrazine, DNSH) is presented.

2. Experimental

2.1. Chemicals

Cetyltrimethylammonium bromide (CTAB), sodium tetraborate, phosphoric acid and methanol were from Merck (Darmstadt, Germany). The acetonitrile used was from KEBO (Spånga, Sweden). Sodiumdodecyl sulfate (SDS) was obtained from ICN (Aurora, OH, USA). Sodium hydroxide pellets were from Eka Nobel (Bohus, Sweden). The HNE was purchased from Calbiochem (San Diego, CA, USA). Fluka (Buchs, Switzerland) supplied the dansylhydrazine. The ferrous ammonium sulfate and butylated hydroxy toluene (BHT) were obtained from Sigma (St. Louis, MO, USA). All water solutions were prepared using water from an Elgastat UHQII system (Elga, High Wycombe, UK).

2.2. Apparatus

2.2.1. MEKC–LIF

A Prince auto sampler (Lauerlabs, Emmen, The Netherlands) was used to inject samples hydrodynamically and to control a Spellman model CZE1000R high voltage supply unit (Plainsview, NY, USA). The separation capillary used was from Polymicro Technologies (Phoenix, AZ, USA). The capillary had an I.D. of 25 μm and an O.D. of 150 μm. The injection end of the capillary was glued into a 220-μm I.D. capillary with an O.D. of 365 μm to enable use of the autosampler. A detection window was created 84 cm from the injection end by removal of the polyimide coating. Two buffer systems were used. The first consisted of 10 m*M* B_4O_7 buffer, pH 9.3, with 30 m*M* SDS. Here the applied voltage was +30 kV. The second buffer system consisted of 15 m*M* phosphoric acid adjusted to pH 7.0, with 30 m*M* CTAB and 30% (v/v) ACN. The applied voltage was −30 kV. The injected sample volume corresponded to about 0.5% of the capillary length.

An Innova Argon 304 (Coherent, Palo Alto, CA, USA) full frame argon ion laser was used for detection, in an optical arrangement similar to that

described by Yeung et al. [18]. The 351.0 and 351.5 nm emission lines from the laser were used to detect the DNSH derivatized HNE, with an UG11 shortpass filter to filter out light from other light sources in the room. Emitted light was collected at 90° to the excitation beam, and focused onto an R212-UH photomultiplier tube (Hamamatsu, Hamamatsu City, Japan) using a microscope objective. Scattered light was filtered out using a KV389 longpass filter and Raman emission from the buffer was filtered out using a longpass filter at 470 nm. All filters used were from Schott (Mainz, Germany). The selected excitation and emission wavelengths are based on values given in [19].

2.2.2. MEKC–UV

A capillary electrophoresis system ($HP^{3D}CE$, Wilmington, DE, USA) equipped with a diode array detector set at 350 nm was used for the MEKC–UV analysis. The capillary was an untreated fused-silica column from Polymicro Technologies, (Phoenix, AZ, USA), that was 65 cm long (55 cm to the detection window)×50 μm I.D.×375 μm O.D.

2.3. Animals

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

2.4. Sample preparation

The heads of the rats were stored at −70°C immediately after decapitation. After thawing at room temperature the brains were quickly excised and homogenized (Ultra-turrax, IKA, Germany) in ice-chilled 5 m*M* phosphate buffer, pH 7.0. The resulting tissue homogenates were approximately 20% by mass. In some of the samples oxidative stress was induced by the addition of 100 μ*M* ferrous ammonium sulfate (mixing for 30 min at room temperature). BHT, an antioxidant, was added to a final concentration of 0.075% in order to prevent unwanted oxidation. The homogenates were stored at −80°C until further use. Prior to analysis the samples were deproteinized by the addition of methanol (1.5 times the sample volume) and were then centrifuged at 14 000 *g* for 4 min. The clear supernatant was transferred to a borosilicate test tube for derivatization with DNSH.

2.5. Derivatization procedure

The general derivatization procedure consisted of mixing sample, organic solvent, phosphoric acid and DNSH reagent. Five factors that could potentially affect the reaction between HNE and DNSH were evaluated using a 2^{5-2} reduced factorial design, with three center point experiments to obtain an estimate of the experimental error. The response used was the peak area of the resulting HNE–DNSH reaction product. The investigated parameters were the reaction temperature, the volume fraction of organic solvent, the amount of acid in the reaction mixture, the amount of excess reagent, and the reaction time. The selected variables are summarized in Table 1. The reaction temperature was varied between 20 and 60°C. Methanol was used as the organic solvent and its volume fraction was varied between 1/3 and 2/3. The amount of acid present was varied between 2 and 8%. Stronger aqueous solutions of phosphoric acid were made in order to add the desired amount of acid while maintaining the volume fraction of methanol. Solutions containing 3, 6, 10, 12 and 24% phosphoric acid were thus prepared and added to the reaction mixture as appropriate. 10, 20 and 30 m*M* solutions of DNSH in methanol were used, corresponding to 417-, 833- and 1250-fold excesses of DNSH, respectively. The reactions at 20°C were performed in a temperature-controlled room. The reactions at elevated temperatures were performed in a heated cupboard (Memmert, Schwabach, Germany) at 40 or 60°C.

Derivatization was performed as follows. A 25-μl volume of HNE–methanol solution was transferred

Table 1
Factors screened for their influence on the derivatization of HNE with DNSH

Factors	Experimental domain		
	(−) level	(+) level	(0) level
Temperature (°C)	20	60	40
Volume fraction org. solv.	1/3	2/3	1/2
Amount of acid (%)	2	8	5
Times excess reagent	417	1250	833
Reaction time (min)	30	90	60

to a test tube. This solution was mixed with 50, 75 or 100 μl of aqueous phosphoric acid solution with 50, 25 or 0 μl of methanol to obtain the desired volume fraction of methanol. To this mixture 25 μl of DNSH reagent was added. The tubes were sealed and allowed to react for a length of time determined by the experimental design. All derivatizations were performed in darkness using aluminium foil to shield the reaction mixture from light. Before injection into the MEKC–LIF system all samples and standards were diluted five-fold by mixing 50 μl of the derivatized sample with 50 μl separation electrolyte and 150 μl deionized H_2O.

3. Results and discussion

3.1. Screening experiments

The aim of the screening experiments was to identify factors that affect the derivatization. The reaction between HNE and DNSH is illustrated in Fig. 1. According to our results, the reaction temperature should be kept at the lower level, the organic solvent at the higher level and the amount of acid present should be kept low. At the 95% confidence level, the excess amount of reagent and the duration of the derivatization seem to be of less importance. These results were considered in the further experiments, where the reaction mixture consisted of sample, 2/3 organic solvent, 2% acid and about a 100-fold excess of DNSH. The derivatization time was 30 min and the reaction was performed at 20°C.

3.2. Separation and analysis

A MEKC–LIF separation of DNSH derivatized HNE and the corresponding blank is shown in Fig. 2.

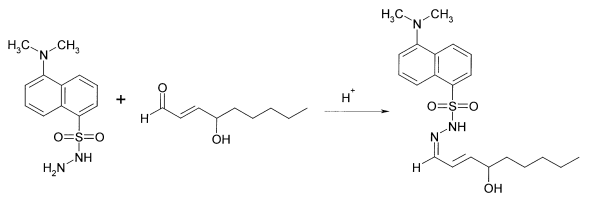

Fig. 1. Derivatization reaction between HNE and DNSH.

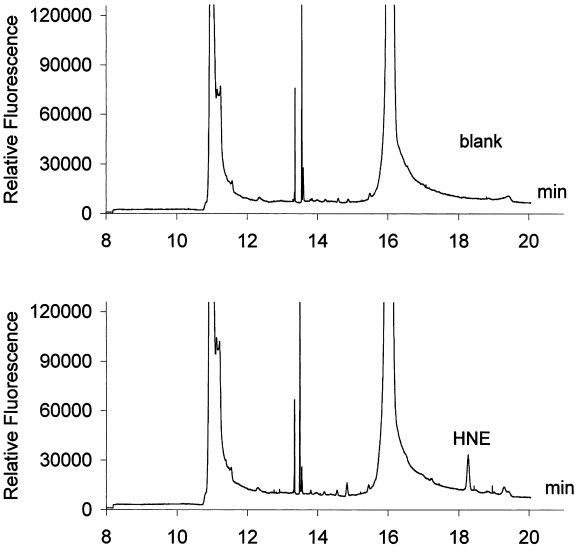

Fig. 2. Separation of DNSH derivatized HNE and the corresponding blank using an electrolyte consisting of 10 m*M* B_4O_7 buffer, pH 9.3, with 30 m*M* SDS. The standard concentration was 60 μ*M*, corresponding to 20 fmol injected.

The separation electrolyte used was a 10 m*M* B_4O_7 buffer, pH 9.3, with 30 m*M* SDS. Side reactions of the DNSH reagent give rise to numerous additional peaks in the chromatogram. Some of these are unstable and diminish significantly with time. Migration time repeatability was good within the same day (RSD_{time}=0.4%, n=4) in the SDS system. However some instability in the elution times between days, in combination with the wide distribution of the extra reagent peaks, prompted us to attempt to change the selectivity of the separation. Through scouting experiments it was concluded that a change of surfactant gave the most significant change of selectivity. A new separation was developed using CTAB as surfactant. As CTAB has a lower critical micelle concentration than SDS more organic solvent could be added to the separation electrolyte while maintaining micellar conditions. This was advantageous to keep hydrophobic substances in solution. But the CTAB system, although having more repeatable migration times, was not preferred because it increased baseline noise. A benefit from testing two systems, with different selectivity, was that the purity of the HNE peak could be verified. The limit of detection (3 S/N) in the SDS system was approxi-

mately 30 n*M* or 0.3 fmol in the injected sample. This corresponds to about 1 μ*M* in the original rat brain homogenate. This is, according to [20], slightly higher than the basal levels of HNE that can be expected in healthy tissues, but lower than levels that may occur during oxidative stress. Fig. 3 shows chromatograms of two samples of derivatized brain homogenates from the same rat: one that was protected from oxidation by the addition of BHT (a) and one in which oxidative stress was induced by the addition of iron (b). A chromatogram of standard derivatized HNE is also shown (Fig. 3c). As can be seen in Fig. 3a the basal level of HNE is below our limit of detection. In contrast, in the sample where lipid peroxidation has been induced there is a large peak originating from HNE. An estimate of the concentration can be obtained through a comparison with the peak in the standard sample in Fig. 3c, where the original HNE concentration before derivatization and dilution was 24 μ*M*. The superior sensitivity, in terms of LOD, of the LIF detector was confirmed when the same samples were run on the system with UV detection — no peaks originating from HNE could be discerned at these concentrations.

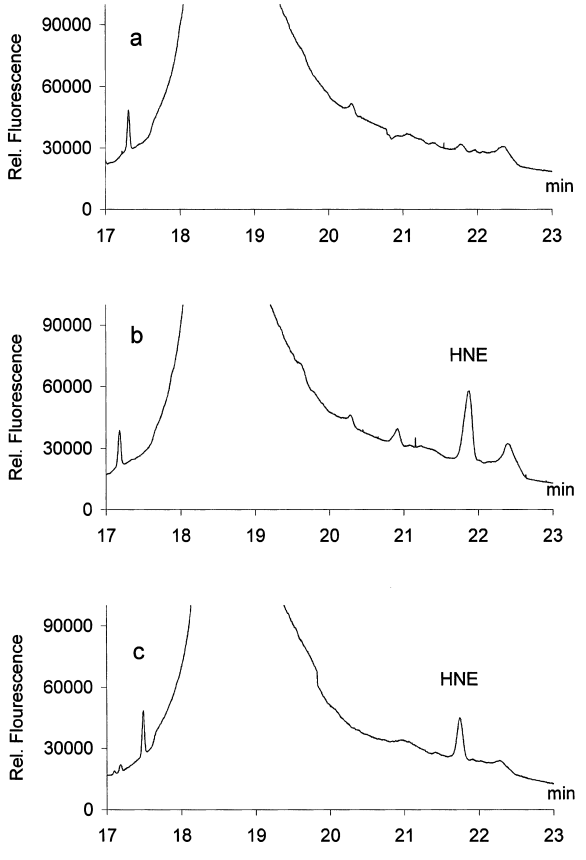

Fig. 3. Separation of DNSH derivatized samples. (a) Rat brain homogenate protected from oxidation by the addition of BHT. (b) Rat brain homogenate where lipid peroxidation has been induced. (c) Standard 4-HNE sample at 24 μ*M*, corresponding to 8 fmol injected.

Since two of the advantages of this method are the small sample volume requirements, and the simplicity of the sample purification and derivatization procedures, a considerable reduction in the amount of tissue used should be possible. A future prospect using this method is thus to study discrete areas or structures of the brain. Some further investigations are required before an accurate quantification can be performed, for example free HNE is known to react rapidly with proteins [20] which may influence its levels in vivo. However, this method should be useful for analysis of systems where an elevated level of lipid peroxidation is expected.

4. Conclusions

In this paper the derivatization, separation and detection of HNE using the fluorescent reagent DNSH and MEKC–LIF have been demonstrated. Screening experiments showed that the outcome of the derivatization is influenced by the reaction conditions. The sample treatment and derivatization procedure are very simple to perform and the applicability of the method has been shown by derivatization and analysis of rat brain homogenates.

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