Toxic aldehydes formed by lipid peroxidation I. Sensitive, gas chromatography-based stereoanalysis of 4-hydroxyalkenals, toxic products of lipid peroxidation

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Abstract

An efficient analytical method is presented that does not only allow to detect and quantify 4-hydroxyalkenals, but for the first time provides a tool to look at the enantiomeric ratio of these interesting lipid peroxidation products. It involves acetylation as the only derivatization step, which can be carried out under mild conditions with acetic anhydride and gas chromatography on a chiral permethyl cyclodextrin phase. All biologically important homologues (C_5–C_9) can be selectively observed in a single chromatographic run. The resolution allows a reliable quantification of the enantiomers. The method was successfully applied in the stereoanalysis of 4-hydroxynonenal formed in rat liver microsomes after treatment with ADP/Fe^{2+}.

1. Introduction

Lipid peroxidation (LPO) has been suggested to play an important role in the pathogenesis of several diseases [1,2]. This free radical mediated process was studied *in vivo* [3] as well as in

Fig. 1. Structures of naturally occurring 4-hydroxyalkenals 1a–e and of the novel internal standard 2.

model systems like liver microsomes [4], isolated hepatocytes [5], lipid vesicles [6] and isolated unsaturated fatty acids [7]. Although the whole process is far from being completely understood, it is generally accepted that LPO involves formation of unstable lipid hydroperoxides as a first step [1]. These intermediates rapidly decompose to form a vast variety of mostly aldehydic secondary products. The most prominent representatives are malonic dialdehyde, *n*-hexanal and 4-hydroxynon-2-enal (1e) [4,7] (see Fig. 1).

Compound 1e and the other hydroxyalkenals 1a–d have been observed first in rat liver microsomes after initiation of LPO with ADP/Fe^{2+} or CCl_4 [4]. They exhibit numerous toxic effects, which have been associated with their ability to react as cross-linkers of proteins [8] or DNA [9]. Hydroxyalkenals are known to covalently modify
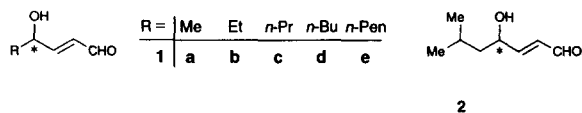
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low-density lipoprotein [10] and glucose-6-phosphatase [8] and to show chemotactic activity towards rat neutrophils [11].

Many analytical procedures have been developed for a sensitive and selective detection of hydroxyalkenals. Modern methods are mostly based on gas chromatography (GC), which is a powerful tool for the efficient analysis of volatile LPO products, such as 1a–e, especially if modern capillary columns are used. Derivatization at the carbonyl function or at the hydroxy group of these reactive compounds has been employed and serves three purposes simultaneously: it protects the sensitive analytes during the workup of biological samples, increases their volatility and enhances the sensitivity of detection. The so far most successful GC-based method employs a "tandem derivatization" including conversion of the carbonyl function into an O-pentafluorobenzyl oxime and trimethylsilylation of the hydroxy group [12]. If combined with sensitive mass-selective or electron-capture detection, it allows the analysis of hydroxyalkenals down to the picomolar concentration range.

The stereochemistry at C-4 of the chiral hydroxyalkenals is an interesting aspect, which has been completely neglected so far, even in studies concerning the biological activity of these toxic lipid peroxidation products, as well as their formation and metabolism. An *in vivo* occurrence of hydroxyalkenals in an optically active form would be a strong hint that enzymes are involved in the biological pathway of these compounds. Still, the established analytical methods do not permit to detect and separately quantify the enantiomers. In this paper we present a procedure for a sensitive and enantioselective analysis, which is based on resolution of R- and S-hydroxyalkenals on a chiral β-cyclodextrin stationary phase. The enantiomers were identified by comparison with material obtained by stereoselective total synthesis. Our analysis can be applied to determine the enantiomeric excess of 4-hydroxyalkenals in biological samples. For the reliable quantification of the analytes, a novel internal standard, the branched-chain hydroxyalkenal 2, has been developed.

2. Experimental

2.1. Chemicals

All reagents were of commercial quality. Organic solvents were dried and distilled prior to use. Deionized water was prepared with a Milli-Q appliance (Millipore, Bedford, MA, USA). Trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Machery–Nagel (Düren, Germany), heptafluorobutyric anhydride (HBFA), trifluoroacetic anhydride (TFA) and acetic anhydride were obtained from Aldrich (Milwaukee, WI, USA). Buffer salts were supplied by Merck (Darmstadt, Germany), biochemicals by Fluka (Buchs, Switzerland).

2.2. Synthesis

Hydroxyalkenals were prepared from commercially available 1,1-diethoxypropyne and the corresponding aliphatic aldehydes (ethanal–n-hexanal) (Aldrich), as published by Esterbauer and Wegner [13]. The new internal standard 4-hydroxy-6-methylhept-2-enal (2) was synthesized from 3-methylbutyric aldehyde analogously and was characterized spectroscopically. NMR spectra have been recorded on an AC 200 system from Bruker (Karlsruhe, Germany), IR spectra on a Perkin-Elmer 1420 IR spectrometer (Perkin-Elmer, Norwalk, CT, USA).

IR (film): $\nu = 3600–3100$ cm^{-1}, 3020–2700, 1680, 1620, 1460, 1380, 1360, 1260, 1120, 970, 730. ^{1}H NMR (C^{2}HCl$_3$, 200 MHz): $\delta = 0.93$ (d, $^{3}J = 6.6$ Hz, 3 H, CH$_3$), 0.98 (d, $^{3}J = 6.7$ Hz, 3 H, CH$_3$), 1.48 (t, $^{3}J = 7.1$ Hz, 2 H, 5-H), 1.61 [s(b), 1 H, OH], 1.70–2.02 (m, 1 H, 6-H), 4.51 (m$_c$, 1 H, 4-H), 6.33 (dd, $^{3}J_{2–3} = 15.5$ Hz, $^{3}J_{1–2} = 7.7$ Hz, 1 H, 2-H), 6.83 (dd, $^{3}J_{3–2} = 15.7$ Hz, $^{3}J_{3–4} = 4.7$ Hz, 1 H, 2-H), 9.59 (d, $^{3}J = 7.9$ Hz, 1 H, CHO).

Optically active 4S-1a and 4S-1e were obtained after reduction of a ketone precursor with S-BINAL-H, a chiral aluminum hydride [14]. The absolute configuration of the synthetic material has been elucidated by oxidative degradation to

the known corresponding α-hydroxycarboxylic acids [14].

2.3. Derivatization and calibration

For calibration, samples of the aldehydes **1a–e** (10, 5, 1, 0.5 and 0.1 mg) were dissolved in 300 ml of toluene–0.1 M triethylamine. Acetic anhydride (100 μl) was added and the solutions were kept in a sealed vial at 100°C for 3 h. After cooling to room temperature, the samples were extracted with phosphate buffer (pH 6.0, 0.5 M, 100 μl) and analyzed by GC. The other anhydrides were used analogously for derivatization. MSTFA was added neat, the samples were heated to 60°C for 30 min and analyzed without further purification.

2.4. Gas chromatography

GC was performed on a GC 2000 Vega Series 2 (Carlo Erba, Milan, Italy), equipped with a flame ionization detector, and on a 5890 Series II, equipped with a 5971 A mass-selective detector (Hewlett-Packard, Palo Alto, CA, USA). Data acquisition and processing were carried out with a Hewlett-Packard ChemStation software (1989 release). Separations were accomplished on a C-Dex-B fused-silica capillary column (30 m × 0.25 mm I.D., film thickness 0.25 μm) (J & W Scientific, Folsom, CA, USA), guarded with a retention gap consisting of uncoated fused silica (1 m × 0.52 mm). Helium was used as carrier gas at 100 kPa. Samples of 1 μl were injected on column. Temperature: 100°C (5 min), 2°C/min at 170°C (5 min).

2.5. GC–MS

The mass-selective detector was automatically tuned before processing sample analysis with perfluorotributylamine (PFTBA) as a standard. Electron impact ionization with 70 eV energy was used. Total ion chromatograms were recorded from 50 to 250 u, dwell time 100 ms. The characteristic fragments chosen for selected ion

Table 1
Characteristic fragments of acetylated hydroxyalkenals employed for MS–SIM detection

Compound	Fragments
1a	100, 71, 81
1b	114, 85, 81
1c	128, 99, 81
1d	142, 113, 81
1e	156, 127, 81

monitoring (SIM) of the aldehydes **1a–e** are given in Table 1.

2.6. Preparation and workup of liver microsomes

Liver microsomes were prepared from male Wistar rats according to literature procedures [15] and diluted in a buffered solution of 50 mM Tris–HCl, 5 mM MgCl$_2$ and 25 mM KCl (adjusted to pH 7.5). For calibration 100, 50, 10, 5 and 1 μg of **1e** and 25 μg of the internal standard **2** were added to 2.5 ml microsome suspension (equivalent to 1 g liver wet mass). The samples were diluted with water (17.5 ml), poured on a column charged with 10 g Chemtube hydromatrix (Varian, Harbor City, CA, USA) and extracted with dichloromethane (40 ml). The eluate was collected in a flask containing 0.1 M acetate buffer (pH 3.0, 2 ml). Dichloromethane was removed under reduced pressure. The residue was applied to a conditioned 200 mg RP-18 extraction column (J.T. Baker, Phillipsburg, PA, USA). The column was washed with cyclohexane (1 ml) and methanol (2 ml). The methanol fraction was evaporated to dryness and derivatized as described.

2.7. LPO experiments

For LPO experiments, 2.5 ml of microsome suspension were diluted with 17.5 ml of a NADPH-generating solution [16] (0.174 mM Na$_2$NADP, 37.2 mM nicotinamide, 4.3 mM glucose-6-phosphate, 6 U/ml glucose-6-phosphate dehydrogenase (EC 1.1.1.49)). LPO was

initiated by the addition of ADP (1.61 mM) and FeSO$_4$ (18 μM). The samples were incubated at 37°C for 30 min. Internal standard **2** (25 mg) was added prior to workup. Preparation, acetylation and analysis by GC–SIM was performed as described above.

3. Results and discussion

3.1. GC on chiral stationary phases

During the last years, a wide variety of chiral polysiloxane derivatives have become commercially available for use in GC. Amide and metal complex phases have been successfully employed to separate several chiral natural products like flavours or pheromones [17]. A third class of growing importance are cyclodextrin phases. Cyclodextrins are cyclic α-D-glucose oligomers, the hexa-, hepta- and octamers (α-, β- and γ-cyclodextrins) are of importance for GC applications. They can be employed as different derivatives (*e.g.* alkylated, acylated), either pure or dissolved in polysiloxanes, such as DB-1701 (7% phenylmethyl-, 7% cyanopropylmethyl-, 86% dimethylpolysiloxane). These composites combine the high stability and chemoselectivity of polysiloxanes with the stereoselectivity of cyclodextrins.

As we aimed at the simultaneous enantiomer separation of all the relevant hydroxyalkenals in one single chromatographic run, we had to find a stationary phase that was sufficiently selective for analytes of different polarity and volatility. The influence of the stereocentre on the overall physical properties of the 4-hydroxyalkenals decreases with increasing chain length, therefore the separation of R- and S-4-hydroxynonenal (**1a**) is especially challenging. Our first choice was a C-Dex-B capillary column, which is coated with a 10% solution of permethyl-β-cyclodextrin in DB-1701. This composite has been used for numerous applications in the literature [18], for analytes with different functional groups and a wide range of polarity.

3.2. Chromatography of 4-hydroxyalkenals **1**

Nearly all of the published procedures for a chromatographic analysis of 4-hydroxyalkenals include one or two derivatization steps. Most successful so far has been a "tandem derivatization" of the carbonyl function as a pentafluorobenzyl oxime and of the hydroxy group as a trimethylsilyl ether, which renders the analytes more volatile, more sensitive to detect and more stable during the workup procedure [12]. The non-derivatized hydroxyalkenals can rapidly react with nucleophiles like –NH and –SH groups in peptides and amino acids, which may cause a severe loss during the analysis in biological samples.

3.3. GC of 4-hydroxyalkenals **1** on a chiral phase without derivatization

The hydroxyalkenals **1a–e** are sufficiently volatile to be examined without derivatization on a permethylcyclodextrin stationary phase. We found, however, that their resolution is significantly influenced by the chain length. While hydroxypentenal (**1a**) could be separated easily, resolution of the non-derivatized longer-chain homologues could not be accomplished (see Table 2).

3.4. GC of 4-hydroxyalkenals on chiral column with derivatization

Derivatization of the carbonyl function was also found to be disadvantageous for a separation of the enantiomers. So we finally opted for transformation only of the polar OH group to obtain more volatile derivatives. These could be analyzed at lower temperatures when the cyclodextrins are less flexible and when their separation properties are consequently better. Furthermore, O-derivatization enabled us to modify the close neighbourhood of the stereocentre. Reagents of different steric bulk were examined to optimize chiral discrimination in the interaction between stationary phase and the analytes. For a first approach we focussed on

Table 2
Chromatography of derivatized 4-hydroxyalkenals on a chiral C-Dex-B stationary phase

Compound	Derivatization				
	$(H_3C)_3Si-$	$F_7C_3C(O)-$	$F_3CC(O)-$	$H_3CC(O)-$	H
1a	–	–	–	+	+
1b	n.d.	n.d.	–	+	(+)
1c	n.d.	n.d.	–	+	–
1d	n.d.	n.d.	–	+	–
1e	–	–	–	+	–

+ = Separation; (+) = partial separation; − = no separation; n.d. = not determined.

well established methods like trimethylsilylation with MSTFA and O-acylations with HFBA, TFA and acetic anhydride. The results of our chromatographic experiments are summarized in Table 2.

Only acetylation of the five hydroxyalkenals **1**, which could be accomplished selectively under
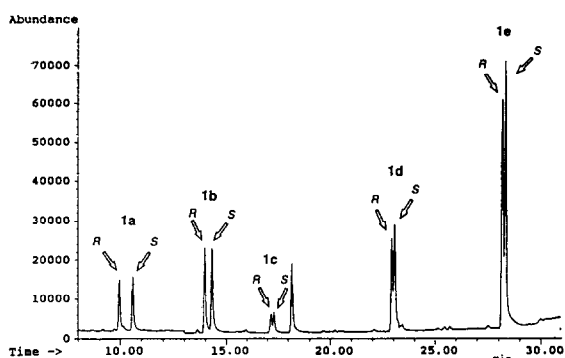

Fig. 2. Separation of derivatized R- and S-4-hydroxyalkenals by GC on permethyl cyclodextrin stationary phase.

mild conditions, led to derivatives that could be separated in one single chromatographic run (see Fig. 2). The resolution factors R_s even of the long-chain homologues are well above the limit (0.8–1) that is generally regarded as critical for secure quantification of the resolved peaks (see Table 3). The samples are stable for several days, if they are stored at $-18°C$ and water is carefully removed.

The peaks were identified by their mass spectra and by comparison with synthetic material. Racemic derivatized **1a** and **1e** were mixed with synthetic optically active material to establish their sequence of elution. It could be demonstrated that the early peaks correspond to the R isomers (Fig. 3).

Interestingly the resolution R_s of the acetylated hydroxyalkenals decreases with increasing chain length only for hydroxypentenal (**1a**), hydroxyhexenal (**1b**) and hydroxyheptenal (**1c**). For the longer-chain homologues, no more significant changes were observed. Peak widths

Table 3
Separation data of acetylated R- and S-hydroxyalkenals **1**

Compound	$t_R(S) - t_R(R)$ (min)	$\bar{\omega}$ (min)	α	R_s
1a	0.674 ± 0.083	$0.0626 \pm 6.80 \cdot 10^{-3}$	$1074 \pm 9.29 \cdot 10^{-3}$	10.8 ± 1.22
1b	0.348 ± 0.013	$0.0738 \pm 6.34 \cdot 10^{-3}$	$1027 \pm 8.54 \cdot 10^{-4}$	4.75 ± 0.54
1c	0.146 ± 0.009	$0.0788 \pm 1.05 \cdot 10^{-2}$	$1009 \pm 4.79 \cdot 10^{-4}$	1.88 ± 0.31
1d	0.136 ± 0.005	$0.0814 \pm 9.61 \cdot 10^{-3}$	$1006 \pm 2.78 \cdot 10^{-4}$	1.70 ± 0.26
1e	0.152 ± 0.015	$0.0790 \pm 1.89 \cdot 10^{-2}$	$1006 \pm 4.06 \cdot 10^{-4}$	2.06 ± 0.70

Mean ± R.S.D. $(n = 5)$.
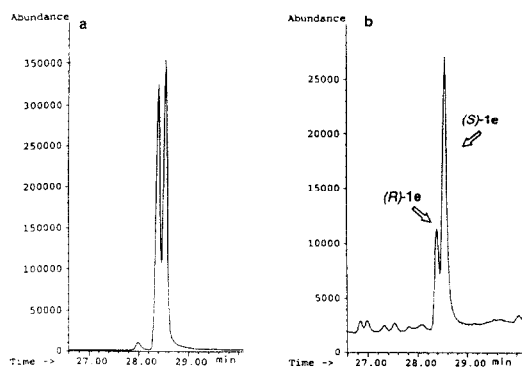
Fig. 3. (a) GC of racemic derivatized **1e**. (b) GC after addition of the S-enantiomer.

Fig. 4. Mass spectrum of acetylated 4-hydroxynonenal (**1e**) with typical fragmentation pattern.

show no correlation to configuration or chain length of the analytes.

3.5. GC–flame ionization detection experiments

With flame ionization detection, 250 ng of the acetylated aldehydes **1a–e** could still be identified and quantified. The analysis functions $m = f(R)$ (m = mass of the analyte, R = detector response) were linear (Table 4).

3.6. GC–MS experiments

Mass-selective detection was used to obtain total ion chromatograms and the mass spectra of acetylated **1a–e**. The mass spectra show three
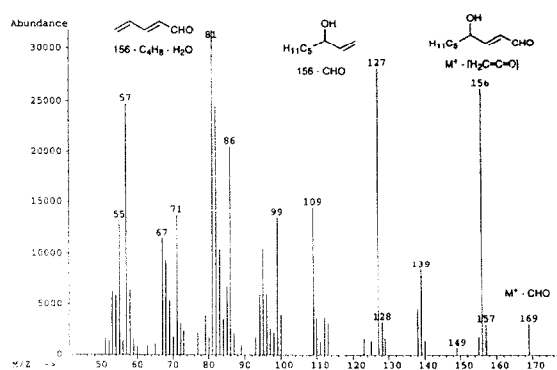
typical fragmentations: loss of the acetyl group, β-cleavage of the alcohol and α-cleavage of the carbonyl group (Fig. 4). The most characteristic fragments have been chosen for SIM.

3.7. Analysis of 4-hydroxyalkenals 1 in biological samples

First experiments have been carried out to apply the described method to the analysis of biological samples. For calibration, aliquots between 100 and 1 μg **1e** were added to an amount of rat liver microsomes equivalent to 1 g liver wet mass. The aldehydes were reextracted with dichloromethane, purified on RP-18 material, derivatized with acetic anhydride and finally

Table 4
Analysis functions for the quantitative analysis of derivatized **1a–e**

	Equation	r	n
Flame-ionization detection			
1a	$R = 714.2\,(\pm 26.62)m - 138.1\,(\pm 58.92)$	0.994	3
1b	$R = 457.3\,(\pm 13.91)m + 187.0\,(\pm 25.47)$	0.927	3
1c	$R = 888.2\,(\pm 4.55)m + 122.5\,(\pm 54.28)$	0.998	3
1d	$R = 1233.7\,(\pm 142.24)m - 538.2\,(\pm 204.12)$	0.993	3
1e	$R = 1118\,(\pm 73.64)m - 260.3\,(\pm 38.17)$	0.974	3
Mass-selective detection			
1e	$R = 41117\,(\pm 5119)m - 52\,683\,(\pm 21\,206)$	0.993	6
Mass-selective detection + I.S. 2			
1e	$R/R_{I.S.} = 0.771\,(\pm 0.038)m/m_{I.S.} + 0.772\,(\pm 0.431)$	0.996	4

m = Mass of the analyte; R = detector response.

subjected to chromatography. For selective detection of **1e** in rat liver microsomes, the fragment at *m/z* 156 was the target ion and those at 127 and 81 the qualifier ions. After extraction and purification, 75% of the free aldehyde **1e** could be recovered in the samples. The detection limit for derivatized **1e** was 660 pg. The analysis function (Table 4) was linear for the whole concentration range, even near the detection limit. In blank samples no peaks with the retention time of derivatized **1e** were observed.

3.8. Use of 4-hydroxy-6-methylhept-2-enal (2) as an internal standard

To improve the precision of quantification even in complex biological samples we decided to use the synthetic branched-chain 4-hydroxy-6-methylhept-2-enal (**2**) as an internal standard. Compound **2** was added to the samples before workup. It could be assumed that **2** would exhibit the same chemical properties as the hydroxyalkenals **1a–e**, especially with regard to solubility or binding to biological matrix components. Calibration data shows a linear correlation between the response ratio and the mass ratio of **1e** *vs.* **2** (Table 4). Deuterated internal standards like 2,3-dideutero-4-hydroxynon-2-enal [19] or 9,9,9-trideutero-4-hydroxynon-2-enal [20] cannot be used in difficult enantiomer separations because they may not coelute with the unlabelled compound. This may affect the quality of separation as even a slight shift of retention times causes peak broadening.

3.9. Detection and stereochemical analysis of 4-hydroxynonenal (1e) in liver microsomes after treatment with ADP/Fe^{2+}

As a first test for the applicability of our method, we investigated rat liver microsomal fractions that had been freshly prepared from male Wistar rats and stored in a NADPH-generating solution. After LPO initiation by treatment with ADP/Fe^{2+}, the samples were analyzed for 4-hydroxynon-2-enal (**1e**) by GC-SIM. We have been able to detect **1e** and to demonstrate that it is formed as a racemate (see
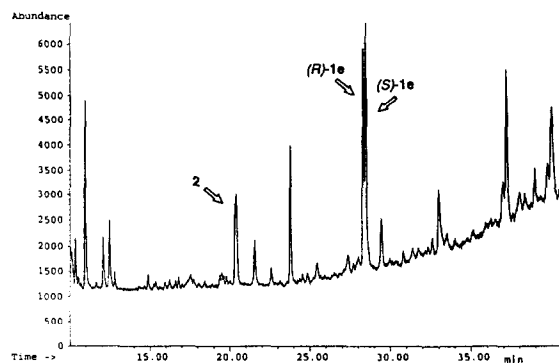

Fig. 5. Formation of racemic **1e** in ADP/FeSO$_4$-treated rat liver microsomes, as demonstrated by GC–SIM after acetylation of the free aldehyde.

Fig. 5), the first concrete experimental evidence on the non-stereoselective formation of this metabolite. This finding strongly supports the generally accepted concept [1] that hydroxyalkenals are formed, at least in liver tissue, without any enzymatic stereocontrol by a free radical mechanism.

4. Discussion and outlook

With our novel sensitive enantiomer analysis, the ratio of *R*- and *S*-hydroxyalkenals in biological model systems can be determined. For the first time it is possible to study the implications of stereochemistry on the generation and metabolism of these toxic LPO products.

However, the scope of our method is still limited, as the sensitivity of detection is considerably lower, compared with established non-selective methods. This is due in part to the simple effect of peak splitting, but more importantly to constraints imposed by the chromatographic system. We had to find a compromise for the choice of the derivatization procedure since the hydroxyalkenal analysis had to be optimized with respect to enantiomer separation. With perfluorinated agents, which improve the detection especially for GC–MS, unfortunately the racemate could not be resolved. We further had to leave the reactive carbonyl group unprotected. As a consequence, loss of hydroxy-

alkenals during the isolation from biological material cannot be ruled out.

Further work will be directed at solutions of these problems. Hopefully, we will be able to expand the scope of this encouraging method to the investigation of animal or human serum and tissue. So far, we have been able to provide a tool to examine the hitherto unexplored field of hydroxyalkenal stereochemistry and thus contribute to a more complete understanding of the complex processes involved in LPO.

5. Acknowledgements

6. References

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