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Determination of malondialdehyde in human blood by headspace-solid phase micro-extraction gas chromatography–mass spectrometry after derivatization with 2,2,2-trifluoroethylhydrazine

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ABSTRACT

Malondialdehyde (MDA) has been proposed as a useful biomarker of lipoperoxidation in biological samples, and more developed analytical methods are necessary. A simple and sensitive gas chromatography–mass spectrometry (HS-SPME-GC–MS) was described for the determination of malondialdehyde (MDA) in blood. Acetone-d_6 was used as internal standard. MDA and acetone d6 in blood reacted for 40 min at 50 °C with 2,2,2-trifluoroethylhydrazine in headspace vial and simultaneously the formed TFEH derivatives were vaporized and adsorbed on polydimethylsiloxane-divinylbenzene (PDMS-DVB). The compounds were desorbed for 1 min at 240 °C and injected in GC–MS. The reaction solution showed good recoveries at pH 4.0. In the established condition, the method detection limit (MDL) was 0.4 μg/L in 0.1 mL blood sample and the relative standard deviation was less than 8% at the concentration of 25.0 and 50.0 μg/L. The mean concentrations of MDA in normal human blood (n = 20) were measured to be 187.9 μg/L (2.61 μmol/L).

1. Introduction

Oxidative stress caused by reactive oxygen species (ROS) damages DNA, lipids and proteins and has attracted a great deal of interest in relation to the etiopathogenesis of several chronic diseases and the aging process [1–14]. A great variety of aldehydes are formed from peroxidized polyunsaturated fatty acids. Malondialdehyde (MDA), three-carbon compound, is one of the major oxidation products, and reactive toward amino groups of proteins and nucleic acids. Therefore, MDA has been inferred to have mutagenic and cytotoxic effects. Due to its relation to free radical damage, MDA has been proposed as a useful biomarker of lipoperoxidation in biological and medical science.

Many assay to determine MDA are based on the reaction with 2-thiobarbituric acid (TBA) [15–30,31]. But TBA reacts with a number of chemical species (sugars, nucleic acids, amino acids, proteins, phospholipids and aldehydes) [32,33] to produce a pink chromophore that can be measured by HPLC-UV or fluorescence detection. Furthermore, the treatment of biological samples to obtain the condensation product is usually carried out in acidic media and at high temperature (about 100 °C) and may generate further oxidation of the matrix with overestimation of the results [34]. To overcome the biases from derivatization of MDA, another derivatizing method was developed, which was based on its reaction with 2,4-dinitrophenylhydrazine (DNPH) at low pH with the formation of DNPH–hydrazone derivatives [35–39]. Phenylhydrazine (PH) [40,41] and 2,4,6-trichlorophenylhydrazine (TCPH) [42] were used based on the same reaction principle with DNPH to form the volatile products. The derivatization requires mild reaction conditions at low temperature, but is not as quick as the TBA assay, and also it involves long extraction procedures and purification of DNPH reagent daily. Recently, specific and highly sensitive method for MDA analysis has been developed relying on HS-SPME and DNPH derivatization [43,44]. In this extraction technique, MDA was adsorbed from a gaseous phase onto the fiber loaded with derivatization reagent, and the derivatives were later thermally desorbed in the injector of GC. The method has many advantages of convenient and rapid extraction, also drawbacks of the low recovery due to low volatility of MDA, and long loading time of SPME fiber with TCPH.

The purpose of the present study was to develop a method of measuring MDA in human blood, based on derivatization with 2,2,2-trifluoroethylhydrazine (TFEH) and detection using HS-SPME-GC–MS, do not involve long preparation of the sample and sample boiling and therefore will avoid the possible artifactual production of MDA. TFEH, a very volatile hydrazine, was tested as a reagent for the formation of volatile hydrazone derivatives to improve low volatility of MDA. Derivatization was performed by the reaction of MDA and TFEH in blood in headspace vial and the

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formed volatile TFEH–hydrazone was vaporized, and simultaneously adsorbed in fiber, and then desorbed in GC–MS.

2. Experimental

2.1. Materials

All organic solvents were used HPLC grade. Sodium chloride, 2,2,2-trifluoroethylhydrazine (70 wt% solution in water), 1,1,3,3-tetraethoxypropane (99%) and acetone-d_6 as internal standard were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Apparatus

All mass spectra were obtained with an Agilent 6891/5973N instrument. The ion source was operated in the electron ionization mode (EI; 70 eV). Full-scan mass spectra (m/z 30–300) were recorded for analyte identification. An HP-InnoWax capillary column (30 m × 0.25 mm I.D. × 0.25 μm film thickness) was used. Samples were injected in the splitless mode. The flow rate of helium as carrier gas was 1.0 mL/min. The injector temperature was set as 240 °C. The oven temperature programs were: initial temperature of 40 °C (held for 1 min) then increase to the final temperature of 150 °C at 30 °C/min. The ions selected by SIM were m/z 81, 131 and 150 for MDA–TFEH and m/z 62, 91 and 160 for acetone-d_6–TFEH (internal standard).

2.3. Preparation of MDA standards

1,1,3,3-Tetraethoxypropane (TEP) was used to prepare a malondialdehyde stock solution. A volume of 10 μL of TEP was accurately diluted to 10 mL with 0.1N HCl in a screw-capped test tube and incubated in boiling water bath for 5 min and then rapidly cooled with tap water (stock solution) [45]. A working solution of MDA was prepared by pipetting 1 mL of the hydrolyzed acetal (stock solution) into a 100 mL calibrationed flask and diluted to volume with ultrapure water. The working solution was 1.0 mg/L MDA.

2.4. SPME fibers

The commercially available SPME fibers were purchased from Supelco (Bellefonte, PA, USA), and 100 μm-polydimethylsiloxane (PDMS), 65 μm-polydimethylsiloxane-divinylbenzene (PDMS-DVB), 85 μm-polyacrylate (PA), 85 μm-carboxen-polydimethylsiloxane (CAR-PDMS) fused-silica fibers were evaluated for the determination of MDA. Fibers were initially conditioned according to the manufacturer's instructions in order to remove contaminants and to stabilize the solid phase. Conditioning was carried out in an extra split/splitless port with helium carrier gas prior to each adsorption.

2.5. Extraction/derivatization procedures

Sample preparation (extraction and derivatization) was carried out in 10 mL headspace vials with carried-lined screw caps. To a solution containing 0.1 mL of blood, 4 mL of NaCl saturated solution, 50 μL of MDA (1.0 mg/L), 200 μL of TFEH solution (70 mg/L) were added. A one-step derivatization/adsorption was carried out in a headspace vial with continuous shaking, and then desorption process was performed at temperature of 240 °C. Derivatization was performed for different SPME adsorption-times (10, 20, 30, 40, 50, 60 and 80 min) at different temperatures (35, 40, 50, 60 and 70 °C) and at different pH (1.0, 2.0, 3.0, 4.0, 5.0, 7.0 and 9.0). pH of the solution was controlled using 1.0N HCl solution or 1.0N KOH solution.

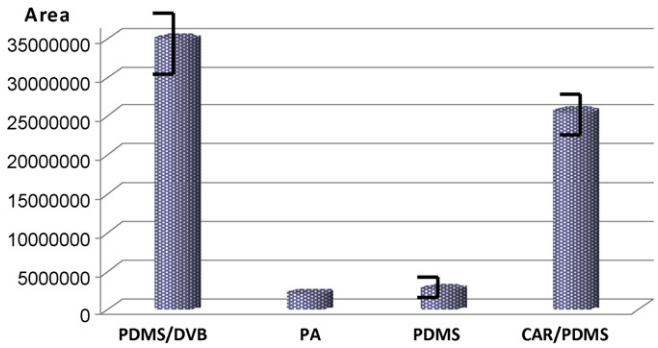

Fig. 1. Extraction efficiencies of TFEH–hydrazone according to SPME fiber.

The optimum conditions of derivatization of MDA were determined by the amounts of the formed TFEH–hydrazone.

2.6. Calibration and quantification

Calibration curve for MDA was established by derivatization after adding 25.0, 50.0, 125.0, 250.0 and 500.0 μL of MDA (0.01 mg/L) and 50.0 μL of acetone-d_6 (0.1 mg/L) in 0.1 mL of blood. The corresponding concentrations of standards were 2.5, 5.0, 12.5, 25.0 and 50.0 μg/L in 0.1 mL blood. The ions selected for quantification were m/z 150 for MDA and m/z 160 for acetone-d_6 as internal standard. The ratio of the peak area of standard to that of internal standard was used in the quantification of the compound.

3. Results and discussion

3.1. Selection of SPME fiber

Four SPME fibers were evaluated to select the suitable fiber for the MDA detection. The extraction condition was 40 min at 50 °C, pH 4 and the desorption condition was 1 min (splitless mode) at 240 °C. The adsorption efficiencies on the SPME fibers were evaluated by comparing the areas of MDA derivative (Fig. 1). The best efficiency was obtained on polydimethylsiloxane-divinylbenzene (PDMS-DVB) among the four fibers (Fig. 1), it was thought because of best interaction of the formed hydrazone ring with divinylbenzene fiber. Therefore, PDMS-DVB was selected as the suitable fiber for the MDA detection.

3.2. Optimization of derivatization conditions

The reaction pH of the MDA with TFEH was studied. The derivatives were tested at pH 1.0, 2.0, 3.0, 4.0, 5.0, 7.0 and 9.0. The other reaction conditions were: reaction temp and time was 40 min at 50 °C, the desorption temp and time was 1 min at 240 °C. The reaction rate of MDA with PFPH was determined by the detection of the product; MDA–TFEH. The results showed good yield in the pH range of 3–5 (Fig. 2).

The reaction temperature of the MDA with TFEH was studied. The derivatives were analyzed at reaction temperature 35, 40, 50, 60 and 70 °C. When the optimal reaction temperature of MDA with TFEH was determined by the yield of the product; MDA–TFEH, it was 50 °C (Fig. 3).

The reaction time of the MDA with TFEH was studied. The derivatives were analyzed at reaction times 10, 20, 30, 40, 50, 60 and 80 min. When the optimal reaction time of MDA with TFEH was determined by the yield of the product, it was obtained in 40 min (Fig. 4).

Conclusively, the maximum yield of MDA with TFEH in blood was obtained after the reaction for 40 min at temperature 50 °C and pH 4.0.

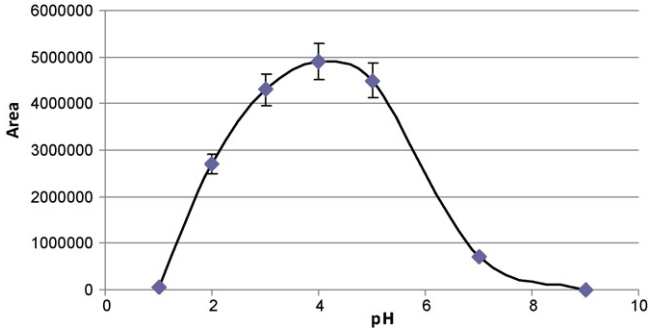

Fig. 2. The effect of reaction pH of MDA with TFEH.

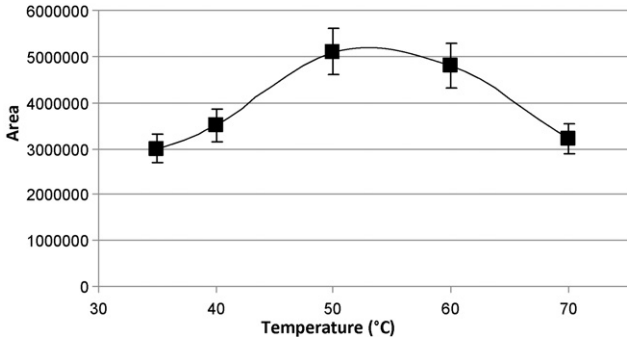

Fig. 3. The effect of reaction temperature of MDA with TFEH.

3.3. Chromatography

The optimum derivatization conditions were applied to the analyses of MDA with TFEH. Fig. 5 shows GC–MS chromatogram after the derivatization of MDA and acetone-d_6 in blood. For the GC separation of the derivatives, the use of a semipolar stationary phase was found to be efficient. The derivatives were well separated from the peaks of the background compounds of blood. The retention times of the acetone-d_6–TFEH and MDA–TFEH were 4.15 and 4.68 min, respectively. There was no extraneous peak observed in the chromatograms at the retention times of analytes.

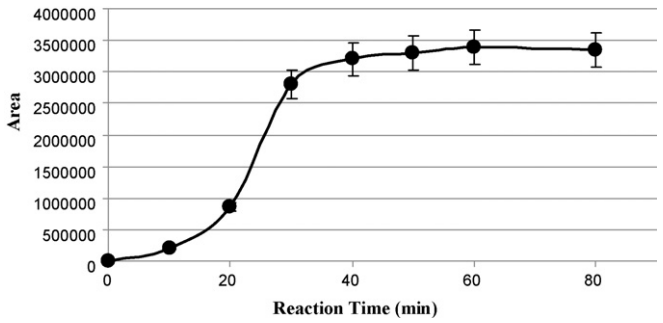

Fig. 4. The effect of SPME adsorption-times of MDA with PFPH.

3.4. Mass spectrometry

The mass spectra of MDA–TFEH and acetond-d_6–TFEH by electron impact ionization at 70 eV are shown in Figs. 6 and 7. The molecular ion at m/z 150 and diagnostic ions at m/z 69, 81 and 131 of Fig. 6 indicated that MDA was derivatized to corresponding MDA–TFEH. The fragment of m/z 131 is accounted for the loss of a fluorine atom from the molecular ion. The fragments of m/z 69 and m/z 81 are accounted for itself of a [CF_3^+] and the loss of a [CF_3^+] from the molecular ion, respectively. The molecular ion at m/z 160 and diagnostic ions at m/z 62, 91 and 142 of Fig. 7 indicated that acetond-d_6 was derivatized to corresponding acetone-d_6–TFEH. The fragment of m/z 142 is accounted for the loss of a [CD_3] from the molecular ion. The fragments of m/z 62 and m/z 91 are accounted

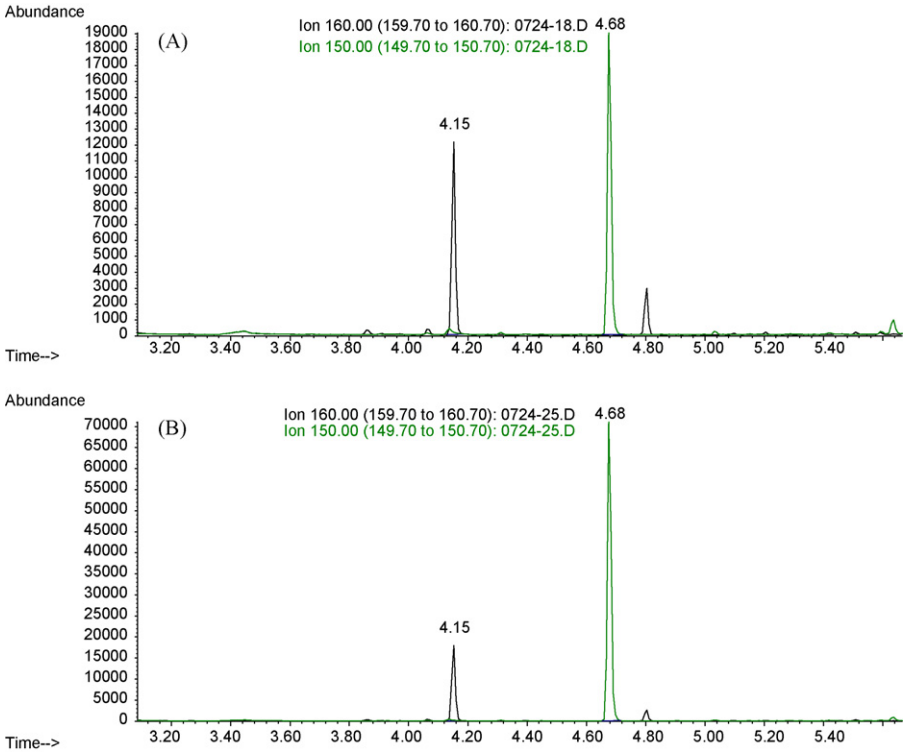

Fig. 5. GC–MS chromatogram of the derivative of (A) control sample, (B) sample spiked in concentration of MDA 50 ng/mL in control sample (4.15 min: acetone-d_6–TFEH, 4.68 min: MDA–TFEH).

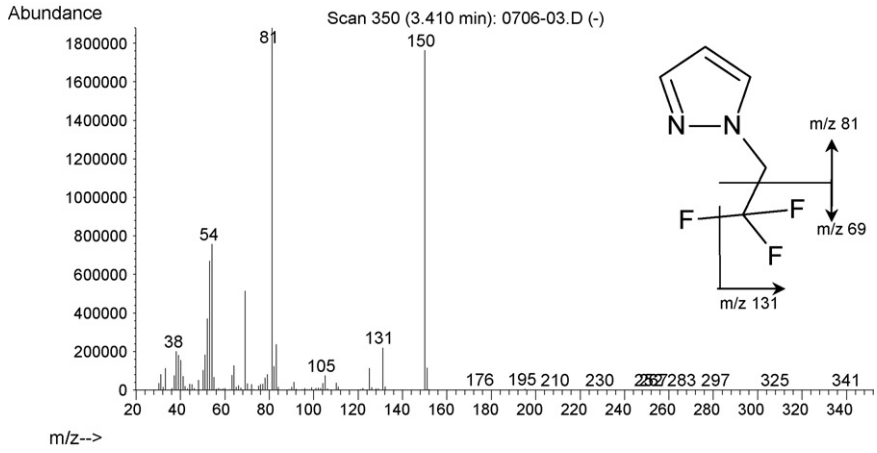

Fig. 6. Mass spectrum of MDA–TFEH.

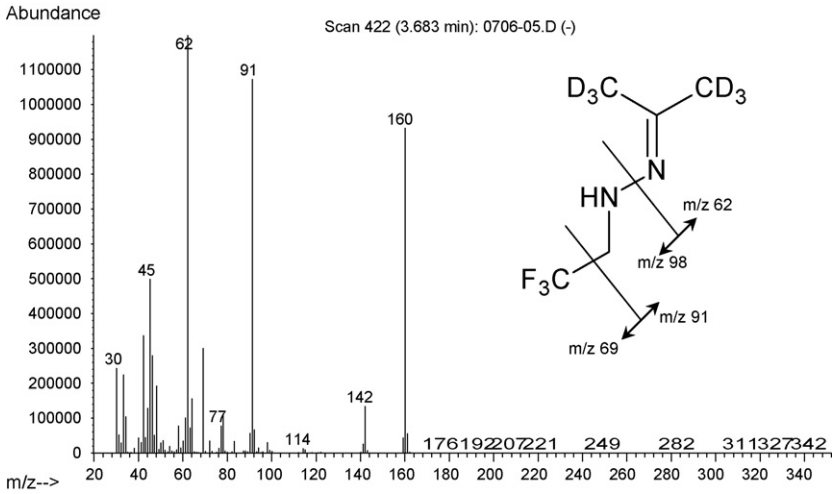

Fig. 7. Mass spectrum of acetone-d_6–TFEH.

for the loss of a [$CF_3CH_2NH^+$] and a [CF_3^+] from the molecular ion, respectively.

3.5. Method detection limits

The method detection limit (MDL) was defined by 3.14 times of standard deviation for replicate determinations ($n = 7$) from samples spiked at the concentration of 0.5 μg/L in blood [46], in which MDL was calculated to 0.4 μg/L. The combination of high derivatization yield and the high sensitivity of the derivative by EI-MS (SIM) permit their determination of MDA at concentrations well below those (0.86 μg/L [21], 3.6 μg/L [24], 14.4 μg/L [37]) cited in this paper.

3.6. Linearity

Examination of typical standard curve by computing a regression line of peak area ratios of MDA–TFEH to acetone-d_6–TFEH on concentration using a least-squares fit demonstrated a linear relationship with correlation coefficients consistently higher than 0.999. The line of best fit for MDA is $y = 0.0085x + 0.0127$ over a range of 5–100 μg/L, where x is the analyte concentration (μg/L) and y is the peak area ratio of the analyte to internal standard. The standard deviation of slope and intercept of calibration curve were consistently lower than 0.0006 and 0.0008, respectively.

3.7. Precision and accuracy

The precision and accuracy of the assay was very good, as shown in Table 1. For five independent determinations of samples spiked in control blood in the concentration of 25.0 and 50.0 μg/L, the accuracy was 99.2–99.4% and the relative standard deviation was less than 8%.

Table 1
Precision and accuracy of MDA.

Compound	Spiked conc. (μg/L)	Measured conc. (μg/L)	Mean ± SD (RSD)[a] (μg/L)
MDA	25.0	23.4, 25.1, 23.6, 26.4, 25.3	24.8 ± 1.3 (5.1)
	50.0	45.3, 46.9, 54.2, 48.9, 53.0	49.7 ± 3.8 (7.7)

[a] SD = standard deviation; RSD = relative standard deviation.

Table 2
Comparison of MDA levels in biological samples.

Reference	Biological sample	Mean ± SD (μg/L)
This	Blood	187.9 ± 105.6 (n = 20)
[18]	Plasma	49.7 ± 9.4 (n = 10)
[24]	Plasma	77.5 ± 44.9 (n = 25)
[42]	Plasma	49.0 ± 12.2 (n = 10)

3.8. Applications

This paper was designed to describe the determination method of MDA in human blood by HS-SPME-GC–MS as biomarker for oxidative stress. We analyzed MDA in blood samples of healthy volunteers. In the analytical results, MDA was detected in the concentration range from 75.4 to 544.2 μg/L in blood samples (Table 2).

4. Conclusions

In this paper we present a simple method for the determination of MDA in human blood based on a derivatization with TFEH and HS-SPME. The method does not require rigorous conditions which may lead to the formation of artefacts, it is sensitive for the determination of low level of MDA. The MDL of MDA was 0.4 μg/L in 0.1 mL blood. The accuracy and precision of the assay was very good. The relative standard deviation was less than 8%. The mean concentration of MDA in blood was measured to be 187.9 μg/L (2.61 μmol/L) in range of 75.4–544.2 μg/L.

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