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Determination of the lipid peroxidation product (*E*)-4-hydroxy-2-nonenal in clinical samples by gas chromatography–negative-ion chemical ionisation mass spectrometry of the *O*-pentafluorobenzyl oxime

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Abstract

(*E*)-4-Hydroxy-2-nonenal (HNE) is a highly reactive product of the free radical-stimulated lipid peroxidation of phospholipid-bound arachidonic acid in cellular membranes. We describe a sensitive and specific method for the determination of HNE in clinical samples. The method is based on the formation of the *O*-pentafluorobenzyl (*O*-PFB) oxime derivative of HNE, which is then extracted and cleaned up by solid-phase extraction. The HNE *O*-PFB oxime is then analysed without further derivatisation by capillary column gas chromatography–negative ion chemical ionisation mass spectrometry (GC–NICI-MS) using selected-ion monitoring. Concentrations down to the pmol range were achieved using deuterated HNE as an internal standard. The method was used to determine HNE in the cerebrospinal fluid and plasma of patients with Parkinson's disease, the plasma of patients with HIV-1 infection and AIDS and in inflamed mucosal biopsy specimens from patients with inflammatory bowel disease.

Keywords: Lipids; (*E*)-4-Hydroxy-2-nonenol

1. Introduction

(*E*)-4-Hydroxy-2-nonenal (HNE) is one of the major products of lipid peroxidation [1], being formed almost exclusively by the peroxidation of membrane bound arachidonic acid [2]. HNE exhibits a variety of cytopathological effects, including enzyme inhibition, the inhibition of DNA, RNA and protein synthesis and the induction of heat shock proteins [3]. HNE is cytotoxic to many cell types, including hepatocytes, mammalian fibroblasts and Ehrlich ascites tumour cells [3]. HNE also induces genotoxic and mutagenic effects and inhibits cell proliferation [3]. It is believed that HNE exerts these effects because it is a highly electrophilic agent with reactivity towards molecules with sulphydryl and amino groups, e.g. proteins [3]. HNE may be a "second toxic messenger" of lipid peroxidation in cellular membranes [3] and it is considered to be the most reliable index of lipid peroxidation [4].

HNE has been implicated in the pathology of a number of diseases, including rheumatoid arthritis [5], inflammatory bowel disease [6], male infertility [7], atherosclerosis [8], thrombosis [9] and cancer [10]. Since HNE exerts many of its biological effects at micromolar concentrations [3], the rapid, sensitive

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and specific determination of this highly reactive molecule is of the utmost importance.

We reported previously the development of a method that combined capillary column gas chromatography–negative ion chemical ionisation mass spectrometry (GC–NICI-MS) for the determination of HNE in biological fluids [11]. The method was based on the formation of the *O*-PFB oxime derivative of HNE and its deuterated internal standard, which, after sample clean-up by solid-phase extraction and purification by HPLC, were derivatised further to trimethylsilyl ethers. Subsequent capillary column GC–NICI-MS, using selected-ion monitoring, allowed quantitation in the low nmol/ml range [11].

Several other GC–MS methods for the determination of HNE have been described recently in the literature using electron impact rather than chemical ionisation. Three methods used the oxime bis-*tert.*-butyldimethylsilyl ether derivative of HNE [12–14]. The other method involved the reduction of HNE to the corresponding alcohol with sodium borohydride followed by the formation of the *tert.*-butyldimethylsilyl (TMS) ether derivative [15].

While all of these GC–MS methods are sensitive and specific, they all suffer from the disadvantage that they require two derivatisation steps and are time-consuming and cumbersome. We report here the development of a more rapid and convenient GC–NICI-MS technique that dispenses with the preliminary HPLC purification of the HNE *O*-PFB oxime and the subsequent need to derivatise further to the TMS ether [11], while losing none of the selectivity and sensitivity of the original method. The method has been used to determine HNE in the cerebrospinal fluid and plasma of patients with Parkinson's disease, the plasma of patients with HIV-1 infection and AIDS and mucosal biopsy specimens from patients with inflammatory bowel disease.

2. Experimental

2.1. 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, USA), ethyl acetate (nanograde) and chloroform (nanograde) were from Mallinckrodt (Paris, KY, USA) and acetonitrile (Unichem) was from Ajax Chemicals (Auburn, Australia). BHT was used as a freshly prepared solution in methanol (10 mg/ml). Disposable reversed-phase cartridges (C_{18} Sep-Pak) were purchased from Waters Associates (Milford, MA, USA). Distilled water was purified by passage through a Barnstead Nanopure system (Barnstead, Boston, MA, USA) and then doubly redistilled in glass. HNE was synthesised by the method of Gardner et al. [16] and stored as a solution in chloroform at −80°C. Standard solutions of HNE were prepared by placing 10-μl volumes in a wide-mouthed centrifuge tube and allowing the chloroform 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 was determined spectrophotometrically at 223 nm using the molar absorptivity, $\epsilon = 13\,750$ [15]. The internal standard, (*E*)-4-hydroxy-9,9,9-trideutero-2-nonenal (deuterated HNE) was synthesised as described by Rees et al. [17] and the concentration was determined spectrophotometrically after dissolution in distilled water [18]. Aliquots of deuterated HNE were diluted with methanol–water (80:20, v/v) containing BHT (50 μg/ml) and stored at −80°C under nitrogen.

2.2. Extraction and derivatisation

To 250 μl of plasma or cerebrospinal fluid or to mucosal tissue suspended in 250 μl of phosphate-buffered saline (~1 mg protein/ml) were added 5 μl of the BHT stock solution and 10 μl (100 ng) of deuterated HNE 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 200 μl of 1.5 *M* sodium acetate buffer, pH 5.0. The biological sample was then added and the mixture was vortex-mixed (1 min). Tissue samples were cooled in ice and sonicated for 30 s (MSE 100-W ultrasonic disintegrator, Measuring and Scientific Equipment, London, UK). The samples were allowed to stand at room temperature for 15 min and then were stored at −80°C until analysis. The mixture was thawed and 1 ml of ethyl acetate was added and the mixture was vortex-mixed and then centrifuged at 900 *g* (2 min). The lower

aqueous layer was frozen by immersing the bottom of the centrifuge tube in a mixture of dry ice–acetone and the ethyl acetate extract was decanted into 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 that had been preconditioned with 3 ml of methanol and washed with 5 ml of water. The column was washed with 3 ml of light petroleum (b.p. 40–60°C) to remove non-polar materials and 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 evaporated under a stream of nitrogen and the residue was dissolved in 25 μl of acetonitrile. The derivative was kept at −80°C until analysis by GC–MS. The injection volume onto the GC column was 5 μl.

2.3. Gas chromatography–mass spectrometry

GC–NICI-MS was performed using a Fisons Trio 2000 Model gas chromatography–mass spectrometry system. GC separations were achieved using a cross-linked fused-silica capillary column (BP-5, 5% phenylmethylsilicone), 22 m×0.22 mm I.D., phase thickness, 1.0 μm (Scientific Glass Engineering, Melbourne, Australia).

Injections (5 μl) were made on-column and the oven was temperature-programmed after sample injection at 50°C for 1 min then 20°C/min to 180°C followed by 25°C/min to 300°C. The carrier gas was helium at a flow-rate of 1 ml/min. The temperature of the transfer line was 310°C. The mass spectrometer was operated in the NICI mode using methane as the reagent gas. The temperature of the ion source was 170°C and the electron energy was 70 V.

2.4. Quantification of HNE

Quantification of HNE was achieved by comparison of the peak areas of the *m/z* 331 ion for the *syn*-isomer of HNE *O*-PFB oxime with the corresponding *m/z* 334 ion of the internal standard, deuterated HNE *O*-PFB oxime. Calibration curves were prepared by the GC–NICI-MS of standard mixtures prepared by adding increasing amounts of HNE (1, 2.5, 5, 10, 25, 50 and 100 ng) to 100 ng of the internal standard in distilled water (250 μl). The samples were extracted and derivatised as described in Section 2.2. The precision of the method was studied by submitting replicate plasma, cerebrospinal fluid (CSF), or mucosal tissue samples to the entire extraction and derivatization procedure. The intra-day precision was obtained by injecting the derivatised extract ten times on the same day. The inter-day precision was obtained by making two injections daily for five consecutive days.

2.5. Patients

Cerebrospinal fluid (CSF) was obtained from four patients with Parkinson's disease (mean age 72.3±9.2 years) who were undergoing lumbar puncture for diagnostic purposes. Blood was taken by venipuncture and plasma was separated by centrifugation.

HIV-1 patients were seropositive, as determined by enzyme-linked immunosorbent assay (ELISA). The patients were classified as having asymptomatic HIV-1 infection (four male patients, mean age 41±3.9 years SEM) or AIDS (seven male and one female patient, mean age 43.1±4.1 years) by the Centre for Disease Control criteria. The control subjects were ten healthy HIV-1 seronegative males, mean age 41.3±7.3 years. Mucosal biopsy specimens were taken from four patients (mean age 42.1±7.3 years) with inflammatory bowel disease who were undergoing routine colonoscopy. Inflamed tissue was taken from the distal colon and control tissue from the uninvolved proximal colon. Tissue specimens were dropped into liquid nitrogen and stored at −80°C prior to analysis.

3. Results

The NICI mass spectra of the *syn*-isomer of HNE *O*-PFB oxime is shown in Fig. 1. The relative ion abundance for the *syn*- and *anti*-isomers was similar.

The ions of *m/z* 331, 301 and 283 were chosen for the selected-ion monitoring (SIM) of the elution of HNE *O*-PFB oxime and the ions of *m/z* 334, 304 and 286 for the corresponding deuterated HNE *O*-PFB oxime.

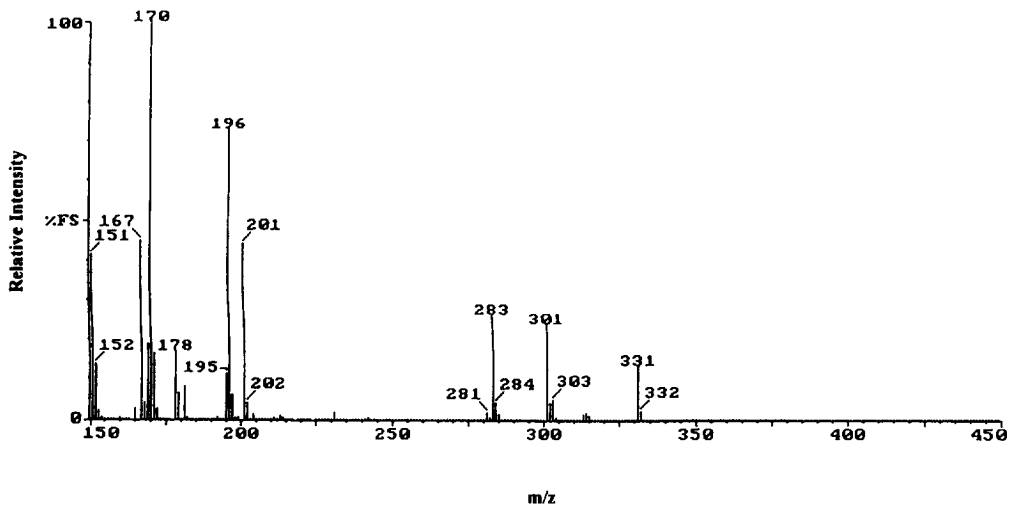

Fig. 1. Negative ion chemical ionisation mass spectrum of the *syn*-isomer of synthetic HNE *O*-PFB oxime using methane as the reagent gas.

The ion of *m/z* 331 would result from the loss of HF from the (vestigial or absent) molecular anion at *m/z* 351. The ion of *m/z* 301 would result from the analogous loss of CF_2 from the molecular anion. The ion of *m/z* 283 would result from the consecutive losses of CF_2 and H_2O from the molecular anion.

In the NICI mass spectrum of the analogous TMS derivative of HNE *O*-PFB oxime reported previously [11], there are analogous ions to *m/z* 331 and 301 at *m/z* 423 and *m/z* 373 (loss of HF and CF_2, respectively, from the molecular anion of *m/z* 423). However, there is no ion corresponding to that of *m/z* 283 in the spectrum of HNE *O*-PFB oxime, since there is no remaining hydroxyl group to enable the consecutive step for loss of H_2O.

The gas chromatographic separation of the *O*-PFB oxime of synthetic HNE gave two isomeric peaks with retention times of 11.59 and 11.75 min, respectively. The peak profiles arising from the monitoring of the ions *m/z* 331, 301 and 283 were approximately twice as large for the *syn*-oxime than for the *anti*-oxime. A similar phenomenon was reported previously for HNE *O*-PFB oxime TMS ether [11]. Accordingly, the *syn*-oxime was chosen for the quantitative determination of HNE as the *O*-PFB oxime. The selected ion chromatogram at *m/z* 331 for HNE *O*-PFB oxime extracted from the plasma of a patient with AIDS is shown in Fig. 2.

The calibration line over the concentration range 1–100 ng/ml of HNE using 100 ng of deuterated HNE as the internal standard and performing SIM of the ions *m/z* 331 and 334, respectively, was linear (the correlation coefficient was 0.9984, $n=10$). The limit of detection at a signal-to-noise ratio of three was 0.1 ng/ml. Mean recoveries from the extraction and derivatisation procedure were 96.5±1.8% at 50 ng/ml; 98.5±12.2% at 10 ng/ml and 97.6±14.2% at 1 ng/ml ($n=10$).

The within-day and day-to-day precision of the method was determined by the analysis of separate 200 μl volumes of a plasma sample from an AIDS patient, a CSF sample from a patient with Parkinson's disease and a mucosal biopsy specimen from a patient with inflammatory bowel disease, after the addition of 100 ng of deuterated HNE to each sample. In plasma, the intra-day concentration of

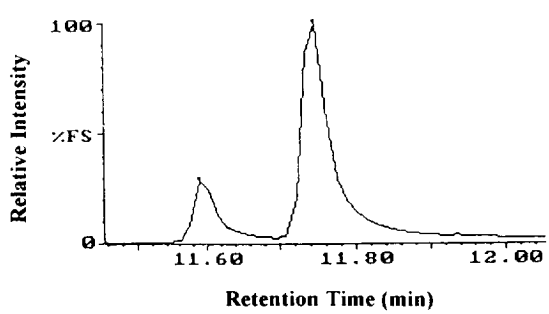

Fig. 2. Selected-ion monitoring profile at *m/z* 331 for HNE *O*-PFB oxime extracted from the plasma of a patient with AIDS.

Table 1
HNE concentrations in the plasma of patients with Parkinson's disease

Patient	Disease duration (years)	Concentration (nmol/ml)	
		CSF	Plasma
1	2	0.20	0.71
2	3	1.39	2.71
3	6	2.41	5.01
4	8	3.14	6.03

HNE was 1.27±0.07 nmol/ml ($n=10$) and the inter-day concentration of HNE was 1.31±0.09 nmol/ml ($n=10$). In CSF, the intra-day concentration of HNE was 12.34±0.12 nmol/ml ($n=10$) and the inter-day concentration of HNE was 13.14±0.17 nmol/ml ($n=10$). In inflammed mucosal tissue, the intra-day concentration of HNE was 3.12±0.23 nmol/mg protein ($n=10$) and the inter-day concentration of HNE was 2.96±0.19 nmol/ng protein ($n=10$).

Table 1 shows the concentration of HNE in the CSF and plasma of patients with Parkinson's disease. The concentration of HNE in the CSF was around half of that in the plasma. The concentration of HNE in the plasma or CSF increased with the number of years following diagnosis.

The concentration of HNE in the plasma of patients with asymptomatic HIV-1 infection and with AIDS compared to healthy age-matched control subjects is shown in Table 2. There was an elevation in the HNE content of the plasma from patients with asymptotic HIV-1 infection and a further increase in the plasma of AIDS patients.

In Table 3 the concentration of HNE in inflammed mucosal biopsy specimens and control tissue is shown. There was a large increase in the concentration of HNE in the inflamed tissue.

Table 2
HNE concentrations in the plasma of patients with asymptomatic HIV-1 infection and with AIDS compared to healthy age-matched control subjects

Sample	n	Concentration (nmol/ml)
Control	10	0.66±0.06
HIV-1	4	0.81±0.07[a]
AIDS	6	1.24±0.18[b]

[a] vs. control; $p<0.05$.
[b] vs. AIDS; $p<0.01$

Table 3
HNE concentrations in mucosal biopsy specimens from patients with inflammatory bowel disease

Sample	Concentration (nmol/mg protein)	
	Inflamed	Control
1	1.31	0.02
2	3.76	0.03
3	2.43	0.10
4	7.99	0.23
5	1.25	0.07
6	4.78	0.03

4. Discussion

The GC–NICI-MS determination of HNE in clinical samples described here is more rapid and convenient than our original method [11]. It avoids both the cumbersome separation of the HNE *O*-PFB oxime derivative by HPLC and the need to derivatise further to the TMS ether prior to GC. The use of an internal standard with three deuterium labels on the terminal carbon atoms of HNE [17] avoided the possibility of proton exchange of the vinyl-perdeuterated analogue of HNE used in the original method [11].

Van Kuijk et al. [14] have reported the determination of HNE in oxidised low density lipoprotein by GC–NICI-MS of the *O*-PFB oxime TMS ether [14]. These workers used an ion of relatively low mass for quantitative SIM (m/z 152) and found it necessary to precipitate the excess *O*-PFB–HCl reagent by the slow addition of sulphuric acid. While the most abundant ions in the mass spectra of HNE *O*-PFB oxime were m/z 201, 196, 170, 167 and 151 (Fig. 1), we selected a higher mass ion (m/z 331) to ensure greater specificity and less interference from contaminants of lower molecular mass.

It has been suggested previously that free radicals may play a role in the pathology of Parkinson's disease [19]. The results obtained here suggest that the production of HNE by free radical-initiated lipid peroxidation may be linked to the progression of the disease. HNE reacts readily with glutathione [3] and glutathione is depleted in the substantia nigra of patients with Parkinson's disease [20].

The finding that there is a significant increase in the concentration of HNE in the plasma of patients with asymptomatic HIV-1 infection and a further

increase in the plasma of patients with AIDS is an important observation. It has been reported previously that an increase in lipid peroxidation occurs in HIV infection [21]. HNE is a powerful immunosuppressant [22] and may be involved in the pathology of HIV-1 infection and its progression to AIDS.

There has been speculation that HNE may be a potential mediator of inflammation in inflammatory bowel disease by acting as a chemoattractant to neutrophils [23]. The significant concentration of HNE found in inflamed mucosal tissue adds weight to this hypothesis.

References

[1] A. Benedetti, M. Comporti and H. Esterbauer, Biochim. Biophys. Acta, 620 (1980) 281.
[2] H. Esterbauer, A. Benedetti, J. Lang, R. Fulceri, G. Fauler and M. Comporti, Biochim. Biophys. Acta, 876 (1986) 154.
[3] H. Esterbauer, R.J. Schaur and H. Zollner, Free Radic. Biol. Med., 11 (1991) 81.
[4] C. Goldring, A.F. Casini, E. Maellaro, B. Del Bello and M. Comporti, Lipids, 28 (1993) 141.
[5] M.L. Selley, D.J. Bourne, M.R. Bartlett, K.E. Tymms, A.S. Brook, A.M. Duffield and N.G. Ardlie, Ann. Rheum. Dis., 71 (1992) 481.
[6] C.F. Babbs, Free Radic. Biol. Med., 13 (1992) 169.
[7] M.L. Selley, M.J. Lacey, M.R. Bartlett, C.M. Copeland and N.G. Ardlie, J. Reprod. Fertil., 92 (1991) 291.
[8] M.E. Rosenfeld, J.C. Khoo, E. Miller, S. Pathasarathy, W. Palinski and J.L. Witztum, J. Clin. Invest., 87 (1991) 90.
[9] M.L. Selley, J.A. McGuiness, L.A. Jenkin, M.R. Bartlett and N.G. Ardlie, Thromb. Haemost., 59 (1988) 143.
[10] S. Toyokuni, K. Uchida, K. Okamoto, Y. Hattori-Nakakuki, H. Hiai and E.R. Stadtman, Proc. Natl. Acad. Sci. U.S.A., 91 (1994) 2616.
[11] M.L. Selley, M.R. Bartlett, J.A. McGuiness, A.J. Hapel, N.G. Ardlie and M.J. Lacey, J. Chromatogr., 488 (1989) 329.
[12] C. Norsten-Höög and T. Cronholm, Anal. Biochem., 189 (1996) 131.
[13] M. Kinter, S. Sullivan, R.J. Roberts and D. Spitz, J. Chromatogr., 578 (1992) 9.
[14] F.J.G.M. van Kuijk, A.M. Siakotos, L.G. Fong, R.J. Stephens and D.W. Thomas, Anal. Biochem., 234 (1995) 420.
[15] C. Des-Rosiers, M.J. Rivest, M.-J. Boily, M. Jetté, A. Carrobé-Cohen and A. Kumar, Anal. Biochem., 208 (1993) 161.
[16] H. Gardner, R.J. Bartelt and D. Weisleder, Lipids, 27 (1992) 686.
[17] M.S. Rees, F.J.G.M. van Kuijk, R.J. Stephens and B.P. Mundy, Syn. Commun., 23 (1993) 757.
[18] H. Esterbauer and W. Weger, Monatschr. Chem., 98 (1967) 1964.
[19] T. Yoshikawa, Eur. Neurol., 33 (Suppl. 1) (1993) 60.
[20] E. Sofia, K.W. Lanse, K. Jellinger and P. Riederer, Neurosci. Lett., 142 (1992) 128.
[21] A. Sönnerborg and C. Jarstrand, Scand. J. Infect. Dis., 20 (1988) 287.
[22] L. Tessitore, L. Matera, G. Bonelli, F.M. Baccino and M.U. Dianzani, Chem.-Biol. Interact., 62 (1987) 217.
[23] C.F. Babbs, Free Radic. Biol. Med., 13 (1992) 169.