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Relevance of a new HPLC assay for 4-hydroxy-2-nonenal in human plasma

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ABSTRACT

Objective: 4-hydroxy-2-nonenal (4-HNE) is one of the major end-products of arachidonic acid peroxidation since proposed as sensitive oxidative stress marker. Despite the relevance of its physiological and pathogenetic roles, there are currently no available easy-to-use analytical methods for the accurate assay of 4-HNE in human plasma. For this reason, the typical values previously reported were few, contradictory, and often related to small groups of subjects.
Methods: We present here a simple and reliable chromatographic method for 4-HNE assay in human plasma. After a deproteinization step, fluorescence derivatization and solid phase extraction (SPE), 4-HNE is finally separated and quantified by a high-performance reversed-phase liquid chromatography (HPLC).
Results: An almost complete recovery (>98%), high sensitivity (limit of detection of 100 pmol/l) and a wide dynamic range (from 1 to 2000 nmol/l) propose this method as particularly suitable for high throughput measurements of 4-HNE in human plasma samples. The typical values of 4-HNE (37 ± 15 nmol/l, mean ± SD) were measured in a group of 96 elderly volunteers.
Conclusions: This simple HPLC method, with its good SPE cleaning and high sensitivity of detection, is proposed as a very reliable and advantageous tool to characterize lipid oxidative damages in several chronic pathological conditions. The typical plasmatic levels measured were very little scattered but much lower than those previously reported by other authors. This discrepancy suggests that, in some cases, it has been analyzed not only the free, but even the bonded fraction of 4-HNE.

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KEYWORDS

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Introduction

The reactive oxygen species (ROS) attack on poly-unsaturated fatty acids (PUFA) of membrane phospholipids may cause the formation of their peroxides. From its next fragmentation originate 4-hydroxy-2-nonenal (4-HNE), a 9-carbon aldehyde characterized by a hydroxyl group in position 4 and one double bond between carbon 2 and 3 (Figure 1). 4-HNE is one of the major end- products of arachidonic acid peroxidation proposed as a marker of oxidative stress sometimes more sensitive than malondialdehyde (MDA) [1].

Biochemistry of lipid peroxidation

The lipids oxidation process begins with the subtraction of a hydrogen atom from a methylene group adjacent to a double bond in a PUFA and resulting in the formation of a radical centered on carbon [2]. From the rearrangement of the double bond, a conjugated diene is formed: oxygen can now react with the carbon-centered radical forming a peroxyl group which, by reacting with another PUFA, can become a hydroperoxide, generate a new carbon radical on the second PUFA, and thus perpetuate the reaction cascade (Figure 2).

Already in these early stages of the process, many lipid peroxides are able to bind covalently nucleic acids and proteins making the so-called "stable adducts". Lipid hydroperoxides may also react further by forming other peroxides and cyclic endo-peroxides or, by fragmentation, α, β-unsatured aldehydes such as 4-HNE, acrolein

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and MDA. These, like all aldehydes, are characterized by a carbonylic group in which, because of the electronegativity of oxygen, there is a strong polarization of the double bond carbon-oxygen that determines the resonance between two limit formulas (resonance carbocation). This chemical character makes the aldehydes particularly reactive with nucleophilic compounds which, in acidic environment, can attack their carbonyl carbon: carbonyl oxygen, instead, by purchasing a proton, forms a hydroxyl group completing the nucleophilic addition reaction (Figure 3).

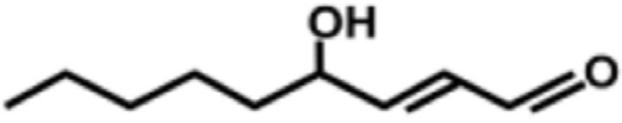

Figure 1. The 4-hydroxy-2-nonenal molecular formula.

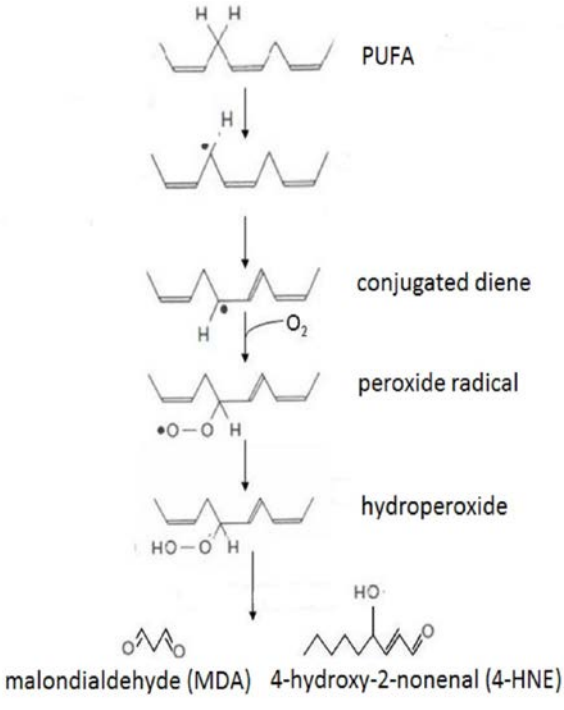

Figure 2. The reaction cascade of lipid peroxidation of poly-unsatured fatty acids (PUFA).

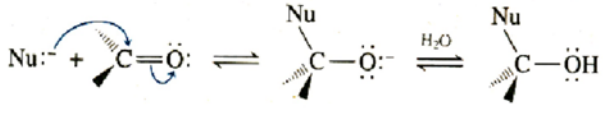

Figure 3. The reaction scheme of nucleophilic addition.

Physiology of 4-HNE

4-HNE is the main aldehyde generated by the attack of ROS on PUFA omega-6 such as arachidonic, linoleic and linolenic acid: by nucleophilic addition, it mainly reacts with sulfhydryl and amino acid groups of proteins (namely cysteine, histidine and lysine lateral chains), peptides, phospholipids and nucleic acids forming stable adducts that modify the structures and, often, the functions of these macromolecules.

Under redox-balance conditions, free 4-HNE plays its physiological role participating in the cell signaling pathway [3-4]; completed its function, 4-HNE is in various ways metabolized, by enzymatic reduction forming 4-hydroxynonenal and 1,4-dihydroxynonene, by direct oxidation forming carboxylic acid, or by enzymatic oxidation catalyzed by aldehyde dehydrogenase, forming trans-4-hydroxy-2-nonenoic acid.

When its endocellular concentrations rises beyond the physiological levels and an oxidative imbalance starts, 4-HNE is initially subtracted from the cell by formation of complexes with glutathione (GSH) through spontaneous nucleophilic additions or by catalysis of GSH-transferase. The 4-HNE-GSH conjugates are subsequently degraded to mercaptic acids and excreted in urine.

When 4-HNE levels go further and endogenous detoxification mechanisms become insufficient, the formation of stable 4-HNE adducts with proteins and nucleic acids increases rapidly triggering mechanisms like autophagy, senescence, or cell cycle arrest. The cell, that initially subsists, with aggravation of the oxidative stress undergoes processes of apoptosis or irreversible cell injury that evolve into necrosis and pathological conditions [5].

Pathogenetic role of 4-HNE

The 4-HNE pathogenetic role began to be revealed by the end of 20th century, when its adducts have been demonstrated within the atherosclerotic plaques [6], in amyloid deposits of Alzheimer's disease [7-8] and in systemic amyloidosis [9]. 4-HNE high levels were also found in parkinsonians [10] and in amyotrophic lateral sclerosis cases [11]. Neuronal proteins were still shown to be particularly sensitive to the cytotoxic action of 4-HNE [12], whose overproduction has also been demonstrated in subjects exposed to pollutants, toxic agents [13] and in multiple chemical sensitivity (MCS) [14].

In vitro studies showed that 4-HNE adducts were present both on proteins of endocellular spaces

and on those of interstitial and extracellular fluids [15]. Additionally, while 4-HNE could also bind phospholipids, mainly phosphatidylethanolamine, it was proposed its key role on the dysfunctions of cell membranes [16].

Effects of 4-HNE on α-synuclein aggregation in Parkinson's disease [17] and changes in the cytochrome C function in the respiratory chain [18] were also highlighted. Of particular relevance was the demonstration that the stabilization of 4-HNE on membrane would not be permanent; indeed, would be possible continuous exchanges between membrane-bonded and free forms of 4-HNE both in intra- and inter-cellular spaces [19]. These were some of the first evidences that 4-HNE was probably not a mere catabolite of the lipid peroxidation process because it could play a specific role in cell signaling pathway.

The pathogenetic involvements of 4-HNE was demonstrated as well in type 2 diabetes [20], obesity [21], Down's syndrome [22], oncogenesis and neurodegenerative diseases [23], in which the adducts seemed to be a *leitmotif*, where the targets appeared only few proteins with remarkable overlaps between different diseases [24]. Recent studies, finally, confirmed the regulating role of 4-HNE in membrane permeability [25] and, more generally, the implication of phospholipid peroxides in modulation of inflammatory and immune processes [26].

Methods for 4-HNE assay

Notwithstanding the interest towards its physio-pathological role, currently there are not accurate and, at the same time, practicable methods for the 4-HNE assay in biological fluids. Some different methods were proposed in previous published works: from the simplest and not always affordable immunochemical and colorimetric assays to the sophisticated techniques such thin layer chromatography (TLC), high pressure liquid chromatography with electrochemical detection (HPLC-ED), liquid chromatography matrix-assisted laser desorption ionization/time of flight (LC-MALDI/TOF), tandem (LC-MSMS) or gas chromatography (GC-MS) mass-spectrometry [19,21,27-30].

Probably just for the wide heterogeneity of these proposed methods, there are actually very scattered and often unrepresentative typical values for 4-HNE in human biological fluids. Aim of our study was therefore to develop and propose a simple, accurate and reliable HPLC method for the determination of 4-HNE in human plasma. After a deproteinization step, fluorescence derivatization and SPE, 4-HNE is finally quantified by a high-sensitivity reversed-phase liquid chromatography. By this method, the typical values of 4-HNE in a group of 96 elderly volunteers were determined.

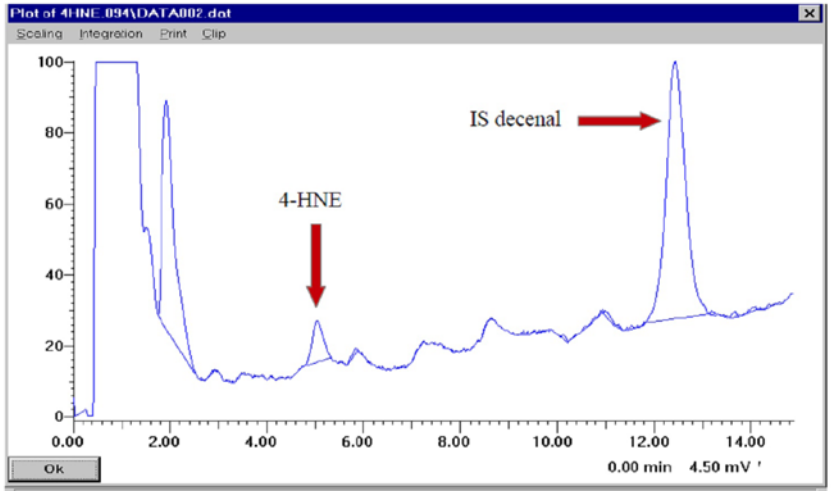

Figure 4. Typical chromatogram of unknown plasma extract.

Materials and Methods

Chemicals and solutions

All chemicals used, perchloric acid, dansyl hydrazine (DNSH) and orthophosphoric acid, were of analytical grade and purchased from Carlo Erba (Milan, Italy). Water, and methanol used for mobile phase and solution preparations were of HPLC grade.

4-HNE and 4-hydroxy-decenal (4-HDE), used as internal standard (IS) were purchased from

Sigma-Aldrich (Milan, Italy), and cartridges for the SPE (Bond Elut C18, 50 mg/ml) from Agilent Technologies (Milan, Italy). Stock solutions of 4-HNE (5 µmol/l) and IS (100 µmol/l) were prepared in water and kept at −80°C until analysis. One aliquot of 4-HNE stock solution was adequately diluted in water and in plasma matrix pooled from donor samples to obtain working spiked solutions ranging from 50 to 500 nmol/l.

Collection of samples

Plasma samples were obtained from apparently healthy elders (n = 96) of 68 ± 7 years (mean ± SD). Blood samples were collected in vacuum sealed tubes containing EDTA. After sampling, the tubes were immediately centrifuged at 3500 rpm for 10 minutes at 4°C and the plasma frozen at -80°C until analysis.

Samples preparation

Both the standards, unknown and spiked plasma samples were processed according to the derivatization and extractive procedures summarized below:

After addition of 50 µl of IS, 500 µl of EDTA plasma were deproteinized with 20 µl of 65% perchloric acid, vortex-mixed and centrifugated (10 min, 3500 rpm); 350 µl of supernatant were added to 350 µl of 20 mmol/l DNSH methanolic solution and 350 µl of 2% orthophosphoric acid aqueous solution. After 20 min of incubation in the dark, the derivatized samples were applied onto SPE cartridges preconditioned with 500 µl methanol, followed by 1 ml water. Cartridges were then washed with 500 µl of methanol/water solution 50% (v/v) and finally eluted with 500 µl methanol. After dilution 1:2 in water, 20 µl of eluates were injected into the chromatograph.

4-HNE was quantified by measuring peak areas corrected vs IS via Unipoint Gilson software: the calibration curve was the linear regression calculated from standard spiked solutions after subtraction of the aldehyde concentrations in the correspondent unspiked samples.

Chromatographic conditions

The HPLC system consisted of a 307 pump model and a 234 autosampler model with a 20 µl loop, both from Gilson (Villiers-le-Bel, France). The separation was performed on a Poroshell 120 EC-C18 column (50 mm × 4.6 mm i.d.) packed with 2.7 um particles (Agilent). The mobile phase was a methanol/water solution 60% (v/v), sonicated before the use. A flow rate of 1.5 ml/min was used giving a pressure of about 180 kg/cm^2 at room temperature. Fluorescence was detected with the excitation/emission wavelenghts pair of 350 and 525 nm. In these conditions, 4-HNE and IS elute respectively with capacity factors (k') of 11.8 and 30.3.

Results

Plasma deproteinization step

The deproteinization step has proved to be crucial for the overall recovery in subsequent derivatization and extraction steps. Whether deproteinization is achieved by acidification with concentrated perchloric acid (acidic deproteinization, 40 µl/ml plasma) or by addition of methanol (organic deproteinization, methanol *vs* plasma ratio of 2:1 v/v) the final recovery was almost complete (>98%). On the contrary, by omitting the deproteinization step and performing a direct plasma derivatization, recovery did not exceed than 42-45%.

The reason for this was only partially investigated: one test of sample alkalinization to inhibit the hypothetical 4-HNE nucleophilic attack on proteins amine groups allowed to exclude the phenomenon of 4-HNE disappearance by formation of adducts. However, the effects on recovery of an increased concentration of DNSH in the reaction mixture was not evaluated: the hypothesis that the derivatizer may be consumed by reaction with aldehydic or ketonic functional groups presents in non-deproteinized samples was anyway very plausible. Between acid and organic deproteinization, the first was preferred for its best volume of supernatant and for the lower dilution of sample.

Derivatization optimization

The derivatization reaction is a nucleophilic addition of the derivatizer DNSH on aldehydic carbonyl of 4-HNE. The most important variables for the deproteinized plasma derivatization step were studied: derivatizer concentration, acidity and composition of the mixture, time and temperature of reaction. The concentration of DNSH was optimized in 20 mmol/L in order to conjugate maximum recovery with minimal interference in HPLC run.

The derivatization reaction is rapid, already complete after 10 min of incubation at room temperature, and proceeds rightly by methanol concentrations in derivatization mixture comprised between 33 and 66%. Acidification of derivatizing solution by addition of diluted phosphoric acid has appeared indispensable for aqueous solutions of

chemical standards only, since the residual acidity present in the deproteinized plasma samples ensure itself the reaction.

SPE setup

In order to optimize the solid-phase extraction step of derivatized samples, different SPE cartridges were investigated: C18, C8, CN stationary phases were namely tested, always in the configuration 50 mg/ml. The C18 packing was the only one that has guaranteed the best purification of the sample together higher recovery. In particular, using C18 SPE, a strong washing step with 50% methanol has allowed to minimize one important interfering compound that elutes with a retention time closely similar to 4-HNE in HPLC run. The derivatized and SPE extracted samples, if not immediately forward processed, were stable for at least 6 days at −70°C and 12 h at room temperature on the dark.

Liquid chromatography optimization

The remarkable cleansing of the samples obtained after three pre-treatment steps, two of which with high selectivity, allowed to obtain quite easily the optimal analytical conditions. It is interesting to remark that the change in the percentage of methanol in mobile phase can significantly affect the retention times. In fact, increasing the methanol concentration from 60 to 70%, the 4-HNE retention time (RT) decreases from 5.1 to 1.6 min.

Figure 4 shows a typical chromatogram obtained from a plasma extract under the above described conditions. Intra- and inter-assay precision was calculated by analysis in triplicate of plasma spiked samples (n = 5): the mean values obtained showed a relative standard deviation less than 3.2% for the intra-assay and less than 5.6 % for inter-assay precision. The accuracy values calculated were within the range of ±5% and the limit of detection (LOD), calculated as signal to noise ratio (S/N = 2), was equal to 100 pmol/l. The analytical dynamic ranged between 5 to 2000 nmol/l.

Application on human plasma

To validate the proposed method, 4-HNE levels were measured in EDTA plasma samples collected from a large group of healthy voluntary elders. The values ranged between 37 ± 15 nmol/l (mean ± SD), and 22, 37, 48 nmol/l were respectively the calculated 25th, 50th and 75th percentile.

Discussion

The relevance of 4-HNE assay in human plasma samples has been repeatedly described in recent years, but reliable analytical techniques are few and often hardly available. While non-cromatographic assays, such as colorimetric [1] or immunochemical [21], do not guarantee the necessary reliability due to their lack of specificity, the chromatographic methods require sometimes instruments and highly qualified laboratory personnel, not always easy to find [18]. Our simple HPLC method, with its good SPE cleaning and high sensitivity of detection, is proposed as a very advantageous tool to characterize lipids oxidative damages in several chronic pathological conditions. In this paper, we have initially applied the new method to the determination of a reliable plasmatic reference interval in a representative group of elderly subjects.

It is interesting to highlight that the 4-HNE concentrations found appeared to be much lower than those previously reported by McGrath (0.2-0.7 μmol/l) [1] and Selley (3.3-14.5 μmol/l) [27] for human plasma. These ranges, although determined by different methods, GC-MS and colorimetry respectively, were under 10 to 300 times higher than those we observed. In our opinion, this could be explained by assuming that previous data may have been referred not only to 4-HNE free form, but rather, in whole or in part, to that covalently bonded on proteins and nucleic acids. This possibility is to be considered when using low specific assay such colorimetry or strong pre-treatment steps (see heating) such in GC techniques. To support our hypothesis, it is interesting to note that direct measurement of 4-HNE plasma proteins adducts by immunoassay [21], although strongly dependent to the antibody specificity, provided just comparable results (0.1-7 μmol/l) with those reported by McGrath [1] and Selley [27].

In conclusion, we hope that a wide application of our method will allow us and other researchers to study more in depth both the pathological and physiological roles of this promising biomarker. Indeed, the measure of the increase of 4-HNE in extracellular fluids will be more and more interesting, in future, together with that of its adducts formation within metabolomic, proteomic and genomic studies.

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