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OCCURRENCE OF 4-HYDROXYALKENALS IN RAT TISSUES DETERMINED AS PENTAFLUOROBENZYL OXIME DERIVATIVES BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Frederik J. G. M. van Kuijk*, David W. Thomas**,
Robert J. Stephens**, and Edward A. Dratz+

Chemistry Department, Division of Natural Sciences
University of California, Santa Cruz, CA 95064

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Malondialdehyde measurements have been the major tool for studying relationships between lipid peroxidation and tissue pathology. Recently, we presented a novel gas chromatography-mass spectrometry method for direct detection of phospholipid peroxides with picogram sensitivity based on transesterfication of phospholipids or triglycerides to form pentafluorobenzyl esters. Under some circumstances the reactive primary oxidation products break down. Therefore, we developed a convenient, high sensitivity method to detect more stable secondary lipid oxidation products, the 4-hydroxyalkenals. The method accomplishes a facile extraction of 4-hydroxynonenal from tissues by forming pentafluorobenzyl oxime derivatives to displace aldehydes from Schiff base linkages. 4-hydroxynonenal was found in heart, liver, adrenal, and testis from rats and was detected to the 10-100 pg level by the current method.

4-Hydroxynonenal has been reported to be a specific chemical indicator for lipid peroxidation in rat liver microsomes (1). The 4-hydroxyalkenals elicit a variety of powerful biological activities, such as inhibition of enzymes (2), inhibition of calcium sequestration by microsomes (3), inhibition of DNA biosynthesis (4). and high reactivity toward molecules with sulphydryl groups such as cysteine, glutathione, and proteins containing SH groups (5-7)

4-Hydroxynonenal was the major aldehyde formed during peroxidation of linoleic, γ-linolenic and arachidonic acid, as well during peroxidation of liver microsome membranes (7). Recently 4-hydroxynonenal has been described as a decomposition product of the 13-hydroperoxide of linoleic acid (8). In most of the previous studies (1,7,9) aldehydes were detected in biological systems by forming 2,4-dinitrophenyl hydrazone derivatives, followed by analysis with a combination of thin layer chromatography (TLC) and high performance liquid

* Current address: Biochemistry Department Medical Faculty, University of Nijmegen, PO Box 9101, 6500 HB, Nijmegen, Netherlands.

** Life Sciences Division, Cell Biology Program, SRI International, Menlo Park CA 94025.

+ Correspondence and reprint requests to current address: Department of Chemistry, Montana State University, Bozeman MT 59717.

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chromatography (HPLC) (7). Recently Lang et al. published a HPLC method for quantitative determination of free 4–hydroxynonenal to the 2 ngram level in biological samples, based on extraction of the aldehyde with dichloromethane and trapping on an Extralut column (10). After cleanup the samples were analysed by HPLC. This method relies on the extraction of free aldehydes, however aldehydes are difficult to extract from tissues since they tend to become covalently bound in Schiff bases (11). Therefore we obtained high extraction efficiency for 4–hydroxyalkenals in biological systems by formation of a derivative analogous to our use of O–ethyloximes for determination of retinoids by HPLC (12). Our procedure is based on the use of O–(2,3,4,5,6–pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA.HCl), to form the O–pentafluorobenzyl oxime derivatives of 4–hydroxyalkenals, followed by formation of trimethylsilyl (TMS) ethers of the hydroxyls. These derivatives also provided high sensitivity for detection by GC–MS with negative ion chemical ionization (NICI). The method was developed using synthetic 4–hydroxyalkenals and was found to be useful for determination of physiological levels of 4–hydroxynonenal in a variety of rat tissues.

MATERIAL & METHODS

Reagents

Chemically synthesized 4–Hydroxynonenal and 4–Hydroxyhexenal were gifts from Prof. H. Esterbauer, Department of Biochemistry, University of Graz, Graz, Austria. O–(2,3,4,5,6–Pentafluorobenzyl)hydroxylamine hydrochloride was obtained from Aldrich Chemical Co. (Milwaukee, WI). N.O–Bis(trimethylsilyl)tri–fluoroacetamide (BSTFA) +1% trimethylchlorosilane (TMCS) was obtained from Regis Chemical Co. Dichloromethane and methanol were HPLC grade (Fisher). PIPES buffer was obtained from Sigma (St Louis, MO,).

Preparation of samples for GC–MS

Standard solutions of 4–hydroxyalkenals were stored in dichloromethane containing 50 ug/ml butylated hydroxy toluene (BHT) at –90C. For derivatization, 200 ul methanol was added and the dichloromethane was evaporated under a stream of nitrogen. If the dichloromethane is evaporated without added methanol, it is possible to loose 4–hydroxyalkenals at this step, because of their volatility. A 200 ul aliquot of 0.1 M PIPES buffer containing 0.05 M PFB hydroxylamine hydrochloride was added to 200 ul methanol containing up to 1 mg 4–hydroxyalkenal. The mixture was vortexed and incubated for 5 min at room temperature. After incubation, 1 ml hexane was added, the mixture was vortexed for 1 min and centrifuged at 1000 g for 1 min. The hexane upper layer was collected, and the extraction was repeated twice. The pooled hexane fractions were evaporated under a stream of nitrogen after which 50 ul of BSTFA reagent was added. The samples were incubated for 5 min at 80 °C to form TMS ethers of the hydroxyls. Derivatives were stored at –20 °C until analysis by GC–MS.

Extraction of 4–hydroxyalkenals from tissues

Tissue samples up to 100 mg were homogenized in a mixture of 400 ul methanol containing 50 ug/ml BHT, 200 ul of a 2 mM EDTA buffer, pH 7.0, and 200 ul 0.1 M PIPES containing 50 mM PFB hydroxylamine. Samples were incubated at room temperature for 5 min, extracted with hexane and processed as described above. If tissues were extracted that are rich in tryglicerides, a different procedure was followed. The pooled hexane fractions were evaporated, dissolved in 0.5 ml acetonitrile, and vortexed for 1 min. The upper phase was

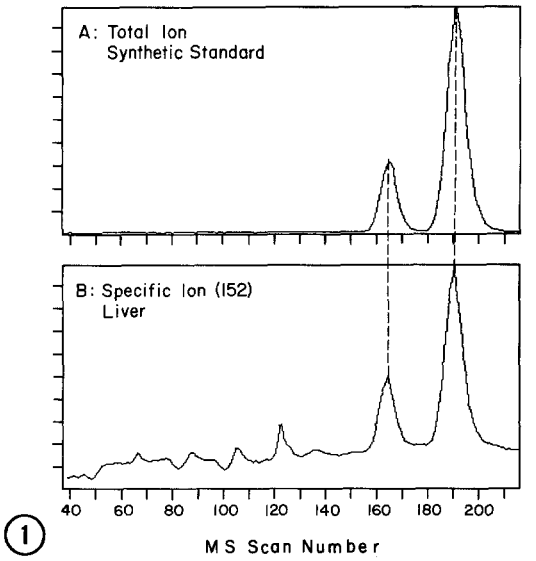
collected and filtered through a 0.2 um Teflon filter. The filtrate was evaporated under nitrogen, the samples dissolved in silylation grade pyridine and mixed with BSTFA to form TMS ethers, as described above.

Gas Chromatography–Mass Spectrometry

GC–MS analysis were obtained using a Ribermag R10–10 C GC–MS system. Chromatography was carried out on a 5 meter DB–5 capillary column (J+W Scientific, Rancho Cordova CA) at 150 °C. A Ros glass needle, on column injector was used at 270 °C. Mass spectra were obtained by NICI with specific ion monitoring at 60 eV using ammonia reagent gas.

RESULTS

Fig. 1a shows a total ion GC/MS chromatogram of the O–PFB oxime, TMS ether derivative of authentic of 4–hydroxynonenal. The structure of this compound is shown in figure 2. The two peaks at scan numbers 165 and 191 in the total ion chromatogram (figure 1a) are due to the syn and anti stereoisomers, which are formed during the conversion of aldehydes into oximes. A full NICI mass spectrum of the O–PFB oxime TMS ether of 4–hydroxynonenal is shown in figure 3. The most abundant specific fragment 152 is the parent carbon chain with loss of the PFB (C7F5H2) group, as shown in figure 2 by a broken line, and loss of a silanol residue



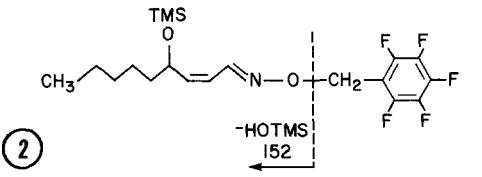


FIGURE 1. Negative ion chemical ionization GC–MS of the O–PFB oxime, TMS ether derivative of 4–hydroxynonenal. (a) total ion chromatogram of synthetic 4–hydroxynonenal, derivatized as described in the materials and methods section. (b) specific ion monitoring for M–C7F5H2–silanol at m/z = 152, after extraction of rat liver in the presence of PFBHA.HCl, and conversion of hydroxyls to the TMS ethers. The mass spectrometer performed 1 scan per 1.15 second.

FIGURE 2. The structure of the O–PFB oxime, TMS ether derivative of 4–hydroxynonenal. The broken line indicates were the molecule fragments to form the most abundant specific fragment, m/z =152, which also involves loss of the silanol group.

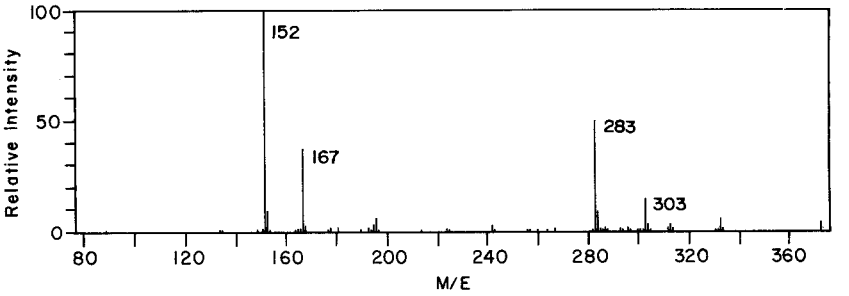


FIGURE 3. Negative ion chemical ionization mass spectrum of 4–hydroxynonenal O–PFB oxime TMS ether found in the gas chromatogram at scan number 192 of figure 1a. The major fragment 152 corresponds to M–C7F5H2–HOTMS.

(HOTMS), which indicates that the aldehyde has a hydroxyl function. The fragments 283, 303, 333, and 373 are specific for 4–hydroxynonenal, but less abundant.

4–hydroxyhexenal was analysed in a similar way, and the analogous fragment 110 was found to be the most abundant specific fragment for this compound. In addition 241, 261, 291, and 331 were found as less abundant specific fragments (data not shown). In both cases 167 was also detected, which is the most abundant non–specific fragment since it is obtained from the C6F5 group, that is lost from the O–PFB oxime as shown in figure 2.

Analysis of rat liver for 4–hydroxynonenal is shown in figure 1b using specific ion monitoring for mass 152. The syn and anti isomers of 4–hydroxynonenal are detected at the same scan numbers and in the same intensity ratio as are found in the authentic standards. Similar results were obtained when rat heart, adrenal, and testis tissues were analysed for 4–hydroxynonenal.

DISCUSSION

Lipid peroxidation has been measured most frequently by malonaldehyde determinations. Bird et al.(13) has reviewed various methods for malonaldehyde detection and their limitations. Malondialdehyde measurements have been of great value if approached with caution but there are many interferences and these methods are not capable of providing information on the other chemical species being produced. Recently, we presented GC–MS methods for direct detection of phospholipid peroxides, which are based on transesterfication to form either fatty acid methyl esters (14) or fatty acid PFB esters (15). The methyl ester method was sufficient to detect oxidized lipids in adipose storage depots of vitamin E deficient rats (van Kuijk, Thomas,

Stephens, and Dratz, unpublished results). The PFB ester method was developed to determine lipid peroxides in small biological tissue samples. It was found that the PFB derivative provided picogram sensitivity using mass spectrometer detection with NICI and specific ion monitoring. Since lipid peroxides tend to be quite short lived in biological systems we felt that our methods to detect phospholipid hydroperoxides should be complemented with a method to determine aldehyde secondary oxidation products.

Based on the use of PFB esters as a sensitive derivatization approach to detect phospholipid peroxides, it was found that an analogous PFB oxime derivative provided enhanced sensitivity for detection of compounds such as 4–hydroxyalkenals. The use of PFB oximes was first introduced by Koshy et al.(16) and Nambara et al.(17) for detection of ketosteroids with high sensitivity by electron capture gas chromatography. Organic aldehyde compounds tend to stick spontaneously to protein and lipid amino groups by Schiff's base linkages which form spontaneously. The presence of hydroxylamine during extraction cleaves the aldehyde Schiff base linkages with protein and lipid amino groups and therefore provides a quantitative extraction of these compounds from tissues (11). The PFB group in the oxime provided 20–100 times more sensitivity for the most abundant specific ion (152) by NICI detection in GC–MS, relative to detection for the most abundant ion (200) by EI. Each aldehyde compound is recovered as two peaks (the syn and anti isomers) on the GC column employed with characteristic relative intensity. Using this methodology we were able to detect 4–hydroxyalkenals at the 10–100 pg level. The paired syn/anti peaks are useful to confirm peak identification of unknown aldehydes when tissues are analysed. Separation of syn/anti isomers can be avoided by using more polar columns if desired (16).

4–hydroxyhexenal was also analysed by the same method as described above. The molecular weigt of all the specific fragments were 42 lower, due to the shorter alkyl chain. It is likely that the most abundant specific fragments of other 4–hydroxyalkenals can be calculated, simply by adding or substracting the appropriate number of CH2 groups.

The analysis as described above is semiquantitative. For quantitative analysis we have prepared deuterated internal standards to allow GC/MS quantitation as described for analysis of vitamin E (18–19). Based on extensive experience with extraction of retinoids, we assume that 4–hydroxyalkenals which are bound to tissue amines as aldimine Schiff bases will extract quantitatively from tissues by forming suitable oxime derivatives. At this point it was not

possible to determine if those unsaturated aldehydes which are bound to –SH groups of proteins (5–7) are released. Presumably, the O–PFB oxime is formed, but it is not clear whether thioether linkages binding the 4–hydroxynonenal to proteins will be hydrolysed under these conditions. Answering this important question must also await the availability of isotopic internal standards. The method presented provides a sensitive new route to study physiological levels of 4–hydroxyalkenals in small tissue samples, and for investigating their effects on cellular metabolism. Studies of the occurrence of 4–hydroxyalkenals in vitamin E deficient and supplemented animals are under investigation.

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REFERENCES

1 Esterbauer, H., Cheeseman, K.H., Dianzani, M.U., Poli, G., and Slater, T.F. (1982) Biochem. J. 208, 129–140.
2 Schauenstein, E., (1967) J. Lipid Res. 8, 417–428.
3 Benedetti, A., Fulceri, R., and Comporti, M. (1984) Biochim. Biophys. Acta 793, 489–493.
4 Seeber, S., Warnecke, P., and Weser, K. (1969) Z. Krebsforsch. 72, 137–143.
5 Esterbauer, H., Zollner, H., And Scholz., N. (1975) Z. Naturforsch. 30c, 466–473.
6 Esterbauer, H., Erlt, A., and Scholz, N. (1976) Tetrahedron 32, 285–289.
7 Esterbauer, H. (1982) in Free Radicals, Lipid Peroxidation and Cancer (McBrien, D.C.H., and Slater, T.F., eds.) pp. 101–128, Academic press, London.
8 Winkler, P., Lindner, W., Esterbauer, H., Schauenstein, E., Schauer, R.J., and Khoschsorur G.A. (1984) Biochim. Biophys. Acta 796, 232–237.
9 Poli, G., Diazani, M.U., Cheesman, K.H., Slater, T.F., Lang, J., and Esterbauer, H. (1985) Biochem. J. 227, 629–638.
10 Lang, J., Celotto, C., and Esterbauer, H. (1985) Anal. Biochem. 150, 369–378.
11 Groenendijk, G.W.T., de Grip, W.J., and Daemen, F.J.M. (1980) Biochim. Biophys. Acta. 617, 430–438.
12 van Kuijk, F.J.G.M., Handelman, G.J., and Dratz, E.A. (1985) J. Chromatogr. 348, 241–251
13 Bird, R.P., and Draper, H.H. (1984) In: Methods in Enzymology, volume 105 (Packer, L.A. ed.) pp. 299–305, Academic Press, Orlando.
14 van Kuijk, F.J.G.M., Thomas, D.W., Stephens, R.J., and Dratz, E.A. (1986) J. Free Rad. Biol. Med. 1, 215–225.
15 van Kuijk, F.J.G.M., Thomas, D.W., Stephens, R.J., and Dratz E.A. (1986) J. Free. Rad. Biol. Med. 1, 387–393.
16 Koshy, K.Y., Kaiser, D.G., and van der Slik, A.L. (1975) J. Chromatogr. Sci. 13, 97–104.
17 Nambara, T., Kigasawa, K., Iwata, T., and Ibuki, M. (1975) J. Chromatog. 114, 81–86.
18 Buntman, D.J., Thomas, D.W., Parkhurst, R.M., Negi, D.S., and Stephens, R.J. (1984) Microchem. J. 29, 66–73.
19 Thomas, D.W., Parkhurst, R.M., Negi, D.S., Luman, K.D., Wen, A.C., Brandt, A.E., and Stephens, R.J. (1981) J. Chromatog. 225, 433–439.