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# Validation of Malondialdehyde and 4-Hydroxy-2-*trans*-Nonenal Measurement in Plasma by NICI-GC-MS[1]

S. Rauli,* M. Del Puppo,* F. Magni,† and M. Galli Kienle*,[2]

*Department of Medical Chemistry and Biochemistry, University of Milan, Milano, Italy; and †Scientific Institute H.S. Raffaele, Via Olgettina, 58, 20132 Milano, Italy

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**Measurement of the concentrations of aldehydes in biological samples has become the object of much effort due to their relevance in relation to the toxic effects of lipid peroxidation, through which a number of aldehydes are derived. We have reconsidered a previously proposed method based on gas chromatographic mass spectrometric analysis of derivatives obtained by the treatment of aldehydes with *O*-pentafluorobenzyl hydroxylamine followed by a trimethylsilylating agent. In view of the possible use of the method for the simultaneous evaluation of the plasma levels of malondialdehyde and 4-hydroxy-2-*trans*-nonenal, we have studied the linearity of the analysis using various internal standards. Commercially available, inexpensive 2,4-dihydroxybenzaldehyde gave optimal results, the correlation coefficient of the calibration curve for plasma being $r > 0.995$ in the 0.1-5 $\mu$M range for both the tested aldehydes. The between-day imprecision (%CV) and accuracy (%bias) of the procedure determined using plasma samples spiked with the two aldehydes and with an internal standard reached maximum values of 3 and 8%, and 5 and 12% for HNE and MDA, respectively. The results obtained on analysis of plasma samples before and after oxidation with copper ions indicate the flexibility of the method for evaluation of the levels of MDA and HNE in plasma samples both under basal conditions and after an oxidative burst.**

**Key words: human plasma, 4-hydroxy-2-*trans*-nonenal, lipid peroxidation, malondialdehyde, NICI-GC-MS.**

Oxidative stress is now considered to be a relevant factor in the pathogenesis and persistence of important pathological conditions such as inflammation, atherosclerosis, diabetes, and ischemia (*1-4*). Following stress, the oxidation of polyunsaturated fatty acids (PUFAs) is enhanced by the formation of alkyl, peroxyl, and alkoxyl radicals, which start the chain process of lipid peroxidation. The final products of this process consist of aldehydes that are sufficiently long-living to be transported and distributed among various tissues (*5*). Therefore, the oxidative stress reaches tissue sites far from the original stress site. The most well-known aldehyde derived through lipid peroxidation is malondialdehyde (MDA), but even more important are other aldehydes, such as 4-hydroxy-2-*trans*-nonenal (HNE) and *n*-hexanal (HEX) derived through microsomal lipid peroxidation (*5*). MDA reactivity with 2-thiobarbituric acid (TBA) is the basis of the most used analytical methods for evaluating lipid peroxidation (*6, 7*). Nevertheless, the specificity of these methods is rather poor because other aldehydes react with TBA in addition to MDA, resulting in products with similar absorption. Moreover, TBA assay conditions such as high temperature and low pH may themselves cause the oxidation of lipids (*6*). An improvement in the specificity of the methods for evaluating lipid peroxidation products has been achieved with the HPLC determination of aldehydes after transformation into either 2,4-dinitrophenylhydrazones (*8*) or treatment with 1,3-cyclohexandione (*9*) to yield fluorescent derivatives. More recently, an additional improvement was obtained with GC-MS methods (*10-12*), some of which allow the simultaneous analysis of various aldehydes. Particularly relevant in this respect is the procedure reported by Luo *et al.* (*13*), which involves the NICI-GC-MS analysis of *O*-pentafluorobenzyl-oximes (PFB-oxime) treated with a silylating agent to hydroxyaldehydes. The authors reported the possibility of applying this method for the quantification of the aldehydes in biological matrices using deuterated benzaldehyde as an internal standard. However, no data have been reported for the evaluation of MDA and HNE in

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[2] To whom correspondence should be addressed at: c/o DIBIT Spettrometria di Massa, Via Olgettina 58, 20132 Milano, Italy. Tel: +39-2-26434924, Fax: +39-2-26434923

Abbreviations: MDA, malondialdehyde; HNE, 4-hydroxy-2-*trans*-nonenal; TBA, 2-thiobarbituric acid; GC-MS, gas chromatography-mass spectrometry; NICI-GC-MS, negative ion chemical ionization-gas chromatography-mass spectrometry; O-PFB-oxime, *O*-pentafluorobenzyl-oxime; O-PFB-oxime-TMS, *O*-pentafluorobenzyl-oxime trimethylsilyl ether; PFBHA-HCl, *O*-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride; DETBA, 1,3-diethyl-2-thiobarbituric acid; BHT, butylated hydroxy-toluene; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; TMCS, trimethylchlorosilane; IS, internal standard.

biological samples by this method (*13*), or on the imprecision and accuracy of this quantitative procedure. We have reconsidered the method, focusing our attention on the quantification of malondialdehyde and 4-hydroxy-2-*trans*-nonenal in human plasma and on an efficient internal standard (IS). From commercially available aldehydes, 2,5-dihydroxy-benzaldehyde was selected as an internal standard because the linearity was optimal with this standard in the tested biological matrix. Due to the good reproducibility and accuracy of the method, we have used it to measure the basal concentrations of the two aldehydes in plasma samples obtained from healthy subjects before and after oxidation in the presence of $Cu^{2+}$.

## EXPERIMENTAL

*Materials*—All chemicals were of analytical grade. HNE was a generous gift from Professor H. Esterbauer (Department of Biochemistry, University of Graz, Austria). 1,1,3,3-Tetraethoxypropan (TEP) was obtained from Aldrich. MDA was prepared by hydrolysis of TEP (*11*). 2,5-Dihydroxy-benzaldehyde was obtained from Merck (Schuchardt, München). PFBHA-HCl, 1,3-diethyl-2-thiobarbituric acid (DETBA), and butylated hydroxy-toluene (BHT) were from Sigma Chemical (St. Louis, MO). *N,O*-Bis-(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) and cupric sulphate pentahydrate were obtained from Fluka Chemie AG (Buchs). Hexane and methanol (HPLC grade), and Triplex®III (EDTA) were from Merck (Darmstadt).

*Sample Preparation*—Standard solutions of 4-hydroxy-2-*trans*-nonenal, malondialdehyde and the internal standard were prepared in methanol. Diluted solutions were prepared on the day of an experiment. Standard curves were obtained by spiking control plasma obtained from healthy volunteers with a fixed amount of 2,5-dihydroxy-benzaldehyde as an IS as described below for plasma samples, and with the amounts of each aldehyde given in the result section. Standard samples were also prepared similarly with distilled water instead of plasma.

Blood (7 ml) was collected in tubes containing 0.34 M EDTA (85 $\mu$l), and the plasma obtained on centrifugation was stored at −40°C until use. According to the procedure suggested by Luo *et al.* (*13*) with minor modifications, to plasma (100 $\mu$l) was added a solution of the internal standard in methanol (5 $\mu$l, 1 nmol for basal plasma; 5 $\mu$l, 5 nmol for oxidised samples), followed by aqueous *O*-(2,3,4,5,6-pentafluoro-benzyl)hydroxylamine hydrochloride (0.05 M, 200 $\mu$l), with stirring. After 30 min at room temperature, 0.5 ml methanol and 2 ml *n*-hexane were added. The samples were vortexed for 1 min, concentrated sulphuric acid (140 $\mu$l) was added dropwise, and, after additional vortexing for 1 min, the samples were centrifuged at 3,000 rpm for 5 min. The upper organic phase was extracted and dried over sodium sulphate. Each extract was evaporated under a stream of nitrogen and stored at −40°C until analysis. Samples were reacted for 2 h at 80°C with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) in 1% trimethylchlorosilane (TMCS) (50 $\mu$l) to transform the derivatives of HNE and IS into trimethylsilyl ethers. The samples were then analysed by NICI-GC-MS under the conditions described below. Percent recovery was over 80% for all the tested concentrations, as determined by comparison of the areas obtained with spiked plasma extracts to those with known amounts of pure standards on NICI-GC-MS analysis as described below.

*Oxidation of Plasma*—Human plasma was incubated at 37°C with $CuSO_4$ (final concentration, 3 mM), aliquots being taken at 0′, 90′, 5 h, and 24 h. Oxidation was blocked with the addition of EDTA (final concentration, 5 mM), and the samples were divided into two parts for TBARS determination and GC-MS, respectively. After storage at −80°C, the former part was treated with 1,3-diethyl-2-thiobarbituric acid (DETBA) as described by Conti *et al.* (*14*) to prepare fluorescent derivatives of the aldehydes, while the latter part was extracted and derivatized for GC-MS analyses as described above.

*NICI-GC-MS*—The optimal conditions for analysis of the derivatives of the tested aldehydes were determined using a capillary column, analysis being performed by negative ion chemical ionisation mass spectrometry (NICI-MS) with ammonia as the reagent gas (ion source, 1.1 Torr pressure), as follows: SPB-5 capillary column (0.32 i.d., 30 m long); helium as carrier gas, 9 psi pressure at the head of the column. The initial temperature of 85°C was maintained for 1 min and then increased to 145°C at 20°C/min (3 min), and to 300°C at 8°C/min. The temperatures of the injector and transfer line were maintained at 250°C; the source temperature was 200°C and the electron energy 100 eV.

Mass spectra of the derivatives of the aldehydes were recorded under scan conditions. Quantification of the aldehydes was carried out using the selected ion monitoring technique (SIM) focusing on one specific ion for each aldehyde and the internal standard.

## RESULTS AND DISCUSSION

The method described here was set up and validated for the simultaneous evaluation of malondialdehyde and 4-hydroxy-2-*trans*-nonenal, which are products of major interest in lipid peroxidation. The O-PFB-oxime of MDA, and the O-PFB-oxime TMSs of 4-hydroxy-2-*trans*-nonenal and of 2,5-dihydroxy-benzaldehyde, used as internal standard (Fig. 1), were prepared as described in detail in the experimental section, and their full NICI mass spectra were obtained (Fig. 2). Other aldehydes examined for their use as internal standards were benzaldehyde, as suggested by Luo *et al.* (*13*), who used pentadeuterated benzaldehyde, and cyclohexanone, as reported by Norsten-Höög and Cronholm (*10*). Nevertheless, they were not used because interfering signals were observed for basal extracts at the retention times of the aldehyde derivatives or due to the low intensity of specific ions in the mass spectrum.

For the measurement of plasma levels, the selected ions were *m/z* 152 for HNE ($[M-C_7H_2F_5-TMSOH]^-$), *m/z* 204 for MDA ($[M-C_7H_2F_5-HFNO-C_2H_3]^-$), and *m/z* 296 for IS ($[M-C_7H_2F_5]^-$). The compositions of ions derived from HNE and MDA derivatives have already been reported by others (*12, 13*). Figure 3 shows ion chromatograms obtained for selected ions after injecting a mixture of derivatized authentic aldehydes (MDA, HNE, and IS). As already reported (*12, 13*), two isomers were evident for HNE (A and B in Fig. 3), and three isomers for MDA were detectable (A, B, and C in Fig. 3), while IS gave a single peak. Despite the very narrow range of the retention times,

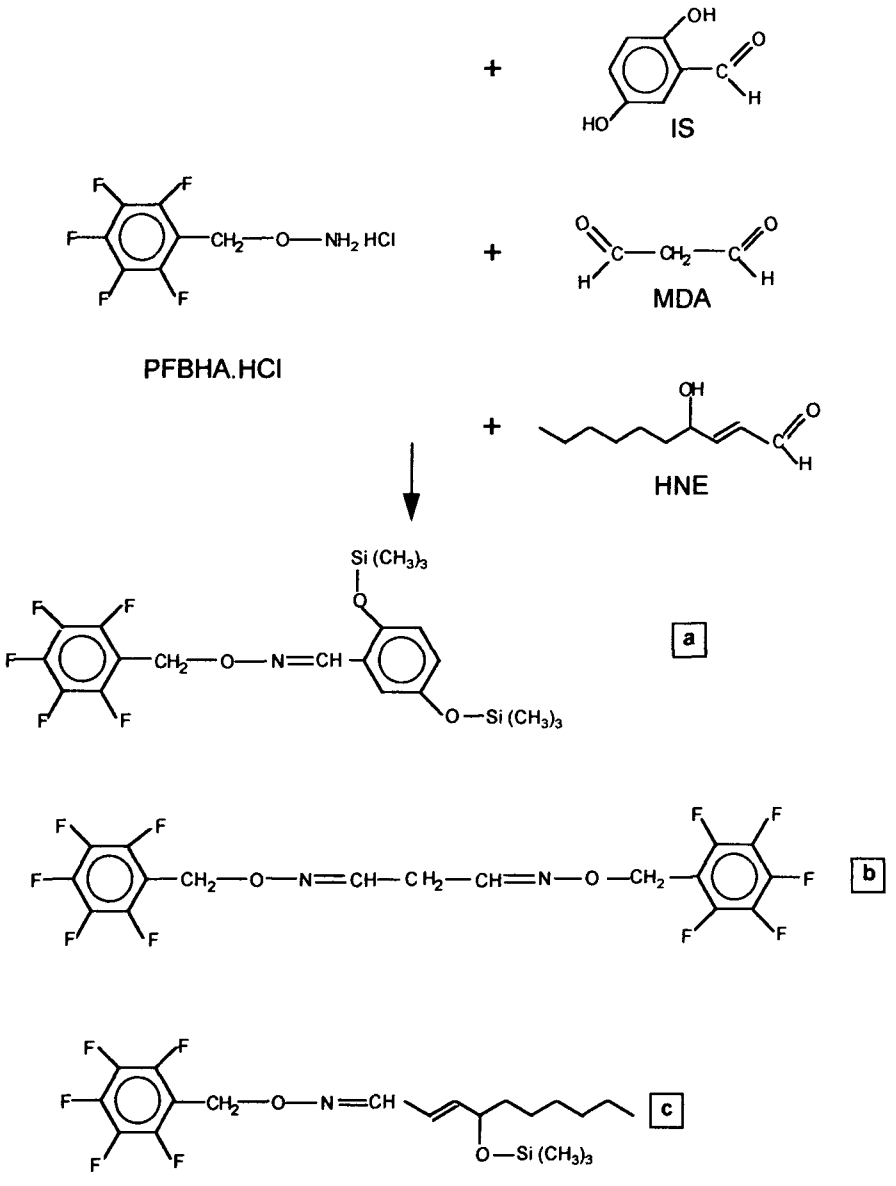


Fig. 1. **2,5-Dihydroxy-benzaldehyde-PFB-oxime-TMS (a), malondialdehyde-PFB-oxime (b), and 4-hydroxy-2-*trans*-nonenal-PFB-oxime-TMS (c).**

efficient separation of the derivatives was achieved with the column used, and the three aldehydes did not interfere with each other.

Signals at the retention times of the two tested aldehydes were always present in the ion chromatograms obtained on analysing plasma samples. In order to confirm that these signals originated from endogenous aldehydes, spiked and basal plasma samples were analysed focusing on three ions for each aldehyde, *m/z* 204, 281, and 442 for MDA, and *m/z* 152, 283, and 333 for HNE, respectively. The ratios of the signal intensities are reported for the isomers of both HNE (Table I) and MDA (Table II). There was a good correspondence between spiked and basal plasma samples for both HNE isomers. For isomer A of MDA (Fig. 3c), the ratios in basal plasma were significantly higher than those in spiked plasma, suggesting the occurrence of interference at the retention time of the MDA derivative that would result in overestimation of the plasma levels if this isomer was used for quantification. Nevertheless, the 442/204 and 281/204 ratios found considering isomers B+C of MDA in basal and spiked plasma were superimposable, confirming that these ratios are representative of the actual concentration of circulating MDA. It is worth pointing out that the ratios presented in Tables I and II appear to be rather different to those determined from the spectra shown in Fig. 2. This is not unexpected because the spectra were obtained by injecting much greater amounts than those injected for the analysis of basal and spiked plasma, which may modify the behaviour of the compounds within the ion source under the chemical ionisation conditions used.

The linearity of the assay was tested in two concentration ranges, from 0 to 500 pmol/ml, using 10 nmol/ml of IS (low range), and from 0.5 to 5 nmol/ml, using 50 nmol/ml of IS (high range). The results obtained for isomers B for HNE and B+C for MDA are shown in Fig. 4. Lines for the two calibration curves were obtained by plotting the intensity ratios of the tested ions against the aldehyde concentration in spiked plasma. In order to test the linearity in the whole range, the intensity ratios were also plotted against the aldehyde/IS molar ratio (Fig. 5). As shown by the high

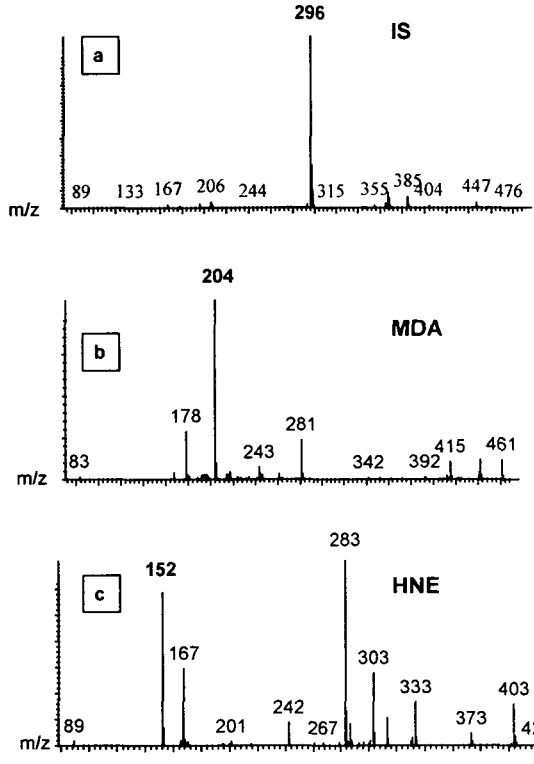


Fig. 2. **Mass spectra of 2,5-dihydroxy-benzaldehyde-PFB-oxime-TMS (a), malondialdehyde-PFB-oxime (b), and 4-hydroxy-2-*trans*-nonenal-PFB-oxime-TMS (c).** Spectra were obtained by NICI-GC-MS under scan conditions, as described under "EXPERIMENTAL." The ions selected for SIM analysis (*m/z*) are denoted in bold type.

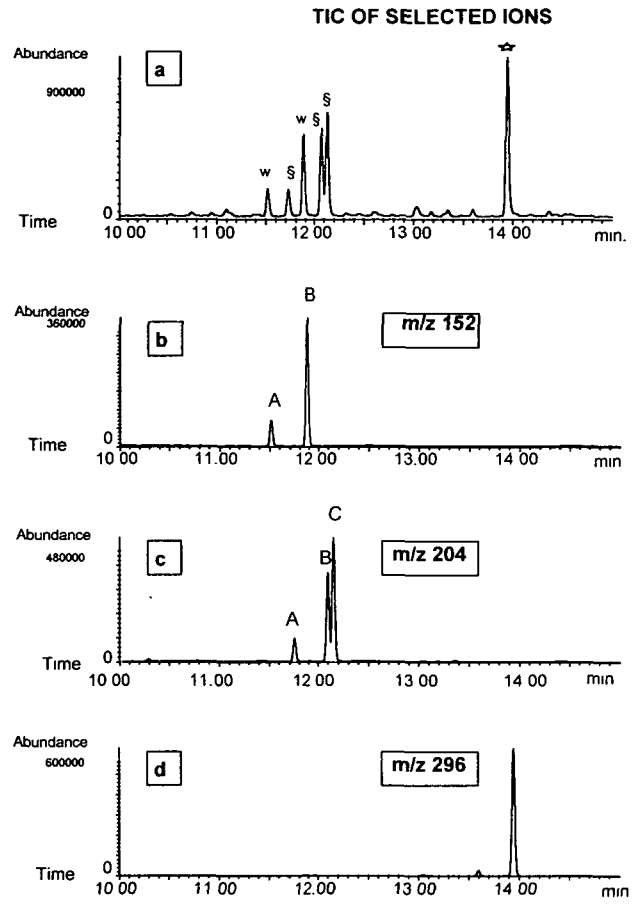


Fig. 3. **Ion chromatograms obtained on SIM analysis of a mixture of MDA, HNE, and IS.** A mixture of 50 pmol MDA, 50 pmol HNE, and 1 nmol IS was derivatized as described under "EXPERIMENTAL" and then analysed by NICI-GC-MS, focusing on the ions at *m/z* 204, 152, and 296. TIC (a) corresponds to the ion current of the three ions. ☆, IS derivative; §, isomers of the MDA derivative; w, isomers of the HNE derivative.

correlation coefficient of the regression line, the assay was linear in the complete range (0–5 $\mu$M). Further expansion of the range gave an acceptable correlation coefficient of the regression line ($r > 0.992$) up to 20 $\mu$M. Therefore, a single reference curve may be used to measure both the basal aldehyde concentration values and those found in samples after oxidative stress. However, if the concentration exceeds 20 $\mu$M, dilution of the plasma samples is required to be in the range of linearity of the method.

In order to evaluate the between-day imprecision of HNE and MDA quantification, peak area ratios determined for the various isomers were calculated with triplicate injections of plasma samples spiked with both compounds and with IS. The coefficient of variation (%CV) was lower than 10% for both HNE and MDA (Table III). The accuracy of the method is shown in Table IV as % bias between the found and theoretical concentrations in plasma samples spiked with HNE, MDA, and IS, prepared for the two calibration curves (low range and high range).

The limit of detection corresponded to the derivative formed from 0.2 pmol of each of the aldehydes. For both HNE and MDA, the injection of 1/25 of a sample (total volume, 50 $\mu$l) gave signals with intensities which were 3-5-fold the noise. This amount corresponds to that injected for the analysis of plasma samples (100 $\mu$l) spiked with 5 pmol of each of the two aldehydes (0.05 $\mu$M).

In the original paper by Luo *et al.* (*13*), pentadeuterated benzaldehyde was suggested as the internal standard. This

TABLE I. **Identification of HNE in basal plasma samples.**

| | Signal intensity ratios in plasma samples | | | |
|---|---|---|---|---|
| | Isomer A | | Isomer B | |
| | 283/152 | 333/152 | 283/152 | 333/152 |
| Spiked plasma | 0.63±0.11 | 1.37±0.64 | 0.39±0.01 | 0.056±0.004 |
| Basal plasma | 0.69±0.08 | 1.15±0.16 | 0.40±0.04 | 0.058±0.007 |

Ratios of signal intensities for isomers of HNE (A and B in Fig. 3b) in basal plasma ($n=12$), and in plasma spiked with HNE ($n=5$). Data are expressed as means±SD.

TABLE II. **Identification of MDA in basal plasma samples.**

| | Signal intensity ratios in plasma samples | | | |
|---|---|---|---|---|
| | Isomer A | | Isomers B+C | |
| | 442/204 | 281/204 | 442/204 | 281/204 |
| Spiked plasma | 0.084±0.003 | 0.103±0.012 | 0.022±0.001 | 0.073±0.004 |
| Basal plasma | 0.102±0.014 | 0.128±0.015 | 0.021±0.002 | 0.079±0.004 |

Ratios of signal intensities for isomers of MDA (A and B+C in Fig. 3c) in basal plasma ($n=12$), and in plasma spiked with MDA ($n=4$). Data are expressed as means±SD.

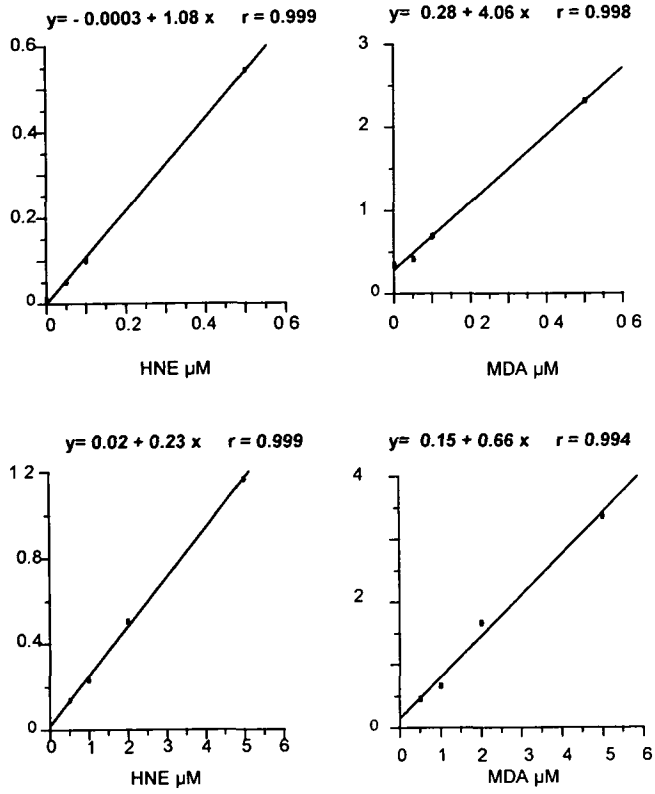


Fig. 4. **Calibration curves of HNE and MDA in spiked plasma.** The intensity ratios are plotted against $\mu$M of the tested aldehydes. The upper graphs are for the low range of calibration (0-0.5 $\mu$M with 1 nmol IS/sample), and the lower ones for the high range (0.5-5 $\mu$M with 5 nmol IS/sample). The lines are for isomer A for the HNE derivative and isomers B+C for the MDA derivative.

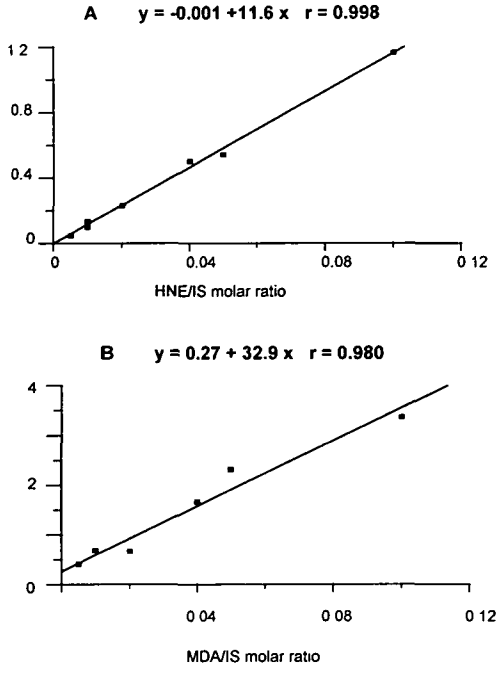


Fig. 5. **Calibration curves of HNE and MDA in plasma samples.** The intensity ratios (same results as used for the lines in Fig. 4) were plotted against the aldehyde/IS molar ratio to check the linearity in the complete range.

TABLE III. **Between-day imprecision of the NICI GC-MS method for the determination of HNE and MDA.**

| | HNE *m/z* 152 | | MDA *m/z* 204 | |
|---|---|---|---|---|
| | Isomer A | Isomer B | Isomer A | Isomers B+C |
| Day 1 | 22.6±0.46 | 144±5.0 | 76±4.2 | 687±53 |
| Day 2 | 26.0±3.45 | 147±15.5 | 76±5.9 | 713±76 |
| Day 3 | 26.0±2.22 | 153±7.9 | 83±12.6 | 682±89 |
| Mean | 24.9 | 148 | 78.3 | 694 |
| SD | 2.0 | 4.6 | 4.0 | 17 |
| C.V.% | 7.9 | 3.1 | 5.2 | 2.4 |

Within-day values are means±SD obtained with triplicate injections of a plasma sample (0.1 ml) spiked with HNE and MDA (10 pmol), and with IS (1 nmol).

TABLE IV. **Accuracy of HNE and MDA quantification.**

| Theoretical concentration ($\mu$M) | %Bias[a] | | | |
|---|---|---|---|---|
| | HNE[b] *m/z* 152 | | MDA[b] *m/z* 204 | |
| | Isomer A | Isomer B | Isomer A | Isomers B+C |
| 0.1 | 2.4 | −7.3 | −6.5 | 0.2 |
| 0.5 | 0.04 | 0.5 | 0.6 | 0.3 |
| 0.5 | 4.4 | 1.4 | −11 | −6.6 |
| 1 | −17 | −8.0 | −9.7 | – |
| 2 | 11 | −5.0 | 11 | 15 |
| 5 | −1.1 | 0.4 | 0.7 | −2.4 |

[a]Calculated as % difference between concentrations found and theoretical concentrations in spiked plasma samples. [b]For isomer identification, see Fig. 3, b and c.

compound is rather expensive. Besides, the use of a single-labeled IS for the measurement of various compounds appears not to be necessary. The use of one isotopomer for each tested compound might be the best in terms of high reproducibility of the analysis. Nevertheless, the reported results confirmed that the selected internal standard is useful for quantification of both the tested aldehydes. Since 2,5-dihydroxy-benzaldehyde is an inexpensive commercial compound, its use as the IS makes the method easy to perform in laboratories where facilities for the synthesis of stable labeled compounds are not available. Moreover, its use may be suggested also when other aldehydes have to be measured provided that interference of the IS with the tested aldehyde can be proven.

While this work was in progress, other procedures were suggested for the measurement of aldehydes derived through lipid peroxidation. Bruenner *et al.* (*15*) reported greater specificity with electron ionisation of oxime-*tert*-butyldimethylsilyl derivatives than with electron-capture ionisation of *O*-pentafluorobenzyl oxime derivatives. Despite the fact that electron ionisation is a more commonly used technique, the method described by the authors requires the use of deuterated isotopomers as ISs. De Zwart *et al.* (*16*) have reported the analysis of a number of aldehydes in urine obtained from rats treated with carbon tetrachloride. These authors suggested the use of 3-bromofluorobenzene as an internal standard and presented data on the linearity of the assay. The retention time of the derivative of the IS (≤5 min) is much shorter than that reported for MDA (>20 min). Therefore, it cannot be considered useful when the purpose of the analysis is to

TABLE V. **Aldehydes determined in basal and oxidized plasma samples.**

| Oxidation time (h) | Concentration ($\mu$M) | | |
|---|---|---|---|
| | TBARS[a] | NICI-GC-MS | |
| | | MDA | HNE |
| 0 | 0.6 | 0.2 | 0.02 |
| 1.5 | 2.9 | 2.7 | 0.06 |
| 5 | 44.7 | 44.3 | 3.8 |
| 24 | 98.1 | 83.6 | 1.9 |

The concentrations of MDA and HNE were determined by NICI GC-MS, and those of TBARS by a fluorimetric method, as described under "EXPERIMENTAL." [a]As MDA.

measure the levels of MDA and HNE in plasma.

As an example of the application of this method, we report here data obtained on analysis of plasma samples either stored in the presence of BHT to avoid oxidation or after oxidation with $Cu^{2+}$ as described in the experimental section (Table V). Samples were analysed both with NICI-GC-MS and with the fluorimetric detection of aldehyde derivatives obtained with DETBA (*14*). The latter method was claimed to be specific because it reduces or eliminates the effects of compounds with spectra dissimilar to that of the MDA-DETBA adduct with the synchronous fluorescence technique (*14*). An increase in the MDA concentration with the oxidation time was observed with both methods. Nevertheless, both the basal values and those at 90 min were lower for the NICI-GC-MS evaluation. The difference may be due to the known formation of MDA from lipoperoxides during sample preparation (*6*), caused by the rather high temperature and low pH for the fluorimetric method. The difference disappears with longer reaction times, when the amount of lipoperoxides oxidised during sample preparation is likely to become irrelevant compared to the high amount of MDA formed during oxidation.

In order to check the possibility of applying the method for the analysis of plasma samples obtained from pathological subjects, we analyzed extracts from hypercholesterolemic and diabetic rats. Ion chromatograms did not show additional peaks, as compared to those shown in Fig. 3 obtained on analysis of plasma samples.

In conclusion, we report here a modified procedure for measurement of the plasma levels of MDA and HNE by NICI-GC-MS. The procedure appears to be simple enough to be applied to the analysis of several samples at one time.

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