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Journal of Chromatography B, 775 (2002) 121–126

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Short communication

Rapid, fluorimetric–liquid chromatographic determination of malondialdehyde in biological samples

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Received 8 March 2002; received in revised form 24 April 2002; accepted 25 April 2002

Abstract

Current chromatographic methods of estimation of malondialdehyde, a marker of oxidative lipid injury, often require extensive extraction procedures, column cleaning or specialized equipment. A rapid and sensitive HPLC method is described for the determination of MDA in plasma and urine. The mobile phase consisted of 40:60 ratio (v/v) of methanol to 50 m*M* potassium monobasic phosphate at pH 6.8, pumped at a rate of 1.0 ml/min on a Hewlett-Packard Hypersil 5 μ ODS 100×4.6 mm placed in a column warmer set to 37 °C. Samples of plasma and urine were treated with the antioxidant, butylated hydroxytoluene and heat derivatized at 100 °C for 1 h with thiobarbituric acid at an acid pH. Samples were extracted with *n*-butanol and 10 μl of the extract was injected at 1 min intervals using an autosampler. The Hewlett-Packard model 1046A programmable fluorescence detector was set at excitation of 515 nm and emission of 553 nm. Retention time was 1.87 min, however absence of interfering peaks, allowed analysis to be carried out in increments of 1 min per sample. Within day variability in estimation was between 8.6% and 10.3%. Between days variability was 3.6–7.9%. Recovery was between 88 and 101%. Samples of urine and plasma from ten normotensive volunteers were 1.94±0.79 μmol/g creatinine and 0.69±0.13 μmol/l respectively and were similar to those reported in the literature. MDA degrades at room temperature at a rate of 10% per hour. It is therefore, suggested that the total assay time be limited to 1 h beginning with sample preparation.

Keywords: Malondialdehyde

1. Introduction

Oxidative stress has emerged as a major pathophysiologic mechanism in mediating various disease states and a wide variety of tools are available to monitor this stress [1]. The formation of lipid hydroperoxides by oxidative lipid damage leads to dysfunction of membrane-bound receptors, and these compounds possess cytotoxic and mutagenic properties which are thought to play a major role in ageing and atherosclerosis [2]. One such byproduct of lipid peroxidation, malondialdehyde (MDA), is formed by β-scission of peroxidized polyunsaturated fatty acids and is commonly measured by derivatization with thiobarbituric acid (TBA) to yield a red compound [3]. The colorimetric test is simple, however, it has low sensitivity and low specificity due to interfering chromogens [3]. HPLC-based assays of MDA–TBA adduct are more specific, but have various drawbacks. For example, some HPLC

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1570-0232/02/$ – see front matter
PII: S1570-0232(02)00273-8

assays require extensive column regeneration procedures and have a short column life [4]. Others require 30 min washing of the column after each injection [5]. Furthermore, these UV based assays often have interfering peaks [4]. Some authors have suggested protein precipitation with acetonitrile [6] or phosphotungstic acid [7] during sample preparation. MDA is mainly protein bound in vivo [3] and removal of protein through precipitation prior to analysis may artifactually reduce levels. Extensive sample preparation requiring pelletization, resuspension and washing cycles may result in sample loss as well [7]. Use of cartridges to isolate the MDA–TBA adduct may introduce additional variability especially in the absence of an internal standard [8]. This drawback is also shared by liquid–liquid extraction. An extensive analysis of various extracting agents demonstrated that *n*-butanol was found to be the most desirable choice, however prolonged time periods are needed for evaporation [9]. It is unknown if *n*-butanol extracts can be injected directly on the column or if ethyl acetate, a more volatile solvent, can serve as a reasonable alternative to *n*-butanol. A new method that utilizes methyl-MDA as internal standard has recently been described and uses capillary zone electrophoresis [10] and others have reported methods that have used fluorimetric detection systems for analysis [11–13]. Thus, a method that is rapid, sensitive and allows a large throughput is needed for the determination of MDA in biological samples. The purpose of the present experiment was to develop such a test for analysis of MDA and characterize its performance in human biological samples.

2. Experimental

2.1. Reagent preparation

Ethanol (95%) and HPLC grade methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA), all other chemicals were purchased from Sigma (St. Louis, MO, USA). Chemical solutions were prepared using distilled deionized water unless otherwise indicated.

Butylated hydroxytoluene (BHT) solution was prepared in 95% ethanol to a final concentration of 0.05% BHT. A 0.44 *M* phosphoric acid solution (H_3PO_4) was obtained by diluting 1 ml concentrated phosphoric acid to 100 ml final volume. 2-thiobarbituric acid (TBA) was dissolved in water on a stirring hot-plate at 50–55 °C to a concentration of 42 m*M*. Forty percent ethanol solution was obtained by diluting 420 ml 95% ethanol to final volume of 1000 ml.

2.2. Standards

Standards and quality control samples were prepared using 97% 1,1,3,3-tetraethoxypropane (TEP) with the stock standard solution containing 5 μ*M* TEP in 40% ethanol solution. Standards were prepared through serial dilution of the stock standard with ethanol solution to obtain concentrations of 5, 4, 2, 1, 0.5, 0.25, 0.125 and 0 (blank) μ*M* TEP. The standard curve was prepared freshly for analysis each day as well as quality control samples 0.125, 0.5 and 2.0 μ*M* TEP.

2.3. Sample preparation

Sample derivatization was carried out in 2 ml capacity plastic centrifuge tubes fitted with screw-on caps. To a 50 μl aliquot of sample or TEP standard, 50 μl BHT solution, 400 μl H_3PO_4 solution, and 100 μl TBA solution were added. Sample tubes were capped tightly, vortex mixed, then heated for 1 h on a 100 °C dry bath incubator. Following heat derivatization, samples were placed on an ice-water (0 °C) water bath for 5 min to cool, with 250 μl *n*-butanol subsequently added to each vial for extraction of the MDA–TBA complex. Tubes were vortex mixed 5 min then centrifuged 3 min at 14 000× min^{-1} to separate the two phases. Aliquots of 100 μl were removed from the *n*-butanol layer of each sample and placed in HPLC vials for analysis without evaporation.

2.4. Chromatographic apparatus and conditions

The chromatographic system consisted of a Hewlett-Packard Chromatographic Series 1100 autosampler and isocratic pump and Hewlett-Packard model 1046A programmable fluorescence detector (Palo Alto, CA, USA). The autosampler was programmed

to inject 10 μl of each sample at 1 min intervals. Pump flow-rate was 1.0 ml/min with mobile phase comprised of methanol–buffer (40:60, v/v). The buffer was 50 m*M* potassium monobasic phosphate (anhydrous) with an adjusted pH of 6.8 using 5 *M* potassium hydroxide. The fluorescence detector was set at an excitation wavelength of 515 nm and emission wavelength of 553 nm. Photomultiplier tube and lamp flash frequency were optimized to give the most signal-to-noise ratio. The column was a Hewlett-Packard Hypersil 5 μ ODS 100×4.6 mm with a 5 μ ODS guard column (Altech Associates, Deerfield, IL, USA) placed in a column warmer set to 37 °C. Peak areas were determined using a ChromJet integrator (Thermo Separation Products, San Jose, CA, USA).

2.5. Assessment of recovery, extracting agent and adduct stability

Increasing amounts of TEP (0, 0.5, 1 and 2 μmol/l) were supplemented in water or plasma specimen from a single patient and extracted with either ethyl acetate or *n*-butanol. *N*-butanol extraction was performed as described above. For ethyl acetate extraction, 500 μl of the solvent was added to the derivatized sample, vortexed, centrifuged and supernatant transferred to a microcentrifuge tube. This was carried out three times followed by vacuum evaporation of supernatant and reconstitution in water.

In order to evaluate MDA–TBA adduct stability, the standard curve was re-injected 2.3 h following initial injection. Additional samples were injected at timed intervals to calculate the decay constant by first order reaction models.

2.6. Calculations

Standard curves were created by linear regression of peak area obtained by integration versus known concentrations of MDA. Concentrations of quality controls and unknown samples were estimated by applying the linear regression equation of the standard curve to the unknown sample peak-area.

The recovery of MDA was estimated by calculating the percent difference between known concentrations of MDA and that obtained by applying the linear regression equation [(observed−expected)× 100%/expected concentration]. Inter-day and intra-day coefficient of variation were obtained by one-way analysis of variance at each of the three concentrations of MDA, 0.125, 0.5 and 2 μmol/l. The limit of detection was calculated by the method of Anderson [14].

The decay rate was calculated using a first order decay model. The ratio of initial to re-injected standard curve slopes was log transformed then divided by elapsed time to yield the elimination rate constant. Fitting a slope to timed injections of MDA confirmed the first order reaction of the decay.

2.7. Subjects

The study was approved by the Institutional Review Board for Human Subjects at Indiana University. Urine and plasma samples were obtained after written informed consent in 10 normotensive volunteers attending a Veterans Administration outpatient medicine clinic. Urine samples were collected without preservatives and plasma was collected under EDTA and frozen at −86 °C until analysis.

3. Results and discussion

3.1. Chromatography

Chromatograms of a TEP standard and that from urine are shown in Fig. 1. Because of the short retention time and the absence of interfering peaks, rapid injections of specimens was possible.

Sample preparation and chromatographic conditions had to be adapted to meet certain criteria. MDA is bound to proteins [3,15] and if a protein precipitation step, such as with trichloroacetic acid, is carried out prior to derivatization, risk of losing the analyte of interest exists. Furthermore, not removing proteins from derivatized samples, would either necessitate extensive column washing following each analysis or result in a short column life. In this experiment protein was not precipitated and samples were derivatized after addition of butylated hydroxytoluene to prevent de novo formation of MDA. The derivatized sample was then extracted with *n*-butanol. Thus, the present method should be consid-

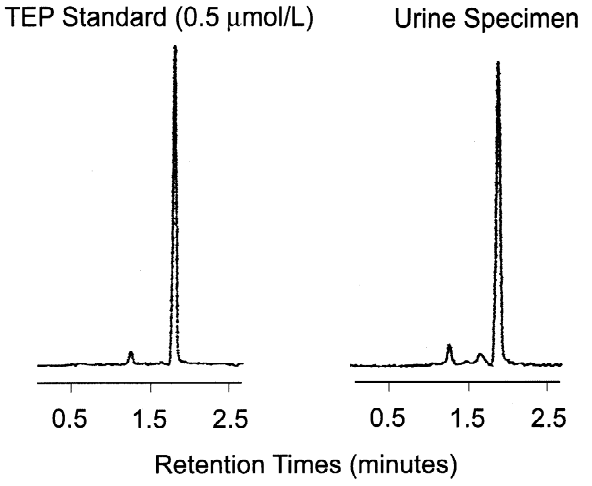

Fig. 1. Chromatogram obtained with 1,1,3,3-tetraethoxypropane (TEP) in double distilled water after derivatizing with thiobarbituric acid (left) and after derivatizing urine of a volunteer with thiobarbituric acid.

ered a measure of total content of MDA in the sample.

3.2. Linearity and extraction solvent

Calibration curves were created for seven different concentrations of MDA (0–5 μ*M*/l) by plotting the peak area versus the nominal MDA concentration. The correlation coefficient was 0.99 or better (Fig. 3). There was a small positive intercept noted in the blank sample, as has been reported by other authors.

There was a highly significant linear relationship between MDA concentration in water and plasma and peak area obtained by fluorescence (Fig. 2A). Furthermore, the slopes of peak area to MDA concentration were parallel in plasma and water when *n*-butanol was used as an extracting solvent (Fig. 2A). This suggests that no matrix effect exists with *n*-butanol used as an extracting solvent. However, plasma extracted with ethyl acetate did not have the expected positive intercept. Furthermore, ethyl acetate was not able to extract the MDA–TBA adduct efficiently from either plasma or water matrix. (Fig. 2B).

3.3. Recovery, limit of detection, and intra-day and inter-day coefficient of variation

Within assay and between assays coefficient of variation for three different concentrations is reported in Table 1. Within day variability in estimation was between 8.6 and 10.3%. Between days variability was 3.6–7.9%. Recovery was between 88 and 101%. The limit of detection was noted to be 0.128 μmol/l. Although a clear peak was noted at 0.125 μmol/l, a significant peak was also noted in

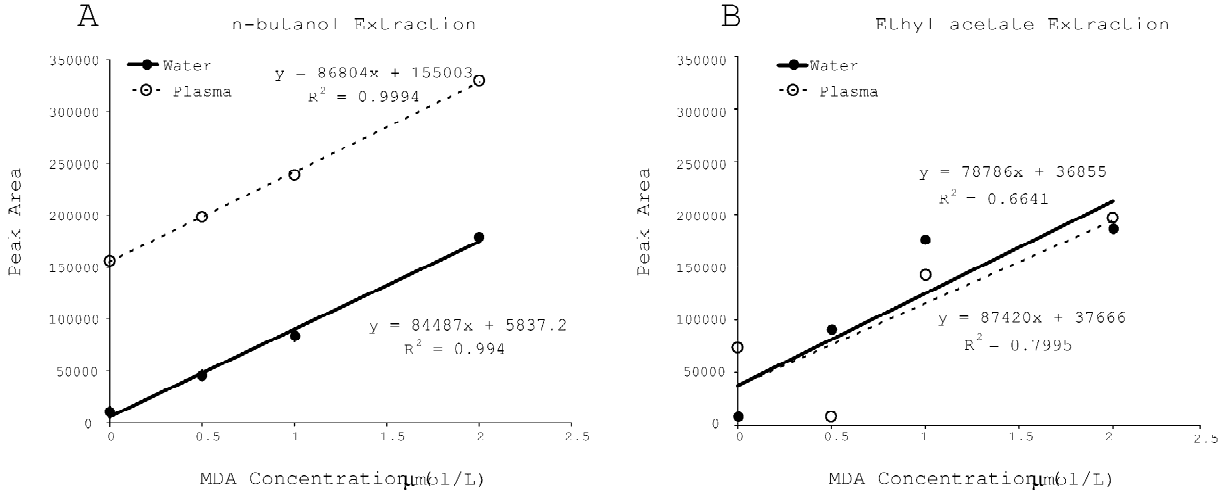

Fig. 2. Extraction efficiency of *n*-butanol and ethyl acetate are shown. In panel A, there is a strong linear relationship between MDA concentration in water as well as plasma. The slopes of plasma and water are parallel but with a positive intercept. Extraction with ethyl acetate is erratic and the plasma sample identical to *n*-butanol does not show a positive intercept. Thus, *n*-butanol is the superior extracting reagent.

Table 1
Recovery and variability of malondialdehyde measurements

Concentration of MDA (μmol/l)	Intra-day coefficient of variation (%)	Inter-day coefficient of variation (%)	Recovery (%±SD%)
0.125	10.3	4.6	99±12
0.5	8.6	7.9	101±9
2.0	10.8	3.6	88±9

the blank sample thus increasing the limit of detection.

A decay in the MDA–TBA adduct was noted to occur (Figs. 3–4). On re-injecting the same sample at timed intervals, or the entire standard curve at 2.3 h there was a first order breakdown noted. From the standard curve, this decay constant was calculated to be $-0.101\ h^{-1}$.

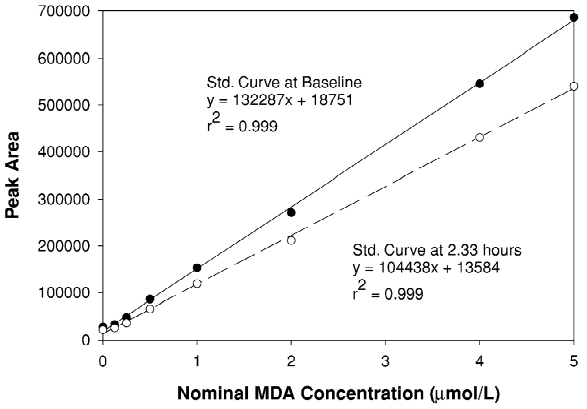

Fig. 3. Standard curve of malondialdehyde shows a highly significant linear relationship. Re-injection of the same standard curve after 2.33 h shows a highly significant linear relationship, except the slope is less steep. This suggests degradation of MDA (see Fig. 4).

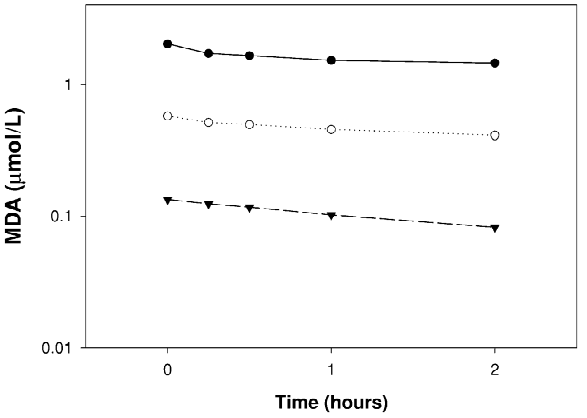

Fig. 4. Samples containing 2, 0.5 and 0.125 μmol/l MDA were injected at timed intervals and plotted on a log scale. A log–linear decline in the concentration is noted, which explains the reduced slope seen in Fig. 3.

3.4. Human studies

Ten male Veterans attending the medicine outpatient clinic for various ailments were recruited for the study. They underwent a single 24 h ambulatory blood pressure monitoring to assure normal blood pressure and serum chemistries to assure normal renal function. The average age was 53±14 years, ambulatory BP 116.7±10.9/72.4±7.1 mm Hg, heart rate 72.6±9.6, body weight 90.6±22.4 kg and height 71±3.4 inches. Three were smokers, nine were Caucasian with one African–American. The concentration of MDA in the plasma of normal volunteers was 0.69±0.13 μmol/l and is similar to the 0.60±0.13 obtained by Wang et al. [4] and Suttnar et al. [6]. Urine concentration of MDA in normal volunteers was 1.94±0.79 μmol/g creatinine which is higher than 0.89±0.35 nmol/mg creatinine reported by Knight et al. in 121 healthy men [16]. Obesity, comorbid conditions, diet, smoking, and older age of our population may account for these differences. However, Guichardant et al. reported MDA level by a fluorimetric HPLC method of 0.23±0.02 μmol/mmol creatinine in healthy volunteers. Our results expressed in the same units were 0.22±0.089 μmol/mmol creatinine [8]. Thus, methodologic differences may account for the variability between studies.

4. Conclusions

In conclusion, we report a new method for measurement of MDA that overcomes the current shortcomings of extensive column washes, reduced col-

umn life and protein precipitation steps. *N*-butanol was found to be a suitable extracting solvent. The current method is rapid and is reproducible both intra-day and between days. However, because MDA degrades approximately 10% per hour the best performance of the assay requires analyzing samples within an hour of derivatization.

Acknowledgements

Nursing assistance of Rebecca R. Lewis, R.N. is gratefully acknowledged.

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