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Short Communication

Malondialdehyde measurement in urine

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Abstract

Malondialdehyde (MDA) is an end product of lipid peroxidation and is a frequently measured index of these processes. The thiobarbituric acid (TBA) test is commonly used to measured MDA, but its specificity is questionable due to the presence of interfering chromogens. Wade and van Rij described in 1988 a method which removes these chromogens by HPLC. However, the sensitivity and the resolution of this method was not adequate for measurements of MDA in urine. We have improved this method by replacing TBA with diethylthiobarbituric acid (DETBA). The less polar MDA–DETBA complexes were isolated on Bakerbond cartridges and quantified by HPLC without interference. MDA was detectable using a fluorescence or ultraviolet detector at picomole levels. This technique was applied to urine samples obtained from ten burns patients on different days following their hospitalization. Urinary MDA in burns patients was very high and reached 18.6 μmol/mmol creatinine in one patient compared with a mean value of 0.23 μmol/mmol creatinine in healthy controls. Maximum MDA levels were attained on the third day for the majority of patients and remained, on average, much higher than normal even after 20 days. Using this method, picomole quantities of MDA can be easily and specifically detected in urine samples. This method is useful for assessing an oxidative stress.

1. Introduction

Malondialdehyde (MDA) is one of the most widely studied products of lipid peroxidation. Its presence in foods or biological samples is important as it is an indicator of the overall sum of lipid peroxidation which has occurred. In urine its presence reflects the lipid oxidation products coming from the diet and formed in the tissues [1,2]. Lipid peroxidation induced tissue damage has been implicated in many nutritional, physiological and pathological conditions.

More recently conjugated MDA compounds were identified in urine: N-(2-propenal)ethanolamine [3], N-(2-propenal) serine [4], N-epsilon-(2-propenal)lysine (the major urinary metabolite of MDA) [5–7], and guanine-malondialdehyde [8]. These urinary compounds resulted from MDA reactions with amino acids [9–11], proteins [12–14] and nucleic acids [15,16].

The thiobarbituric acid (TBA) test has been widely used to measure lipid oxidation since it was introduced by Kohn and Liversedge [17] in 1944. The red complex structure proposed by Sinnhuber *et al.* [18] and confirmed by Nair and

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Turner [19] was an 2:1 adduct of TBA and MDA which consist of two spectrally equivalent tautomeric structures. However, the specificity of the TBA reaction is questionable [2,20,21] because of the presence of interfering chromogens. Recently Wade and Van Rij [22] published a method applicable to biological samples which eliminated these interfering chromogens by isolating and quantifying these MDA–TBA chromogens by HPLC. Our experience showed that the resolution was not high enough for urine samples. We replaced the TBA reagent by diethyl TBA (DETBA) which has already been used for MDA determinations in lipids extracted from plasma [23]. A cleanup and a concentrating step was added to improve both selectivity and sensitivity. Urines of 10 burns patients and 6 controls studied by Berger *et al.* [24] were used to validate this technique and to examine the oxidative status of these patients.

2. Experimental

2.1. Reagents and chemicals

Acetonitrile, methanol and water (HPLC grade) were obtained from Merck (Darmstadt, Germany). Disposable extraction cartridges (Bakerbond octadecyl) (6 ml) were purchased from Baker (Gross Gerau, Germany). 1,3-Diethyl-2-thiobarbituric acid (DETBA), 2-thiobarbituric acid, deferoxamine mesylate were bought from Sigma (St. Louis, MO, USA). 2,6-Di-*tert.*-butyl-*p*-cresol (BHT) and 1,1,3,3-tetraethoxypropane (TEP) were obtained from Fluka (Buchs, Switzerland). Creatinine was determined with kit (Jaffé method [25]) and analyzer (Hitachi 704) from Boehringer (Boehringer Mannheim, Germany).

DETBA solution, (10 m*M*) was prepared by dissolving 100 mg DETBA in 10 ml ethanol and dilution with 40 ml sodium phosphate (0.1 *M*, pH 3.0). A TEP standard stock solution was used to generate malondialdehyde *in situ* under acidic conditions. A 50-μl volume of TEP in 25 ml of a solvent mixture ethanol–water (42:58, v/v) was prepared freshly each month (7.9 m*M*). TEP working solutions (39.6 and 4.8 μM) were prepared each day by successive dilution. A 15 m*M* deferoxamine mesylate solution was prepared by dissolving 9.852 g in water. A 360 m*M* BHT solution was prepared by dissolving 792 mg in 10 ml ethanol.

2.2. HPLC instrumentation

The HPLC system consisted of a Hewlett-Packard Chemstation, Model 1090M equipped with an autosampler and a diode-array detector. MDA–DETBA complexes were separated on a 30×3.9 mm I.D. Nova-Pak C_{18} column filled with 4 μm packing, obtained from Millipore (Volketswil, Switzerland).

2.3. Urine samples

Urines were collected for 24 h from healthy people who had not taken any drugs for the last two weeks. Urines from burns patients were collected for 24 h, immediately frozen and stored at −40°C until their analysis.

2.4. Measurement of MDA

DETBA reaction on urinary samples and cleanup step

MDA measurements were done in triplicate using 200 μl urine aliquots for each determination. Samples were incubated at 100°C for 1 h with 10 μl BHT (1.8 m*M* final concentration), 2 μl deferoxamine mesylate (15 μM final concentration) and 2 ml DETBA. They were cooled at 4°C and the complexes MDA–DETBA isolated on a 6 ml C_{18} Bakerbond cartridge. Cartridges were prewashed with 10 ml methanol and equilibrated with 15 ml water. The reaction mixture (2.2 ml) was then layered on the cartridge and washed through with 5 ml water (pH 3.0 with 3 *M* HCl). DETBA complexes were subsequently eluted with 2 ml methanol and solvent evaporated under nitrogen.

MDA–DETBA analysis

MDA–DETBA complexes were dissolved in 200 μl acetonitrile and 100 μl aliquots were then

injected on the HPLC column. The solvent gradient started with a mixture of acetonitrile–water containing 0.1% triethanolamine (10:90, v/v) followed by a linear increase in acetonitrile to 60% at 20 min. The system then returned to the initial conditions and stabilized for 15 min prior to a new injection. The flow-rate was 1.0 ml/min. Detection was by either UV absorption with a diode-array detector (maximal spectral absorption at λ 539 nm) or with a fluorescent detector (excitation wavelength 515 nm and emission wavelength 548 nm). Complexes are quantified using 1,1,3,3-tetraethoxypropane as external standard.

2.5. Assessment of linearity and recovery

The linearity of response of TEP standard was tested between 10 and 4000 pmol.

Increasing amounts of TEP (31.4, 63.3, 317, 660, 1320, 1980 and 2640 pmol) were added to 100 μl urine to test the linearity and the recovery of TEP in urine. The MDA response linearity of increasing volumes of urine (10 to 400 μl) was also established.

3. Results and discussion

The MDA excreted in urine can originate both from lipid peroxidation in the diet and from factors which increase lipid peroxidation *in vivo* [1,3]. *In vivo* MDA is formed nonenzymatically such as from products of lipid peroxidation, or cellular inflammation reactions, as well as enzymatically such as through lipoxygenase and cyclooxygenase activity. During lipid peroxidation, active species such as free radicals are produced. These compounds can damage tissues, and are thus hazardous to health. The measurement of MDA in urine can thus give a global index of the body's oxidative status.

A typical HPLC profile of TEP standard (Fig. 1) showed two partially resolved peaks eluting at 15.62 min and 15.84 min, respectively. Both peaks had the same UV spectra with a maximum wavelength absorption at 539 nm (Fig. 2) and corresponded to the two tautomeric forms previously described [19]. The use of DETBA instead of TBA results in less polar complexes which can be roughly separated on C_{18} Bakerbond cartridges. Thus, HPLC profiles are cleaner and both selectivity and sensitivity are improved. Picomole levels are detectable using a fluorescence or UV detector.

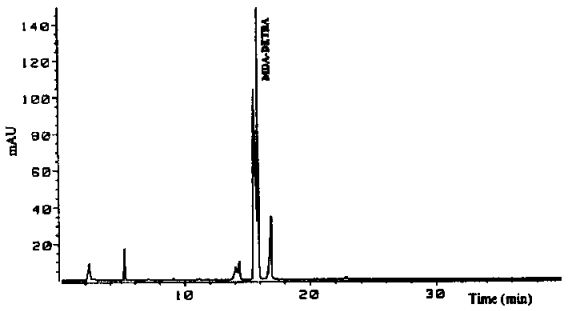

Fig. 1. Typical HPLC profile of TEP standard (317 pmol). MDA–DETBA complexes were detected by UV absorption at 539 nm using a diode-array detector. A 100 μl sample was injected onto a 30 cm × 3.9 mm I.D. Nova-Pak C_{18} column filled with 4 μm packing. A 20-min linear gradient of acetonitrile in 0.1% triethanolamine was used as described in the Experimental section.

3.1. Linearity

Calibration curves of the TEP standard were plotted over the range 0.16–3950 pmol. The regression line of TEP ($y = 8.55x - 316.71$; $r^2 = 0.9979$; $S_{yx} = 938.7$ (S is the square root of the residual mean square error); y in arbitrary area unit; x in picomol) showed an excellent linearity according to the regression coefficient which was

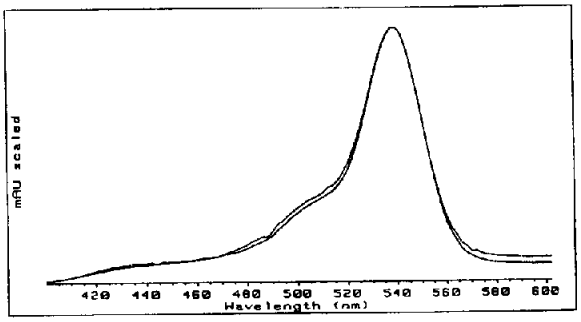

Fig. 2. UV spectra of MDA–DETBA complex obtained by HPLC using a diode array detector.

close to 1. The variation between assays was 4.9% (variation coefficient) as judged by changes in the slope of the different calibration curves ($n = 13$).

MDA detected in increasing volume of urine (10–400 μl) (x-axis in μl) showed a linear increase in MDA as assessed by the regression line ($y = 0.874x + 4.947$; $r^2 = 0.9956$; $S_{yx} = 8.7$; y in pmol).

3.2. Recovery

The response of MDA in 100 μl urine overloaded with increasing amounts of TEP standard remained linear up to the highest concentration tested (2640 pmoles). The regression line was ($y = 1.012x + 128.71$; $r^2 = 0.9959$; $S_{yx} = 65.7$; y in pmol MDA recovered; x in picomol of MDA added). The intercept indicates the amount of MDA present in 100 μl of urine and the slope close to 1 shows a complete recovery of the TEP standard added.

3.3. Reproducibility

The variability of repeated injections of the same urinary sample on HPLC was less than 0.5% (10 determinations) and the overall analytical variability between ten identical samples measured the same day was within 3 to 6% and the variation between different days was 6% (10 determinations).

3.4. Stability

MDA measurements done at different time for a same urine sample frozen at −40°C indicated no significant difference of the MDA concentration for at least 3 months storage. The MDA–DETBA complexes are also stable for at last 48 h.

3.5. Biological application

MDA was determined in 24-h urine from healthy people and from severely burned patients (between 20 and 55% of total body surface) at different days following their hospitalization. Results (Fig. 3) were corrected according to the urinary creatinine content. Urinary MDA was approximately 20 times higher than normal during the first week postburn, attained a maximum on the third day and remained, on average, about an order of magnitude higher even after 20 days postburn. However, seven of ten patients measured on the 30th day after hospitalization had reverted the level of MDA excretion to normal (results not shown). These results indicate that the burn injuries have induced an appreciable oxidative stress as has been already previously reported [26]. Moreover, we observed for one patient, not included in the data presented, a 24-h MDA urinary excretion of 23.6 μmol/mmol creatinine. The urinary MDA values decreased slowly and tended to reach the basal values along with health recovery. Burn injury initiates an inflammation-induced hypermetabolic state that can lead to severe multiple organ failure [26]. Oxidants are major products of inflammation and lipid peroxides have been shown to increase in the plasma of burns animals and patients [26–29]. In these patients, persistent inflammatory status was confirmed by plasma acute phase protein determinations C-reactive protein was analyzed by nephelometry with a

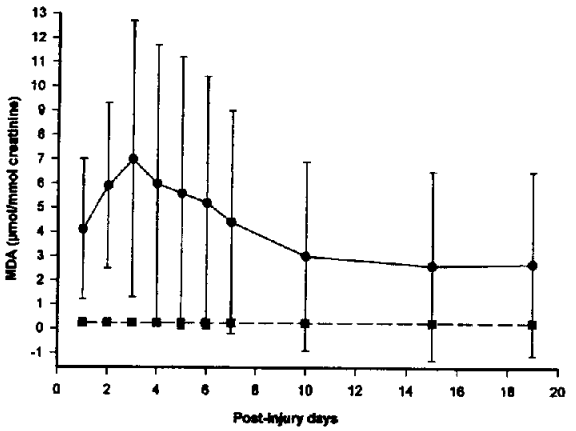

Fig. 3. Urinary MDA levels in burns patients at various days following their hospitalization compared to those in healthy people. The curve (—●—) represents the mean ± S.D. of urinary MDA excretion from 10 patients. The curve (—■—) represent the mean excretion of urinary MDA from 6 healthy people. For the control the S.D. was 0.02 and each determination was done in triplicate.

Behring Nephelometer (methods and reagents from Behring, Marburg, Germany) (data not shown).

Urinary MDA appears to be a very sensitive biochemical parameter and may well be useful in assessing whole body oxidation status.

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