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Short refereed paper

Synthesis and characterization of 4-hydroxy-2-nonenal derivatives for gas chromatographic analysis with electron capture detection (GC-ECD)

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4-Hydroxy-2-nonenal (HNE) has been prepared from the corresponding dimethylacetal (HNE-DMA), in turn synthesized by a conventional approach with a few modifications of the experimental protocol and some improvements in the purification of the final product. In order to exploit the sensitivity of gas-chromatography with electron capture detector (GC-ECD) in the analysis of HNE derivatives, reaction of HNE with 2,4,6-trichlophenylhydrazine (TCPH) and 3,5-dichlorophenylhydrazine (DCPH) was tested. Reaction with TCPH afforded a mixture of products, whereas with DCPH a single major product was formed that was prepared on a millimolar scale and purified. ^{1}H-NMR analysis established that the derivative of HNE with DCPH is HNE 3,6-dichlorophenylhydrazone, that can be used as standard for GC-ECD analysis.

Keywords: 4-Hydroxy-2-nonenal (HNE), gas-chromatography with electron capture detector (GC-ECD), gas chromatography-mass spectrometry (GC-MS), 3,5-dichlorophenylhydrazine

Introduction

4-Hydroxy-2-nonenal (HNE), one of the most abundant and toxic lipid-derived alkenals generated from peroxidation of ω-6 polyunsaturated fatty acids,[1] is present at micromolar levels in biological tissues.[2,3] Several analytical methods have been developed for quantification of HNE, including high-performance liquid chromatography (HPLC) or gas chromatography-mass spectrometry (GC-MS) of suitable HNE derivatives.[4,5] In connection with other studies related to evaluation of malonaldehyde, another important biomarker of lipoperoxidation,[6] we decided to investigate gas-chromatographic analysis of HNE levels using electron capture detection (GC-ECD). For this analytical method, derivatives with halogenated reagents such as pentafluorophenyl or trichlorophenylhydrazines are required.[6–8]

4-Hydroxy-2-nonenal dimethylacetal (HNE-DMA) has been prepared as source of HNE by a conventional approach with a few improvements of the experimental protocol. Also, the purification of HNE-DMA by column chromatography has been revised and, after acid hydrolysis, the reaction of HNE with halogenated phenylhydrazines such as 3,5-dichlorophenylhydrazine (DCPH) and 2,4,6-trichlophenylhydrazine (TCPH) has been studied.

Materials and Methods

The ^{1}H-NMR spectra were recorded on a Bruker AM 500 spectrometer operating at 500.13 for ^{1}H. The central peak of $CDCl_3$ signals (7.27 ppm for ^{1}H) was used as internal standard. The chemical shifts are reported in

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Abbreviations: HNE, 4-hydroxy-2-nonenal; DCPH, 3,5-dichlorophenylhydrazine; TCPH, 2,4,6-trichlophenylhydrazine; GC-MS, gas chromatography-mass spectrometry; GC-ECD, gas chromatography with electron capture detector; HNE-DMA, 4-hydroxy-2-nonenal dimethylacetal; HNE-DCPH, 4-hydroxy-2-nonenal 3,5-dichlorophenylhydrazone; RT, retention time

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parts per million and coupling constants (*J*) are given in Hertz. GC analysis were performed on a HP-5 MS column (15 m × 0.25 mm, 0.25 μm), using helium as carrier gas (0.7 ml/min) with a temperature program from 100°C to 290°C (10°C/min) by a Gas-Chromatograph Trace GC Ultra (Thermo) connected to an electron capture detector or to a mass spectrometer (Trace DSQ, Thermo) operating at 70 eV with electronic impact. Fumaraldehyde bis(dimethyl acetal), pentylmagnesium bromide (2 M solution in diethyl ether), reagents and solvents were supplied by Sigma-Aldrich Italia.

*Fumaraldehyde dimethylacetal (**2**)*

Fumaraldehyde dimethylacetal (**2**) was obtained by partial acid hydrolysis of fumaraldehyde bis(dimethylacetal) (**1**), by a modification of a described procedure.[9] Fumaraldehyde bis(dimethylacetal) (**1**; 0.200 g, 1.135 mmol) was added to Amberlyst-15 catalyst in acid form (40 mg) in diethyl ether (5 ml) under magnetic stirring at room temperature. Stirring was continued for 60 min, then the reaction mixture was filtered through a bed of anhydrous sodium carbonate and sodium sulphate 1:1, w:w. Average yields were between 55–65%.

*4-Hydroxy-2-nonenal dimethylacetal (HNE-DMA, **3**)*

The compound was obtained as described[10] using pentylmagnesium bromide as a 2 M solution in diethyl ether, instead of *in situ* preparing the Grignard reagent. The product was purified by column chromatography using Florisil® as stationary phase; by elution with 20% ethyl acetate in petroleum ether 50–70% average yields of HNE-DMA were obtained.

*4-Hydroxy-2-nonenal 3,5-dichlorophenylhydrazone (HNE-DCPH, **5**)*

HNE (**4**) was generated by hydrolysis of HNE-DMA (**3**) (50 mg, 0.3 mmol) in 1 ml of 1 mM HCl (30 min). Aliquots (5 ml) of a DCPH solution (0.09 mmol/ml in 1 mM HCl) were added to the HNE solution and the reaction mixture was maintained for 60 min at room temperature. The resulting derivative HNE-DCPH, after addition of H_2SO_4 (96%, 30 ml) at 0°C, was extracted with ethyl acetate (3 x 5 ml). The organic solution was treated with anhydrous Na_2SO_4 and, after filtration, evaporated under a stream of nitrogen to afford a yellow solid. A small amount of pure derivative (5 mg) was obtained by repeated HPLC separations on a reversed phase Discovery C-18 column (250 x 4.6 mm, 5 μm; Supelco) using 30% water in acetonitrile solution as eluent. The organic solvent of the combined fractions was evaporated and the resulting aqueous phase was extracted with ethyl acetate. ^{1}H NMR ($CDCl_3$) δ 7.46 (1H, broad s, NH), 7.41 (1H, d, J = 9.3 Hz , –C*H*=N–), 6.93 (2H, d, J = 1.8 Hz, *ortho*-aromatics), 6.84 (1H, t, J = 1.8 Hz, *para*-aromatic), 6.46 (1H, dd, *J* = 9.3 and 15.7 Hz, =*H*C–CH=NH), 5.98 (1H, dd, *J* = 6.5 and 15.6 Hz, –CHOH–C*H*=), 4.30 (1H, dt, *J* = 6.5 and 6.5 Hz, –C*H*OH), 1.63–1.56 (8H, m, 4 x CH_2), 0.92 (3H, t, *J* = 6.9 Hz,–*C*H_3).

Results

HNE-DMA was synthesized by a conventional procedure[9,10] starting from commercially available fumaraldehyde bis (dimethylacetal) (**1**) as reported in Figure 1. A few improvements of various experimental protocols have been realized in order to keep yields reproducible within a 30–45% yield range of obtained HNE-DMA (**3**). The controlled hydrolysis of fumaraldehyde bis (dimethylacetal) (**1**) in the presence of Amberlist (in acid form) was originally described in acetone solution with addition of water. We have carried the hydrolysis in a diethyl ether solution of the compound (**1**) and stopped the reaction by removing the acidic resin and filtering on a 1:1 mixture of sodium carbonate and anhydrous sodium sulphate. This prevented further hydrolysis of fumaraldehyde dimethyl acetal (**2**) and afforded a solution ready for reaction with pentylmagnesium bromide (2 M solution in diethyl ether). Finally, purification of HNE-DMA (**3**) by silica gel chromatography invariably caused some hydrolysis to HNE (**4**) and it was found that purification with Florisil® could improve recovery of required product (**3**).

In order to exploit the quantitative determination of HNE by the sensitive GC-ECD analysis, standard derivatives of HNE with halogenated phenylhydrazines were required. DCPH and TCPH were selected and the formation of the

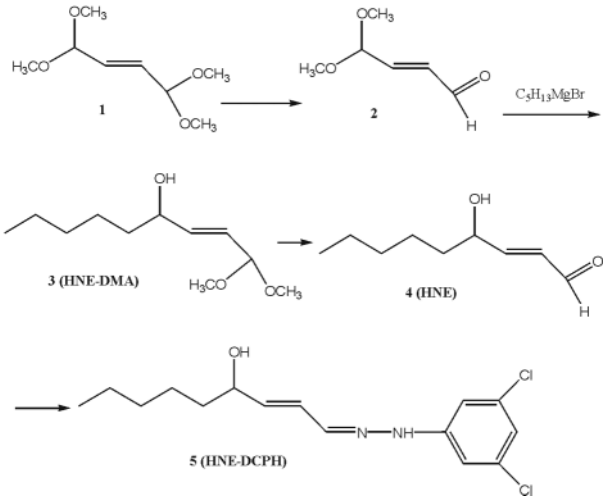

Fig. 1. Synthesis of HNE-DMA, HNE and HNE-DCPH.

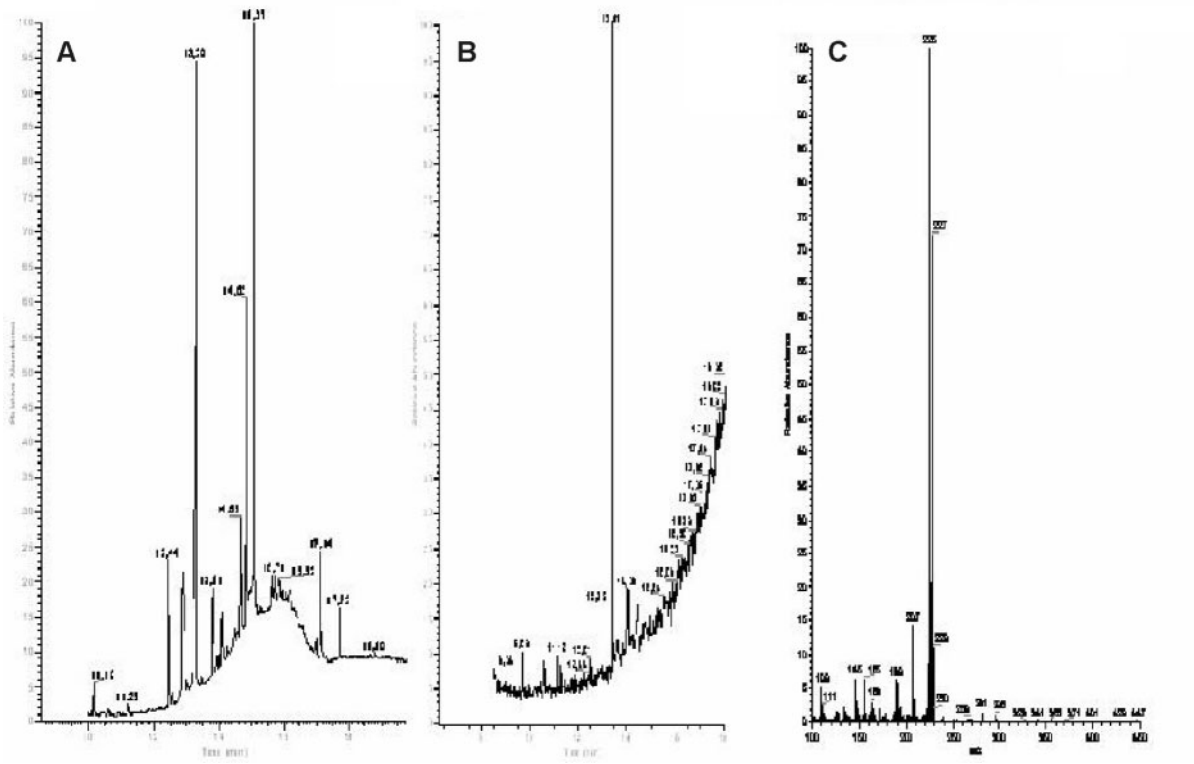

Fig. 2. (a) GC spectrum of HNE-TCPH; (b) GC spectrum of HNE-DCPH; (c) MS spectrum of HNE-DCPH.

related phenylhydrazones investigated. Preliminary experiments with TCPH gave contrasting results and GC analysis showed a mixture of products with two predominant peaks (Fig. 2a). For the peak at RT 13.34 min, MS analysis showed a main fragment corresponding to a trichlorophenylanilium ion (*m/z* 194) typical for hydrazone derivatives. In the second peak at RT 15.07 min, the most abundant fragment at *m/z* 259 was attributed to a N(1)-2,4,6-trichlorophenyl-5-methylenepyrazolinium ion. On the other hand, the reaction of HNE with DCPH gave a predominant product (Fig. 2b) and MS analysis showed a main peak at *m/z* 225 accompanied by other fragments at *m/z* +2 and +4, as expected by an ion containing two chlorine atoms (Fig. 2c). GC-ECD analysis of HNE-DCPH derivative shows a detection limit lower than one picomole injected (Fig. 3).

Discussion

MS analysis of HNE-DCPH did not show the expected 3,5-dichlorophenylanilinium ion at *m/z* 161, while the main fragment was an ion at *m/z* 225 that could correspond to a cyclization product (a pyrazolinium or equivalent six

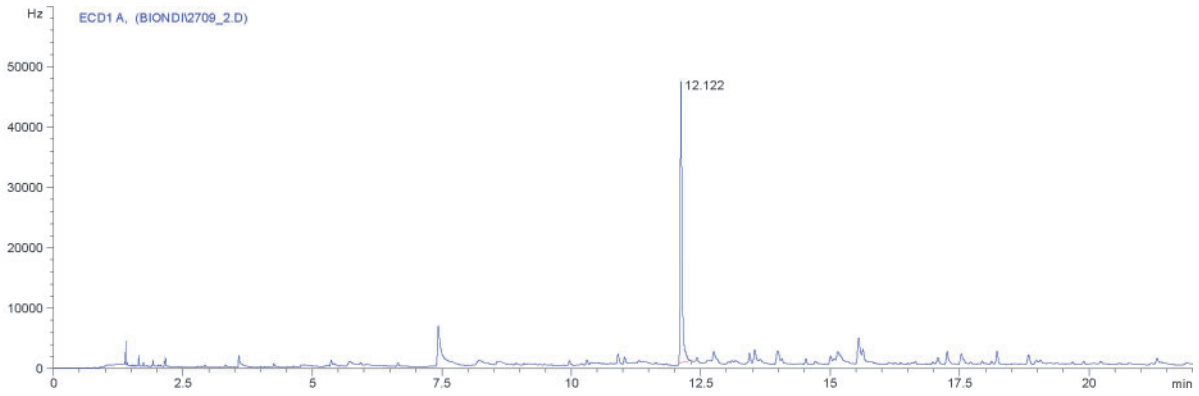

Fig. 3. GC-ECD analysis of HNE-DCPH.

member pyridazinium ion). This result is different from the GC-MS analysis of reaction of HNE with TCPH, where two main products were observed by GC and the 2,4,6-trichlorophenylanilium ion (*m/z* 194) was characteristic of one peak only. These considerations prompted us to elucidate the structure of HNE-DCPH and, for this purpose, we have performed reaction of HNE with DCPH at the millimolar scale. The reaction product, *i.e*. HNE-DCPH, was isolated and characterized by ^{1}H-NMR spectroscopy. This analysis clearly showed a hydrazone structure for HNE-DCPH derivative (**5**) and the observed ion at *m/z* 225 probably is formed under GC-MS conditions of analysis that cause cyclization of the 3,5-dichlorophenylhydrazone to a pyrazolinium or pyridazinium ion. Moreover, the reaction of HNE with DCPH gave a single product and this suggests that DCPH is the reagent of choice for GC-ECD analysis of HNE. Our preparation of a pure standard allowed us to establish that GC-ECD analysis of HNE-DCPH derivative shows a detection limit lower than one picomole injected (Fig. 3). Work is in progress to apply this derivatization procedure to HNE determination in food ageing studies and in biological samples.

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