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DETERMINATION OF THE LIPID PEROXIDATION PRODUCT *trans*-4-HYDROXY-2-NONENAL IN BIOLOGICAL SAMPLES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND COMBINED CAPILLARY COLUMN GAS CHROMATOGRAPHY–NEGATIVE-ION CHEMICAL IONISATION MASS SPECTROMETRY

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SUMMARY

trans-4-Hydroxy-2-nonenal (HNE) is an aldehyde end-product of lipid peroxidation in biological systems which is capable of producing a range of powerful biological effects. We wish to describe a sensitive and selective strategy for the determination of HNE in biological samples. The method is based on the formation of the O-pentafluorobenzyl (O-PFB) oxime derivatives of HNE and its deuterated internal standard which, after sample clean-up by solid-phase extraction and purification by high-performance liquid chromatography (HPLC), were derivatised further to trimethylsilyl ethers. Subsequent capillary column gas chromatography–negative-ion chemical ionisation mass spectrometry (GC–NICIMS) using selected-ion monitoring allowed quantitation in the low ng/ml range. The use of an internal standard and the O-PFB oxime derivatives circumvented the problems encountered previously by other workers because of the volatility and instability of HNE. The *syn*-isomer of HNE O-PFB oxime followed the *anti*-isomer on the HPLC and GC columns used, giving a distinctive pair of peaks of characteristic relative proportion. Moreover, the NICI mass spectra of the geometrical isomers were significantly different, providing further evidence to validate the identity of any endogenous HNE recovered. The method was used to identify and quantify HNE in platelets, monocytes, plasma and oxidised low-density lipoprotein.

INTRODUCTION

trans-4-Hydroxy-2-nonenal (HNE) (Fig. 1) is produced during lipid peroxidation in rat hepatic microsomes stimulated by NADPH-Fe [1], ADP-Fe [2,3]

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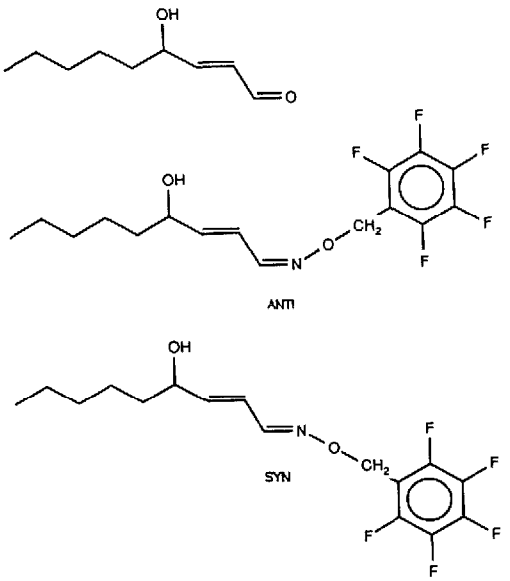

Fig. 1. Structure of *trans*-4-hydroxy-2-nonenal (HNE) and its *syn*- and *anti*-O-pentafluorobenzyl (O-PFB) oxime derivatives.

and carbon tetrachloride [2,3]. HNE is produced in the liver of mice after bromobenzene intoxication [4]. It is also formed by the non-enzymatic oxidation of arachidonic, linoleic and γ-linoleic acid [5] and human low-density lipoprotein (LDL) [6]. HNE has been detected in lipid-containing foodstuffs such as fried meat [7]. HNE has also been identified as a major toxic component of cooking oils associated with the toxic-oil syndrome outbreak in Spain [8].

HNE is capable of producing a variety of potent biological effects such as inactivation of glucose-6-phosphatase [9], adenylate cyclase [9], 5′-nucleotidase [9] and cytochrome P-450 [10], lysis of red cells [1], the reduction of superoxide anion radical production by polymorphonuclear leukocytes [11], chemotaxis of neutrophils [12], potentiation of platelet aggregation [13], cytotoxicity against Ehrlich ascites tumour cells [14], genotoxicity and cytotoxicity in rat hepatocytes [15] and Chinese hamster ovary cells [16], inhibition of the proliferative response of human peripheral blood lymphocytes to phytohemagglutanin and alloantigens [17], inhibition of c-myc oncogene expression [18] and modification of LDL, inducing enhanced uptake by macrophages and cytotoxicity to proliferating fibroblasts [19].

HNE is highly reactive towards sulphydryl (SH) groups. It reacts easily with cysteine to form thiazolidene derivatives [20] and with glutathione to form Michael addition products [21]. It reacts with SH proteins such as DNA polymerase [14] and microtubular protein [22]. HNE also reacts with the amino (NH_2) groups of lysine and tyrosine residues of LDL [23]. It reacts with the N^2 amino group of deoxyguanosine to form Michael-type adducts [24] and with the amino group of phosphatidylethanolamine and phosphatidylserine to form fluorescent chromolipids [25].

The potential physiological and pathological significance of HNE is illustrated by the evolution of a specific rat glutathione transferase 8-8 to detoxify this class of compounds [26] and a cardiac export system for glutathione S-conjugates that metabolises HNE in the rat heart [27].

HNE is formed exclusively from the arachidonic acid derived from polar phospholipids during microsomal lipid peroxidation [28]. This arachidonic acid is also the major source of the malonaldehyde produced during lipid peroxidation [29]. Malonaldehyde is generally determined by its reaction with 2-thiobarbituric acid (TBA) [30]. This method has been extensively used in studies on the relationship between lipid peroxidation, toxicity and pathology. HNE is a highly lipophilic compound and when it is formed from the phospholipids of the cell membrane it is likely to remain there and reach concentrations high enough to inhibit membrane-bound enzymes [2]. Malonaldehyde is a hydrophilic compound which does not remain in the membrane but immediately diffuses into the surrounding aqueous phase [31]. HNE is much more reactive than malonaldehyde [2]. These findings suggest that the measurement of HNE concentrations may be a much more reliable index of the cytotoxic effects of lipid peroxidation than the TBA test.

In studies on rat liver microsomes and LDL, HNE was detected by derivatisation to form the 2,4-dinitrophenylhydrazone which was then separated by thin-layer chromatography and analysed by high-performance liquid chromatography (HPLC) [1–4,6]. This method suffers from the disadvantage that it is tedious and time-consuming and lacks the sensitivity to measure physiological concentrations of HNE. A method for the determination of free HNE in biological samples and foodstuffs, based on the extraction of HNE with dichloromethane or water, clean-up by solid-phase extraction and analysis by reversed-phase HPLC using ultraviolet detection, has been described [7]. The main disadvantage of this approach is that underivatised HNE is rather labile and volatile so it can be completely lost during the removal of solvents [7].

A recently described semiquantitative method for the analysis of HNE in tissues is based on the gas chromatography–mass spectrometry (GC–MS) of the O-pentafluorobenzyl (O-PFB) oxime trimethylsilyl (TMS) ether derivative [32]. The pentafluorobenzyl group in the oxime provides enhanced sensitivity to detection by negative-ion chemical ionization (NICI). The formation of the O-PFB oxime derivative before extraction has the advantage that Schiff base linkages with protein and lipid amino groups are cleaved providing a more efficient extraction of these compounds from tissues [32].

The extremely broad spectrum of biological activity exhibited by HNE and its potential as a specific chemical indicator for lipid peroxidation make the development of a quantitative assay for HNE in biological material of the utmost importance. This paper describes the development of a specific method for the quantitative determination of HNE by capillary column GC–NICIMS of the O-PFB oxime TMS ether derivative of HNE using deuterated HNE as an internal standard.

EXPERIMENTAL

Materials

O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine hydrochloride (O-PFB·HCl) and 2,6-di-*tert.*-butyl-*p*-cresol (BHT) were purchased from Sigma (St. Louis, MO, U.S.A.), bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Pierce (Rockford, IL, U.S.A.), ethyl acetate (nanograde) and pentane (nanograde) from Mallinckrodt (Paris, KY, U.S.A.) and acetonitrile (Unichem) from Ajax Chemical (Auburn, Australia). BHT was used as a freshly prepared solution in methanol (10 mg/ml). Propiolaldehyde diethylacetal was purchased from Aldrich (Milwaukee, WI, U.S.A.) and lithium aluminium deuteride from ICN Biomedicals (Cambridge, MA, U.S.A.). Disposable reversed-phase cartridges (C_{18} Sep-Pak) were purchased from Waters Assoc. (Milford, MA, U.S.A.). Distilled water was purified by passage through a Barnstead Nanopure system (Barnstead, Boston, MA, U.S.A.) and then doubly redistilled in glass. HNE prepared by synthesis [33] was a gift from Professor H. Esterbauer (Department of Biochemistry, University of Graz, Austria). It was stored as a solution in chloroform at −80°C. To prepare standard solutions of HNE, 10-μl aliquots were placed in a wide mouthed centrifuge tube and the chloroform was allowed to evaporate at room temperature. The residue was then vortex-mixed for 30 s with 2.5 ml of distilled water and the exact concentration determined spectrophotometrically at 223 nm using the molar absorptivity, $\epsilon = 13\,750$ [33]. The internal standard, [2,3-$^2H_2$]*trans*-4-hydroxy-2-nonenal ([2,3-^{2}H]HNE), was synthesised by the reaction of hexanal with propiolaldehyde diethylacetal magnesium bromide in anhydrous tetrahydrofuran at −5°C to give 4-hydroxy-2-nonynal diethyl acetal [33]. The triple bond was reduced by reaction with excess lithium aluminium deuteride in anhydrous diethyl ether at −20°C, followed by deuterolysis of the resulting lithium aluminium complex with deuterium oxide and finally saponification of the [2,3-$^2H_2$]*trans*-4-hydroxy-2-nonenal diethylacetal with dilute citric acid to [2,3-^{2}H]HNE. The final product was extracted with chloroform and purified by preparative HPLC using methanol–water (60:40, v/v) as the mobile phase at a flow-rate of 2 ml/min on a C_{18} reversed-phase column. The isotopic integrity of [2,3-^{2}H]HNE was confirmed by ^{1}H nuclear magnetic resonance spectroscopy (NMR) and by MS. The concentration of [2,3-^{2}H]HNE was determined spectrophotometrically after dissolution in distilled water [33]. Aliquots were then diluted with methanol–water (80:20, v/v) containing BHT (50 μg/ml) and stored at −80°C.

High-performance liquid chromatography

The HPLC system consisted of a Waters Model U6K injector, 6000A solvent delivery system, 480 spectrophotometer, 720 system controller and 730 data module (Waters Assoc., Milford, MA, U.S.A.). A Rainin C_{18} reversed-phase column, 250 mm × 4.6 mm I.D., particle size 5 μm (Rainin, Woburn, MA, U.S.A.) was used for the separation of HNE O-PFB oxime. A Newguard column (15 mm × 3.2 mm) (Brownlee Labs., Santa Clara, CA, U.S.A.) was used to protect the column. The mobile phase was 70% acetonitrile in distilled water at a flow-rate of 1 ml/

min. Before chromatography the mobile phase was filtered through a 5-μm filter (Millipore, Bedford, MA, U.S.A.) and degassed by sonication.

Gas chromatography–negative-ion chemical ionisation mass spectrometry

GC–NICIMS was performed using a VG Micromass 70-70 mass spectrometer (VG Analytical, Manchester, U.K.) interfaced to a Hewlett Packard 5792A gas chromatograph (Hewlett Packard, Avondale, PA, U.S.A.). A VG 11-250 data system was used to control the mass spectrometer and for data acquisition and processing. GC separations were achieved using a cross-linked fused-silica capillary column (BP-5, 5% phenylmethylsilicone), 25 m $\times$ 0.33 mm I.D., phase thickness 0.5 μm (Scientific Glass Engineering, Melbourne, Australia). A retention gap of deactivated vitreous silica (SGE, 2 m) was used to preserve the column's integrity and to accommodate larger than usual injections without significant loss of chromatographic resolution. The temperature of the column oven was programmed initially at 90°C for 4 min then increased at 25°C/min until a final temperature of 210°C was reached for elution of derivatised HNE. The carrier gas was helium at a flow-rate of 1 ml/min. A Hewlett Packard 19290A cool on-column injector was used. The mass spectrometer was operated in the NICI mode using ammonia as the reagent gas (pressure 60 Pa). The ionization energy was 100 eV and the filament emission current 500 μA. The source temperature was 200°C. Selected-ion monitoring (SIM) was accomplished by electrical cycling at constant magnetic field between masses *m/z* 303.08, 333.12 and 373.17 for the HNE derivative and *m/z* 306.09, 336.13 and 376.18 for the corresponding [2,3-^{2}H]HNE derivative (^{13}C isotope). The dwell times were 50 ms per mass channel.

Extraction and derivatisation

To 250 μl of biological material (see below) were added 5 μl of the BHT stock solution (to prevent the formation of additional HNE by oxidation during sample work-up) and 5 μl (50 ng) of the internal standard, and the mixture was vortex-mixed (3 min). O-PFB·HCl (2.5 mg) was weighed into a 10-ml glass-stoppered centrifuge tube and vortex-mixed (1 min) with 200μl of 1.5 *M* sodium acetate buffer, pH 5.0. The biological sample was then added and the mixture vortex-mixed (1 min), cooled in ice and sonicated for 30 s (MSE 100-W ultrasonic disintegrator, Measuring and Scientific Equipment, London, U.K.). The samples were allowed to stand at room temperature for 15 min and stored at −80°C until analysis. After thawing, 700 μl of ethyl acetate were added and the mixure was vortex-mixed and then centrifuged at 900 *g* (2 min). The upper ethyl acetate layer was removed with a pasteur pipette drawn out into a capillary and the mixture extracted with a further 700 μl of ethyl acetate. The ethyl acetate extracts were combined in a 5-ml glass-stoppered centrifuge tube and evaporated to dryness under a stream of nitrogen. The residue was extracted in 3 ml of distilled water by vortex-mixing (30 s) and then applied to a disposable C_{18} reversed-phase cartridge preconditioned with 3 ml of methanol and equilibrated with 5 ml of water. After washing the column with 3 ml of light petroleum (b.p. 40–60°C) to remove non-polar materials which could interfere with the analysis, the HNE O-PFB oxime was eluted with 3 ml of ethyl acetate into a 5-ml stoppered centrifuge tube

with a tapered base. The ethyl acetate was removed under a stream of nitrogen and the residue taken up in 25 μl of HPLC mobile phase by vortex-mixing and centrifuging. The injection volume into the HPLC system was 25 μl. Peak identification was achieved by reference to HPLC profiles of the O-PFB oxime derivative of synthetic HNE. Effluent fractions corresponding to HNE O-PFB oxime were collected in a 5-ml stoppered tube and extracted four times with 1 ml of pentane by vortex-mixing and centrifuging. The combined extracts were dried over anhydrous sodium sulphate and evaporated to a volume of 100 μl under a stream of nitrogen. The pentane extract was transferred to a small melting-point capillary tube and the remaining solvent removed carefully under a vacuum (water pump). The residue in the capillary tube was dissolved in 10 μl BSTFA and the tube sealed in a flame and heated at 70°C for 10 min to allow the formation of the TMS ether of the hydroxyl group of HNE. The derivatives were stored at −20°C until analysis. The silylation mixture was concentrated to 4–6 μl under a rotary pump vacuum and 2–5 μl aliquots were analysed by GC–NICIMS.

Nuclear magnetic resonance spectroscopy

The proton magnetic resonance spectra were obtained at 300°K on a Varian XL-200 NMR spectrometer (200.057 MHz) operated in the pulsed Fourier transform mode, 32K data points and a pulse width of 90°. Solutions of the HPLC-purified minor (0.5 m*M*) and major (1.0 m*M*) HNE O-PFB oxime isomers required 224 pulses for a signal-to-noise ratio suitable for analysis.

Quantification of standard HNE and biological extracts

HNE was quantified by comparison of the peak areas of the *m/z* 303 ion for the *syn*-isomer of HNE O-PFB oxime TMS ether with the *m/z* 306 ion of the *syn*-isomer (^{13}C isotope) of the internal standard O-PFB oxime TMS ether. Calibration curves were obtained by GC–NICIMS of standard mixtures produced by adding increasing amounts of HNE to constant amounts of the internal standard in distilled water (200 μl) to give $^2H_0/^2H_2$ ratios of 0.0, 0.05, 0.1, 0.2, 0.35, 0.5, 0.75, 1.0 and 1.5. The samples were extracted and derivatised as described above.

Biological material

Plasma and platelet-rich plasma were prepared by centrifugation of venous blood from healthy adult volunteers. Monocytes were isolated from human blood by centrifugal elutriation [34]. Lipoproteins from normolipidemic individuals were prepared from freshly drawn blood anticoagulated with citrate and ethylenediaminetetraacetic acid (EDTA). The plasma density was adjusted to 1.019 with a high-density salt solution (sodium chloride and potassium bromide, containing EDTA) [35]. After centrifugation the pooled infranatant was adjusted to $d=1.063$, recentrifuged and LDL ($d=1.019$–1.063 g/ml) collected. The LDL (200 μg protein per ml) was oxidised by incubation with 5 μ*M* copper sulfate in modified Tyrodes buffer without EDTA for 20 h at 37°C [36].

RESULTS AND DISCUSSION

The HPLC separation of the O-PFB oxime of synthetic HNE is shown in Fig. 2A. Two isomeric components were obtained with retention times of 14.5 and 15.5 min, respectively. The *syn/anti* orientation (Fig. 1) of the major and minor isomers was determined by NMR. The isomers of [2,3-^{2}H]HNE O-PFB oxime were separated by preparative HPLC on a C_{18} reversed-phase column. Separate analysis of each peak by 200 MHz ^{1}H NMR spectroscopy revealed that in the second, larger peak, the formyl proton adjacent to the oxime resonated significantly downfield from the corresponding proton from the first peak (δ 7.70 and 7.04 ppm, respectively). Based on an extensive study of NMR spectra of related compounds reported previously [37], the larger peak of retention time 15.5 min was assigned as the *syn*-isomer and the smaller peak of retention time 14.5 min as the *anti*-isomer.

An HPLC separation from a platelet extract is shown in Fig. 2B. The peaks due to [2,3-^{2}H]HNE O-PFB oxime were well separated from adjacent peaks allowing the material to be isolated from interfering contaminants. The amount of internal standard added (50 ng) was generally much greater than the endogenous HNE to ensure that the diagnostic paired peaks were detectable by HPLC and also to act as a carrier during the subsequent isolation procedures.

The NICI mass spectra of the *syn*- and *anti*-isomers of HNE O-PFB oxime TMS ether are shown in Fig. 3. The ion fragmentations probably arise from dissociative attachment of thermalised electrons to the molecules since qualitatively similar spectra were obtained when methane or isobutane was used as the reagent gas. The relative ion abundances are quite different for the geometrical isomers

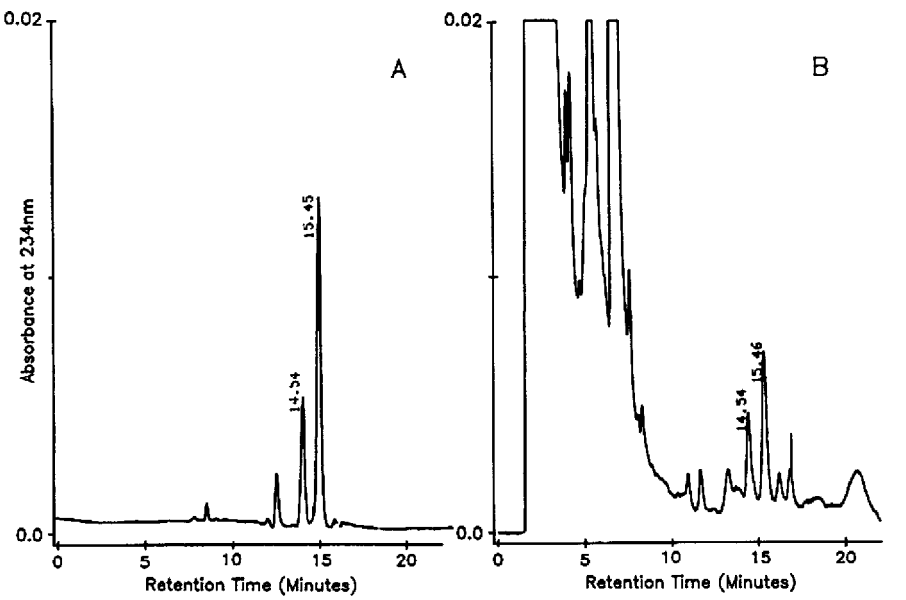

Fig. 2. HPLC separation of synthetic HNE O-PFB oxime (A) and HNE O-PFB oxime extracted from human platelets (B). The amount of [2,3-^{2}H]HNE added to the platelet extract was 50 ng.

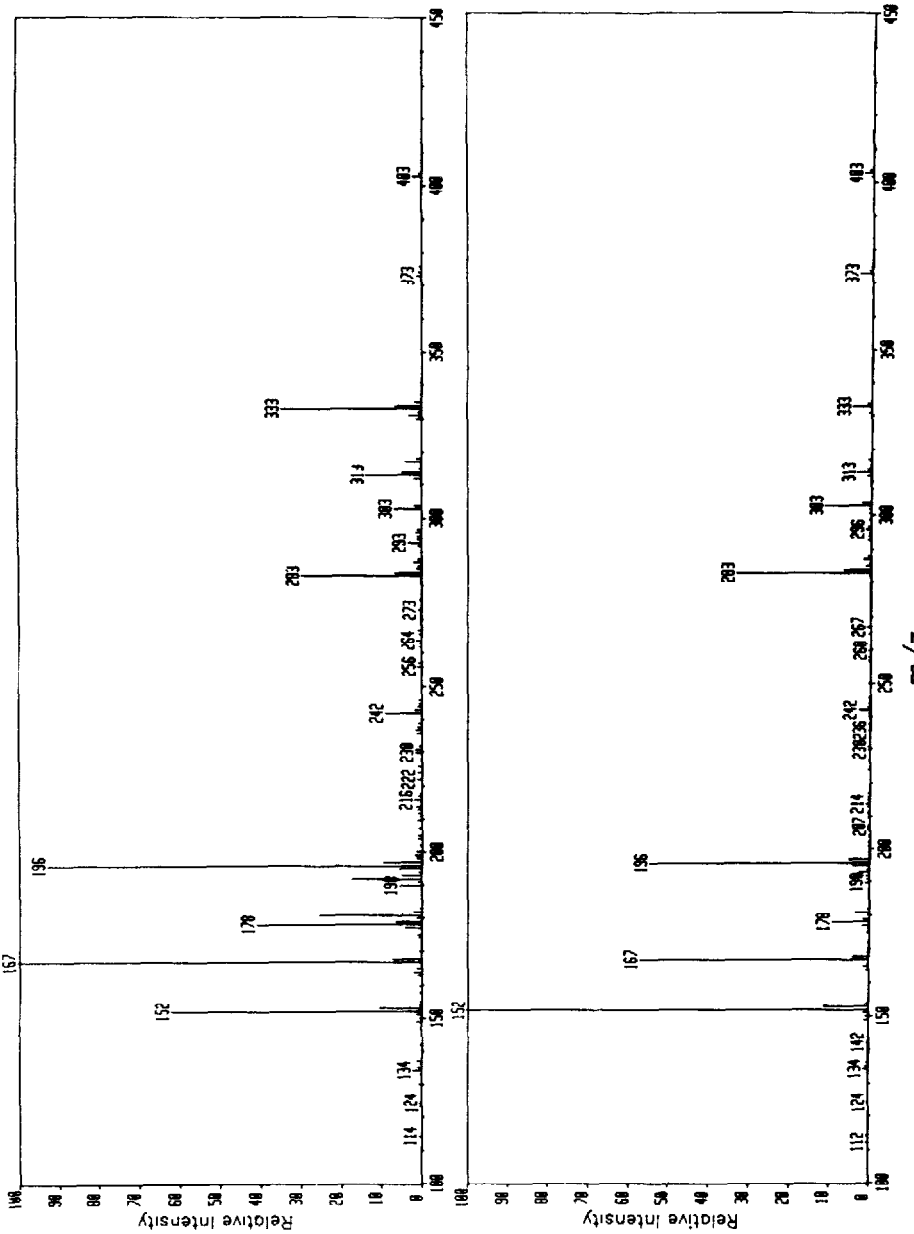

Fig. 3 Negative-ion chemical ionization mass spectra of the *syn-* (bottom) and *anti-* (top) isomers of synthetic HNE O-PFB oxime TMS ether (mol. wt. 423) using ammonia as the reagent gas.

and it is evident that the selected monitoring of appropriate ions can provide further dimensions of information for validating the identity of low levels of endogenous HNE.

Although the most abundant ions in the mass spectra have $m/z < 200$ (Fig. 3), the ions in the high-mass end of the spectra have much greater specificity and information content. Thus, ions of m/z 303, 333 and 373 were chosen for monitoring the elution of HNE O-PFB oxime TMS ether since these ions accentuate the differences in the mass spectra of the two geometrical isomers. Bearing in mind the unequal amounts of the two isomers (Fig. 2), typical ratios observed of the peak profiles of the *anti*- and *syn*-oximes for selected ions of m/z 303 were 0.25:1, for ions of m/z 333, 3.32:1 and for ions of m/z 373, 0.26:1. Illustrative peak profiles for derivatised HNE obtained from human platelets are shown in Fig. 4.

Because in general the amount of internal standard added greatly outweighed that of the endogenous HNE in the present experiments, the spectral signals for the former were attenuated by selecting those daughter ions arising from the naturally occurring ^{13}C-isomer of the derivatised [2,3-^{2}H]HNE, namely ions of m/z 306, 336 and 376. This strategy had the advantages of effectively inflating the spectral signals for small proportions of HNE and of diminishing any cross-talk between the mass channels because of competing ion reactions or isotopic contributions. Further, cyclical monitoring of the matrix of six mass channels demonstrated that the peak centroids for the deuterated internal standard preceded those distinguishing the unlabeled HNE O-PFB oxime TMS ether by 1–2 s, yielding a further diagnostic to validate the identity of the latter.

A typical calibration line for HNE using [2,3-^{2}H]HNE as an internal standard and performing SIM of the ions m/z 303 and 306 for the *syn*-isomers is shown in Fig. 5.

The precision of the method was determined by analysing 200-μl aliquots of the same oxidised LDL sample after the addition to each aliquot of 100 ng of [2,3-^{2}H]HNE. The concentration of HNE was 118.8 ± 5.36 ng/mg of protein ($n = 5$).

Quantitative data for platelets, monocytes and plasma are shown in Table I.

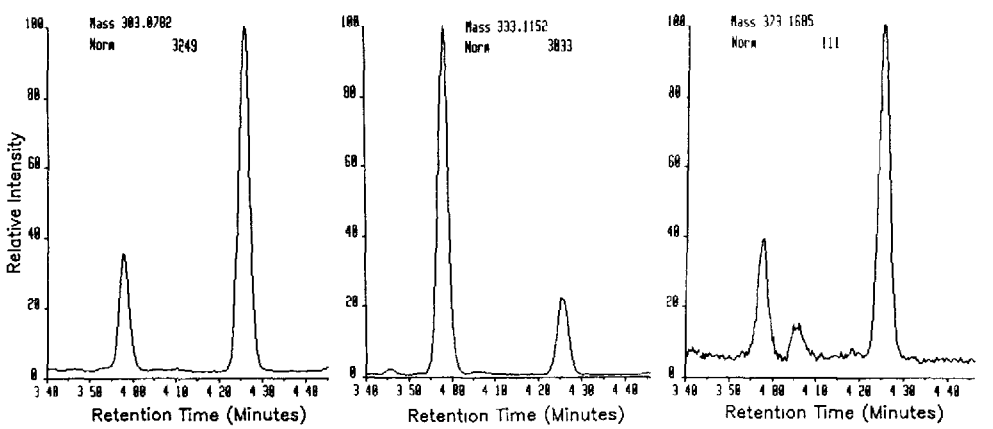

Fig. 4. Selected-ion monitoring profiles of HNE O-PFB oxime extracted from human platelets and derivatised as the TMS ether.

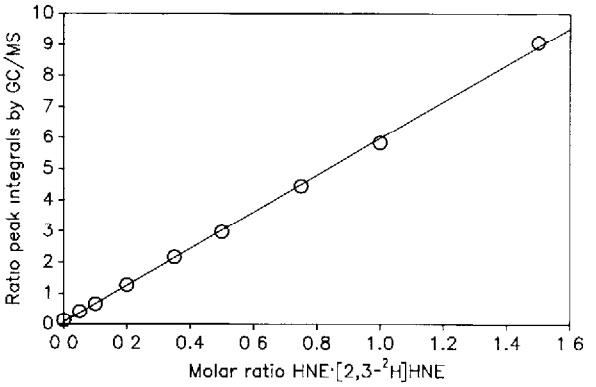

Fig. 5. Calibration line for the analysis of HNE.

TABLE I

HNE CONCENTRATIONS IN PLATELET-RICH PLASMA (PRP), MONOCYTES AND PLASMA OBTAINED FROM HEALTHY VOLUNTEERS

Sample	Concentration (mean ± S.D., $n=4$) (ng per 10^7 cells)
PRP stimulated[a]	1.3 ± 0.9
PRP control	1.4 ± 1.0
Monocytes stimulated[b]	50.5 ± 11.6
Monocytes control	61.2 ± 13.5
Plasma	106.3 ± 65.8 ng/ml

[a]Stimulus: arachidonic acid.
[b]Stimulus: phorbol myristate acetate.

The decreased production of HNE in monocytes stimulated with phorbol myristate acetate compared to the controls may indicate the presence of an HNE metabolising system. The presence of HNE in plasma confirms the existence of a state of chronic oxidant stress previously reported in normal humans [38].

A procedure based on HPLC and GC–NICIMS has been developed in the present work for the quantification of HNE in biological samples. The method has been used to determine the concentration of HNE in plasma, platelets, monocytes and oxidised LDL. It represents a considerable improvement over previous methods based on HPLC alone in terms of specificity and sensitivity down to 5 ng/ml and offers a new approach to studying the relationship between lipid peroxidation and pathology.

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