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Journal of Chromatography B, 774 (2002) 231–239

JOURNAL OF CHROMATOGRAPHY B

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Measurement of 4-hydroxynonenal in small volume blood plasma samples: modification of a gas chromatographic–mass spectrometric method for clinical settings

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Received 16 July 2001; received in revised form 25 February 2002; accepted 15 April 2002

Abstract

4-Hydroxynon-2-enal (4-HNE) is one of the major aldehydic products of lipid peroxidation (LPO) and is involved in a number of pathophysiological processes. Since LPO products are useful indicators for oxidative stress in vivo, a number of detection methods for LPO products in biological tissues were developed. However, none of these methods is presently used in clinical settings. In order to introduce LPO products as biomarkers in clinical studies a suitable GC–MS method for 4-HNE detection was adapted to meet clinical requirements. As one result, the minimal sample volume could be decreased to 50 μl of plasma so that the method might even be suitable for pediatric purposes. The best internal standard (I.S.) for 4-HNE detection by GC–MS 9,9,9-D_3-4-hydroxynon-2-enal was introduced by van Kuijk et al. [Anal. Biochem., 224 (1995) 420]. However, because of its limited availability, benzaldehyde-ring-d_5, 4-hydroxybenzaldehyde, and 2,5-dihydroxybenzaldehyde were tested to find an alternative. Out of these three, 4-hydroxybenzaldehyde was shown to serve best as I.S. To examine the applicability of the adapted method, tests on the stability of 4-HNE in samples during storage were carried out. It was shown that plasma samples need to be stored at −80 °C or less to avoid greater loss of 4-HNE. Samples with 4-HNE concentrations close to the physiological level were shown to be stable over 22 months at −80 °C. The introduction of a new and easily available I.S., reduction of the sample volume, and information about sample stability provided by this study facilitate 4-HNE determination in most clinical settings. © 2002 Published by Elsevier Science B.V.

Keywords: Clinical settings; 4-Hydroxynonenal

1. Introduction

Reactive oxygen species were shown to be involved in a number of pathological processes. For some of the most common degenerative diseases a direct link could be shown between their pathogeneses and chronic oxidative stress [1–3]. As a consequence, numerous methods were developed for measurement of oxidative stress in vivo in the last two decades. However, up to now none of these methods has found its way into clinical routine settings.

In general, oxidative stress might be measured in different ways. One possibility is the detection of

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1570-0232/02/$ – see front matter
PII: S1570-0232(02)00242-8

reactive oxygen species themselves. However, free radicals might be measured only in the close vicinity of their formation and these data do not give information about cumulative effects. More information about the potential toxicity of increased radical formation is given by the products of free radical action. When radical formation reaches a certain level, lipid peroxidation (LPO) is initiated in cellular systems leading to the formation of large amounts of aldehydic products. Some of theses aldehydes, in particular 4-hydroxyalkenals, represent oxidized omega-6 and omega-3 polyunsaturated fatty acids (PUFA) [4]. 4-Hydroxyalkenals are strong electrophilic reagents which react in tissues with nucleophils, preferentially with sulfhydryl (SH), amino (NH_2) groups and the imidazole group of histidine. Thus, these LPO products are biologically very active and can cause severe disturbances of cell functions at the biochemical and genetic level [5,6]. Therefore, LPO products are so-called ''second toxic messengers'' of free radicals and represent excellent indicators in studies on oxidative stress in vivo.

It has been proposed that most biological effects of 4-hydroxyalkenals are based on the reaction of 4-hydroxynonenal (4-HNE) which is formed exclusively from ω-6 PUFA [4]. Other toxic LPO products commonly determined (propanal, hexanal, malondialdehyde) are formed from both ω-3 or ω-6 PUFAs [4]. However, ω-6 PUFAs are the PUFAs with the highest concentration in mammalian tissues. Therefore, and since 4-HNE was reported to be one of the most toxic aldehydes formed in vivo, it is probably the best indicator for LPO derived from cytotoxic reactions. In order to detect 4-HNE in vivo various analytical methods were developed using different techniques, such as HPLC [7–10], GC–MS [11–16], GC [17], LC–MS [18,19], TLC [20] and immunohistochemistry [21].

GC–MS methods applying negative ion chemical ionisation (NICI) and the use of an internal standard show a much higher sensitivity compared to other quantification techniques. This results in a much lower detection limit for 4-HNE which allows a reduction of the sample volume per analysis. Some methods using GC–MS may fulfill the requirements of clinical application after some modification. The method published by Luo et al. [22] allows a simple and fast processing of samples, as well as analysis of 22 different aldehydes simultaneously. Quantification of aldehydic LPO products, such as propanal, hexanal, and MDA, can easily be achieved. However, an accurate measurement of 4-HNE is not possible with this method, as the recovery of this aldehyde from plasma varies between 60 and 80% even under optimal conditions. Luo et al. [22], therefore, recommended the use of a specific, stable isotopic internal standard. However, since isotopic 4-HNE cannot be obtained commercially and is also very difficult to synthesize, this presents indeed a major problem of 4-HNE quantification with this otherwise excellent method. Therefore, this study attempted to find an alternative and tested benzaldehyde-ring-d_5 (RBA) [22], 4-hydroxybenzaldehyde (4-HBA), and 2,5-dihydroxybenzaldehyde (2,5-DBA) [23] for their suitability as internal standards (I.S.). Furthermore, we tried to reduce the necessary sample size and tested also the stability of 4-HNE samples during storage at different temperatures.

2. Experimental

2.1. Chemicals

O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA·HCl) was obtained from Fluka (Buchs) and *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA)+1% trimethylchlorosilane (TMCS) was purchased from Supelco (Bellefonte, PA; Sylon BFT kit, 144 ampoules of 0.1 ml). (±)-4-Hydroxynon-2-enal (4-HNE, 1 mg/100 μl in hexane) was obtained from Biomol (Hamburg, Germany), 9,9,9-D_3-4-hydroxynon-2-enal (D_3-HNE, 1 mg/ml in acetonitrile) was a gift of F.J.G.M. van Kuijk (Department of Ophthalmology and Visual Sciences, University of Texas Medical Branch, Galveston, TX, USA). Benzaldehyde-ring-d_5 (RBA) was purchased from Cambridge Isotope (Andover, MA, USA), 2,5-dihydroxybenzaldehyde (2,5-DBA) and 4-hydroxybenzaldehyde (4-HBA) were purchased from Merck-Schuchardt (Hohenbrunn, Germany). All other chemicals used were obtained from Sigma–Aldrich (Deisenhofen, Germany). Standard solutions of RBA, 2,5-DBA and 4-HBA were prepared by dissolving 641 μmol of each substance in 10 ml methanol following dilution to the appropriate con-

centration. All solvents used were of analytical grade.

2.2. Collection of blood plasma samples

Blood was collected into potassium–EDTA coated syringes containing 1.6 mg EDTA (Sarstedt) which are commonly used in clinical routine. To stabilize LPO products, syringes were precoated before with 50 μg 2,6-di-*tert*.-butyl-*p*-cresol (BHT). For separation of plasma from cellular components blood was centrifuged at 1900 *g* for 10 min at 4 °C. To test the suitability of the methods for conditions of limited blood availability in some experiments only 100 μl blood was drawn in small, potassium–EDTA coated caps supplied with a capillary (Sarstedt).

2.3. Stability of 4-HNE during storage

Fresh plasma from a healthy volunteer was spiked with 4-HNE according to the following procedure: 19.5 μl of 4-HNE in hexane [1 μg/100 μl] was added to 2.5 ml plasma, mixed well and divided into 50-μl portions. Three portions were stored at different temperatures (−196, −80, −20 °C) for 4 weeks and two were allowed to stay at 4 °C for different periods of time (1 or 3 h) prior to being stored at −196 °C for 4 weeks. All samples ($n=6$ per portion) were treated as described below using D_3-HNE as I.S.

For control of plasma containing 4-HNE in physiological amounts freshly taken plasma from six healthy volunteers was stored at −80 °C for 22 months.

2.4. Sample preparation for GC–MS analysis

For sample preparation I.S. was mixed with 50 μl plasma and 250 μl of 0.05 *M* PFBHA·HCl in water. For I.S. experiments, different amounts of the potential I.S. substances were used (5 ng of D_3-HNE, 3.56 ng of RBA, 2.35 ng of 4-HBA, and 7.08 ng of 2,5-DBA). The mix was incubated at room temperature for 30 min. After incubation 0.5 ml of methanol was added followed by 2 ml of hexane. Additionally, six drops of concentrated sulfuric acid were added dropwise and the sample was mixed and centrifuged again. After separation hexane fractions were collected, dried over anhydrous sodium sulfate and evaporated under nitrogen. The sample was derivatised with 40 μl of Sylon BFT at 80 °C. When D_3-HNE or RBA samples were used as I.S., samples were derivatised only for 5 min. When 4-HBA or 2,5-DBA were used as I.S., samples had to be derivatised for 2 h 15 min to achieve complete derivatisation [23].

2.5. Gas chromatography–mass spectrometry

GC–MS analysis was performed on a Varian GC 3400 gas chromatograph in combination with a Finnigan MAT TSQ 70 mass spectrometer. For separation a ZB5 capillary column (30 m, 0.25 I.D., 0.25-μm film thickness, Phenomenex) was used. The temperature program started at 60 °C. After the conditions were held isothermic for 1 min, the temperature was increased up to 85 °C at 20 °C/min. Thereafter, the temperature was increased to 310 °C at 8 °C/min and held isothermic for 3 min. The temperature of both the injector and the transfer line were set at 250 °C. The source temperature was kept at 120 °C and electron energy was set to 70 eV. Helium was used as carrier gas with a flow-rate of 1 ml/min. The injection volume was 0.5 μl splitless changing to split 20:1 at 0.7 min. Negative ion chemical ionisation (NICI) was applied using methane as reagent gas. MS acquisition was performed in the single ion monitoring (SIM) mode, monitoring m/z 403 and 406 for the unlabeled and labeled 4-HNE derivatives, m/z 256, 447, 369 for RBA, 2,5-DBA, and 4-HBA derivatives. The fragment ions m/z 403, 406 and 369 represent the [M-HF] anion [24], whereas m/z 256 represents the [M-HFNO] anion of the derivatised RBA [22]. The fragment ion m/z 447 corresponds to the [M-30] anion which might result from the loss of two methyl groups of the disilylated derivative of 2,5-RBA.

2.6. Statistical analysis

All data are given as mean±SD. Correlation of I.S. was examined for significance by the Pearson test. A significant correlation was accepted at the $\alpha=0.05$ level. Mean values were examined by the unpaired *t*-test using Welsh's correction. Statistical significance was assumed at $P\leq 0.05$. All tests were

performed using the Prism 2 software package (Graphpad Software Inc.).

3. Results

3.1. Adaptation and reliability tests of the GC–MS method

Full scan NICI mass spectra of the most abundant stereoisomers of the *O*-PFB-oxime (TMS ether) of 4-HNE and the internal standards were recorded (Fig. 1). For single ion monitoring (SIM), characteristic fragmentation ions were chosen for each compound. Retention times (RT), molecular ion (M^-), and the ion chosen for measurement are listed in Table 1. For each substance used as I.S. calibration, curves applying a five-level calibration were recorded per 50 μl plasma (from 2.5 to 25 pmol 4-HNE). Applying an acceptance criterium for linearity of $R^2 > 0.9800$, best linearity was achieved using D_3-HNE. For 4-HBA and 2,5-DBA linearity was still in an acceptable range. In the experiments applying RBA, a poorer linearity was found. A comparison of calibration curves set up in 50-μl samples is given in Table 2. The coefficient of determination, residual standard deviation and process standard deviation of the calibration curves are also given.

Applying a signal-to-noise ratio of 4 ($S/N = 4$), no signal for 4-HNE could be recorded in a blank level ($n = 3$, Fig. 2). However, in all our physiological samples 4-HNE could be detected ($n = 6$ adults; Table 3, Fig. 2). Depending on the I.S., chosen values varied from 2.6 to 76.6 nmol/l.

The precision of the method was determined by calculating the coefficient of variation V_c for five repeated measurements of spiked plasma samples containing 100 and 500 nmol/l HNE (Table 4). Acceptance criterium for V_c is $V_c < 10\%$. Recovery of the method could not be determined as there are no suitable reference standards available.

3.2. Comparison of D_3-HNE, RBA, 2,5-DBA, and 4-HBA

Comparison of different I.S. was carried out with different samples of one patient undergoing liver transplantation in whom pathological levels of 4-HNE were expected. Using D_3-HNE as I.S., 4-HNE concentrations were in the range of 20–120 nmol/l. These 4-HNE values were compared to the results obtained in experiments with the other I.S. substances. Using single point determination, a correlation to the results obtained with D_3-HNE could be achieved only for 4-HBA ($R^2 = 0.8655$; $y = 0.6916x + 2.0496$; $\alpha = 0.05$, $P < 0.0001$; Fig. 3). In contrast, the results gained with 2,5-DBA ($R^2 = 0.0176$) and RBA ($R^2 = 0.0293$) did not show any linear correlation to the values measured with D_3-HNE.

3.3. Stability of 4-HNE during storage

Samples spiked with 4-HNE were stored at different temperatures. There was no difference in recovery between samples stored in liquid nitrogen (−196 °C) and samples stored at −80 °C. In the latter, 8.7 pmol 4-HNE could be found. In those samples kept at 4 °C for 1 h prior to storage in liquid nitrogen, 6.5 pmol 4-HNE was recovered. With 3 h at 4 °C, prior to storage in liquid nitrogen only 4.1 pmol 4-HNE could be found. Samples stored at −20 °C contained only 2.3 pmol 4-HNE per 50 μl plasma after storage (Fig. 4).

In physiological plasma samples stored at −80 °C for 22 months, an insignificant decrease of 4-HNE was found compared with those samples which had been worked up immediately. While stored samples showed a mean 4-HNE concentration of 16.7±3.0 nmol/l, the mean concentration of the samples worked up immediately was 21.9±7.2 nmol/l ($n = 6$).

4. Discussion

The method by Luo et al. [22] provides simultaneous determination of 22 aldehydes. However, with respect to 4-HNE and other unsaturated aldehydes this method does not provide reliable results, when RBA is used as I.S. A stable isotopic I.S. for 4-HNE determination, as suggested by Luo et al. [22], was introduced by van Kuijk et al. [25]. However, this I.S. is difficult to synthesize and commercially not

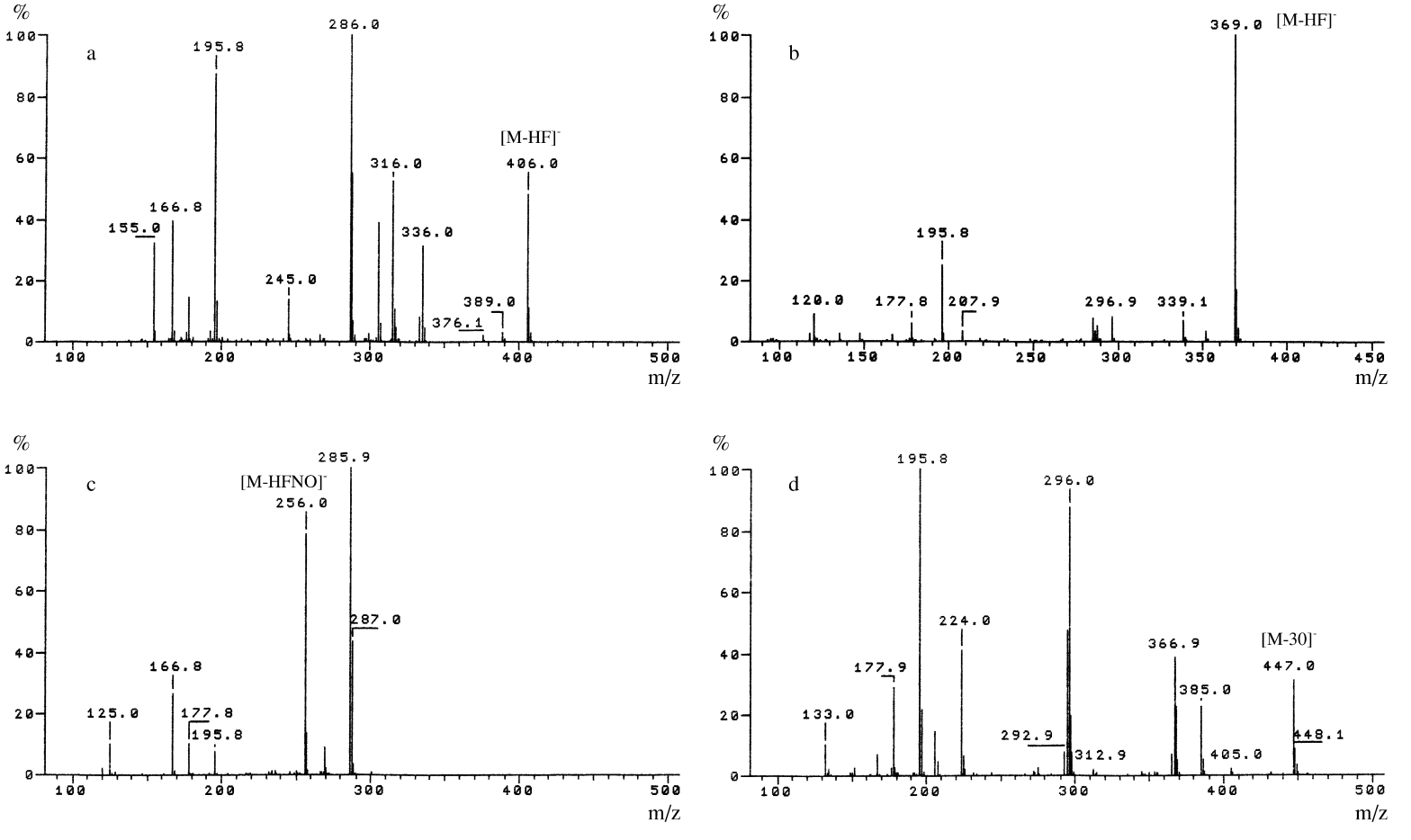

Fig. 1. Mass spectra of (a) the D_3-HNE derivative, (b) the 4-HBA derivative, (c) the RBA derivative, and (d) the 2,5-DBA derivative.

Table 1
Retention times (RT) and characteristic ions of the NICI mass spectra of PFB-oxime–TMS derivatives (isomeres) of the aldehydes

Aldehyde	PFB-oxime–TMS derivative	M^-	Characteristic ion	RT (min)
4-HNE	$C_{19}H_{26}F_5NO_2Si$	423	403[a]	19.99, **20.35***
D_3-HNE	$C_{19}H_{23}D_3F_5NO_2Si$	426	406[a]	19.90, **20.29***
Benzaldehyde-ring-d_5	$C_{14}H_3D_5F_5ON$	306	256[b]	15.85, **15.96***
2,5-Dihydroxybenzaldehyde	$C_{20}H_{24}F_5NO_3Si_2$	477	447[c]	**22.56***
4-Hydroxybenzaldehyde	$C_{17}H_{16}F_5NO_2Si$	389	369[a]	20.66, **20.96***

* Isomere chosen for SIM.
[a] Fragment ion corresponds to $[M\text{-}HF]^-$.
[b] Fragment ion corresponds to $[M\text{-}HFNO]^-$.
[c] Fragment ion corresponds to $[M\text{-}30]^-$ most probably from the loss of two methyl groups.

Table 2
Comparison of calibration curves obtained using 50-μl sample amounts

Internal standard	Sample amount (μl)	Curve function	R^2	Residual SD	Process SD
D_3-HNE	50	$y = 0.0406x + 0.0769$	0.9947	0.0306	0.7537
4-Hydroxybenzaldehyde	50	$y = 0.0026x - 0.0001$	0.9872	0.0031	1.1923
2,5-Dihydroxybenzaldehyde	50	$y = 0.0479x + 0.0239$	0.9825	0.0743	1.5511
Benzaldehyde-ring-d_5	50	$y = 0.0098x + 0.0017$	0.9542	0.02255	2.3010

Calibration was evaluated between 50 and 500 nmol HNE/l plasma applying a five-level calibration. Acceptance criteria for the calibration is $R^2 > 0.9800$ (corresponds to $R > 0.99$).
R^2 = coefficient of determination.

Table 3
Concentrations of 4-HNE in physiological plasma samples ($n = 6$) and their dependence of the internal standard used

Internal standard	HNE range [nmol/l]	HNE median [nmol/l]	HNE mean [nmol/l]	HNE SD [%]
D_3-HNE	2.6–49.9	32.9	30.0	19.6
4-Hydroxybenzaldehyde	26.7–59.4	46.0	44.6	13.5
2,5-Dihydroxybenzaldehyde	29.2–76.6	32.4	42.7	22.7
Benzaldehyde-ring-d_5	18.5–46.6	30.8	30.0	9.2

Table 4
Coefficient of variation V_c for 100 and 500 nmol/l HNE ($n = 5$)

Internal standard	V_c [%][a]	
	100 nmol/l	500 nmol/l
D_3-HNE	2.3	1.4
4-Hydroxybenzaldehyde	1.5	5.3
2,5-Dihydroxybenzaldehyde	2.5	4.4
Benzaldehyde-ring-d_5	21.3	12.0

[a] Acceptance criterium: $V_c < 10\%$.

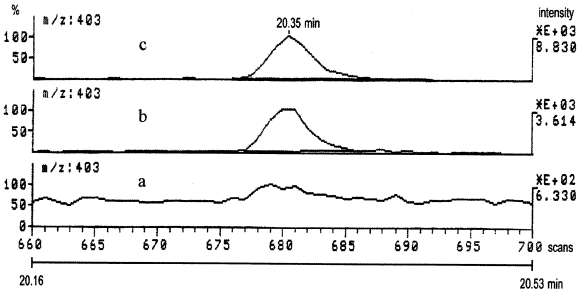

Fig. 2. SIM spectra of 4-HNE (m/z 403) in (a) solvent sample, (b) physiological plasma sample, (c) physiological plasma sample spiked with 2.5 pmol 4-HNE.

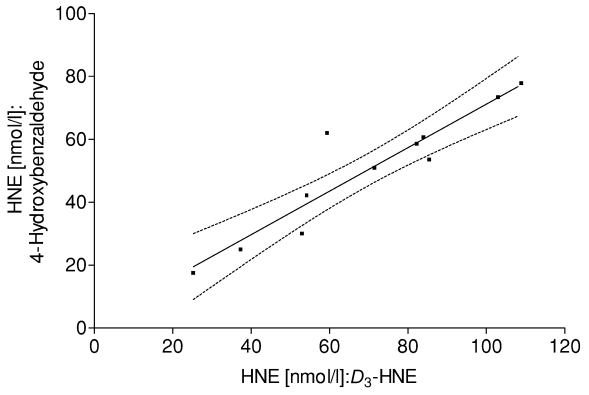

Fig. 3. Correlation between 4-HNE quantification via D_3-HNE and 4-hydroxybenzaldehyde. The 4-HNE concentration determined with 4-hydroxybenzaldehyde as internal standard is plotted against 4-HNE concentration determined with D_3-HNE as internal standard. The dotted slopes give the 95% confidence interval.

available. This study shows that the widely available substance 4-HBA may well be used as I.S. for 4-HNE determination instead of a deuterated compound.

Deuterated substances are often used as I.S. for quantification since they show very similar characteristics compared with the corresponding unlabeled compound. However, quantification will be only successful as long as the labeled compound is stable enough and no exchange of deuterium occurs during sample preparation. D_3-HNE, as proposed by van Kuijk et al. [25], fulfills these requirements for 4-HNE analysis.

In our experiments using the method by Luo et al. [22] excellent results were achieved with D_3-HNE. As alternatives, we tested three other substances showing some structural similarity to D_3-HNE: RBA, introduced by Luo et al. [22], 2,5-DBA as suggested by Rauli et al. [23] and, for the first time, 4-HBA were tested as potential I.S. and compared with results obtained with D_3-HNE. All these I.S. have a molecular structure different from 4-HNE. While 4-HNE is an aliphatic molecule the suggested I.S. are aromatic substances. However, RBA and 2,5-DBA are aldehydes that differ also concerning the number of hydroxy groups. While RBA has no hydroxy moiety, 2,5-DBA is equipped with two hydroxy groups. 4-HBA contains the same number of functional groups as 4-HNE, and therefore, we speculated that 4-HBA might be a more suitable I.S. than the other two compounds suggested by other authors.

Indeed, the linearity of 4-HBA was only slightly less than that of D_3-HNE and the calibration curves showed an acceptable correlation. The residual standard deviation of 4-HBA and RBA is less than that of D_3-HNE, whereas for 2,5-DBA the residual SD is worse. As the residual SD depends on the regression coefficient, the process standard deviation was calculated to be able to compare the data. D_3-HNE indeed shows the smallest process SD followed by 4-HBA.

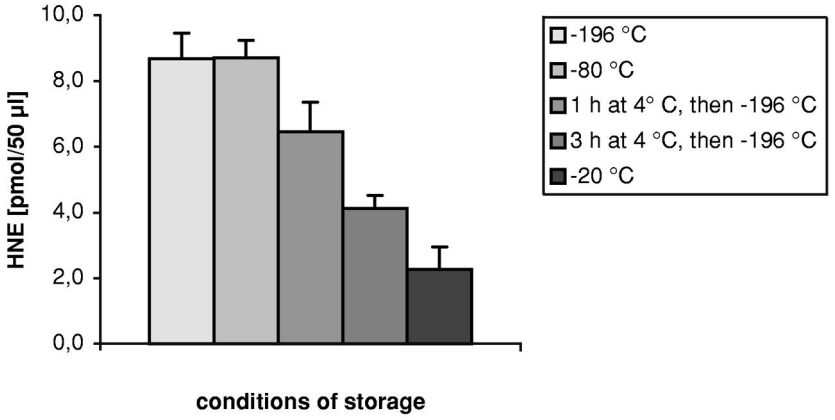

Fig. 4. 4-HNE concentrations found in plasma samples ($n=6$) spiked with 25 pmol/50 µl HNE after storage at different temperatures.

Our acceptance criteria for process SD is to be closest to the values of D_3-HNE because we chose D_3-HNE as the gold standard for this method. Therefore, 4-HBA is considered the best alternative to D_3-HNE.

The coefficient of variation (Table 4) reveals that the precision of D_3-HNE, 4-HBA and 2,5-DBA is within the acceptable range ($<10\%$), only RBA does not meet the criterium.

In experiments where 4-HNE concentrations were measured in human plasma, the results obtained with 4-HBA correlated well with those of D_3-HNE ($R^2 = 0.8655$, Fig. 3). Though this correlation might not be ideal from the point of view of an analytical chemist as the average 4-HNE values found with 4-HBA are about 30% below the values found with D_3-HNE (Fig. 3), it is the best which could be achieved. It is a great improvement to all so far known I.S. suggested to replace D_3-HNE. A disadvantage of 4-HBA may be that it requires longer derivatisation time (2 h 15 min) in comparison to D_3-HNE (5 min). In contrast to 4-HBA, 4-HNE concentrations detected with RBA and 2,5-DBA as internal standard showed very bad correlation ($R^2 = 0.0176$ and $R^2 = 0.0293$) to the values determined with D_3-HNE. Keeping in mind that 2,5-DBA and RBA both show an acceptable coefficient of determination in spiked samples (see Table 2), there is no correlation in real plasma samples. These results show the importance of verifying the suitability of this method by testing the I.S. under realistic conditions. In addition, the calibration curve of RBA showed much less linearity than those of D_3-HNE, 4-HBA or 2,5-DBA in 50-μl samples. Therefore, neither 2,5-DBA nor RBA should be used as I.S. for 4-HNE detection.

While this work was in progress, Ogihara et al. [26] reported the application of a GC–MS method for measurements of aldehydes in newborn infants. The authors employed hydroxylamine hydrochloride and *tert.*-butyl-dimethylchlorosilane for derivatisation of aldehydes according to Norsten-Hoog and Cronholm [16] and introduced 2,2,6,6-D_4-cyclohexanone as I.S. Unfortunately, in their paper no data were given on the reliability of the method used. Compared to 4-HNE, 2,2,6,6-D_4-cyclohexanone shows less structural similarity than 4-HBA or 2,5-DBA. Furthermore, 2,2,6,6-D_4-cyclohexanone has neither an aldehydic functional group nor a hydroxy group. From these structural differences, it appears doubtful that 2,2,6,6-D_4-cyclohexanone is suitable as an I.S. for 4-HNE determination.

4.1. Stability of 4-HNE during storage

Stability of LPO products in biological samples is a major problem [27]. Under experimental conditions, this problem might be solved by designing the experiment in such a way that all samples are treated similarly and are measured after the same time. Unfortunately, this is not possible in clinical studies in which samples are most often collected at different time-points.

In our experiments, the influence of time before freezing, duration of storage, and storage temperature on the 4-HNE concentration in the samples was investigated. As can be seen from our results, there are differences between samples with physiological 4-HNE concentrations and samples spiked with 4-HNE. The spiked samples showed a loss of 4-HNE during storage at 4 and -20 °C compared to samples stored at -80 or -196 °C. In physiological samples there was no significant difference in 4-HNE concentrations in samples worked up immediately compared to those stored for 22 months at -80 °C.

An explanation for the loss of 4-HNE in the spiked samples might be the reactivity of the aldehyde towards other biomolecules. Bruenner et al. [28] showed that more than 99% of 4-HNE–protein adducts occur via Michael addition, and only trace amounts of reversible Schiff base adducts are formed. Common GC–MS and HPLC methods are able to measure either Schiff base bound or free 4-HNE. In spiked or highly oxidized samples, huge amounts of free 4-HNE are present. Once the reaction equilibrium between 4-HNE and proteins within the spiked sample is reached, only trace amounts of the originally present 4-HNE may be detected. Concerning physiological samples, the reaction equilibrium is already present at time of sample collection.

The introduction of a new reliable I.S., information on storage conditions required, and the reduction of the sample size to the volume of capillary collection tubes, lead to a significant improvement of the method developed originally by

Luo et al. [22]. This enhances the possibilities of application in clinical settings.

Acknowledgements

We greatly acknowledge the cooperation with H.J. Bardenheuer, Department of Anesthesiology, University Hospital of Heidelberg, Germany, and we are indebted to F.J.G.M. van Kuijk, Department of Ophthalmology and Visual Sciences, University of Texas Medical Branch, Galveston, TX, USA, who gave us D_3-4-hydroxynon-2-enal as a gift for our experiments.

References

[1] G. Spiteller, Chem. Phys. Lipids 95 (1998) 105.
[2] M.L. Selley, Free Radic. Biol. Med. 25 (1998) 169.
[3] L.L. de Zwart, J.H. Meerman, J.N. Commandeur, N.P. Vermeulen, Free Radic. Biol. Med. 26 (1999) 202.
[4] H. Esterbauer, H. Zollner, R.J. Schaur, in: C. Vigo-Pelfrey (Ed.), Membrane Lipid Oxidation, Vol. 1, CRC Press, Boca Raton, FL, 1990, p. 239.
[5] W.G. Siems, S.J. Hapner, F.J.G.M. van Kuijk, Free Radic. Biol. Med. 20 (1996) 215.
[6] E. Cajelli, A. Ferraris, G. Brambilla, Mutat. Res. 190 (1987) 169.
[7] G. Poli, M.U. Dianzani, K.H. Cheeseman, T.F. Slater, J. Lang, H. Esterbauer, Biochem. J. 227 (1985) 629.
[8] A.L. Bailey, G. Wortley, S. Southon, Free Radic. Biol. Med. 23 (1997) 1078.
[9] C. Goldring, A.F. Casini, E. Maellaro, B.B. Del, M. Comporti, Lipids 28 (1993) 141.
[10] A.E. Holley, M.K. Walker, K.H. Cheeseman, T.F. Slater, Free Radic. Biol. Med. 15 (1993) 281.
[11] M.J. Thomas, T.W. Robison, M. Samuel, H.J. Forman, Free Radic. Biol. Med. 18 (1995) 553.
[12] C. Des Rosiers, M.J. Rivest, M.J. Boily, M. Jette, A. Carrobe-Cohen, A. Kumar, Anal. Biochem. 208 (1993) 161.
[13] B.A. Bruenner, A.D. Jones, J.B. German, Anal. Biochem. 241 (1996) 212.
[14] M.L. Selley, J. Chromatogr. B 691 (1997) 263.
[15] G. Bringmann, M. Gassen, S. Schneider, J. Chromatogr. A 670 (1994) 153.
[16] C. Norsten-Hoog, T. Cronholm, Anal. Biochem. 189 (1990) 131.
[17] H. Tamura, T. Shibamoto, Lipids 26 (1991) 170.
[18] W.J. Blanchflower, D.M. Walsh, S. Kennedy, D.G. Kennedy, Lipids 28 (1993) 261.
[19] N. Deighton, W.J. Magill, D.H. Bremner, E.E. Benson, Free Radic. Res. 27 (1997) 255.
[20] J.K. Beckman, S.A.J. Morley, H.L. Greene, Lipids 26 (1991) 155.
[21] K. Uchida, T. Osawa, H. Hiai, S. Toyokuni, Biochem. Biophys. Res. Commun. 212 (1995) 1068.
[22] X.P. Luo, M. Yazdanpanah, N. Bhooi, D.C. Lehotay, Anal. Biochem. 228 (1995) 294.
[23] S. Rauli, M.D. Puppo, F. Magni, M.G. Kienle, J. Biochem. (Tokyo) 123 (1998) 918.
[24] M. Budzikiewicz, Mass Spectrom. Rev. 5 (1986) 345.
[25] F.J.G.M. van Kuijk, A.N. Siakotos, L.G. Fong, R.J. Stephens, D.W. Thomas, Anal. Biochem. 224 (1995) 420.
[26] T. Ogihara, K. Hirano, T. Morinobu, H.S. Kim, M. Hiroi, H. Ogihara, H. Tamai, Arch. Dis. Child. F 80 (1999) 21.
[27] H. Strohmaier, H. Hinghofer-Szalkay, R.J. Schaur, J. Lipid Mediat. Cell Signal. 11 (1995) 51.
[28] B.A. Bruenner, A.D. Jones, J.B. German, Chem. Res. Toxicol. 8 (1995) 552.