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Technical Report

Increased plasma malondialdehyde levels in glomerular disease as determined by a fully validated HPLC method

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Abstract

Background. Reactive oxygen species and particularly free radical induced lipid peroxidative tissue damage have been implicated in the pathogenesis of various renal diseases. Lipid peroxidation is assessed indirectly by the measurement of secondary products, such as malondialdehyde (MDA), using the widely employed thiobarbituric acid reactive substances (TBARS) method. However, this method lacks sensitivity and specificity. We have therefore developed and validated an HPLC (high-performance liquid chromatography) method for measurement of MDA and applied this to a variety of plasma samples in renal patients.

Methods. The optimized method involves antioxidant treatment of the plasma sample, followed by a protein precipitation step using trichloroacetic acid, acid hydrolysis and formation of an MDA thiobarbituric acid complex. The MDA-$(TBA)_2$ adduct is separated from other interfering compounds by C_{18} reverse-phase HPLC techniques, with visible detection at 532 nm.

Results. The assay was linear over the ranges 0.25–1.0 μM MDA and the detection limit was 0.06 μM MDA. Within-run precision was $<4.5\%$ and between-run precision was $<10.0\%$. MDA plasma concentrations (mean $\pm$ SD) were higher in ESRF diabetic patients (0.32 ± 0.14 μM, $n=20$), non-diabetic ESRF patients (0.32 ± 0.09 μM, $n=20$), and CRF patients (0.14 ± 0.06 μM, $n=40$) compared to healthy controls (0.11 ± 0.03 μM, $n=40$), ($P<0.001$, $P<0.001$ and $P=0.008$). Levels were similar in healthy controls with normal renal function and transplanted patients (0.12 ± 0.03 μM MDA, $n=40$), ($P=$ NS). No correlation was observed between MDA and creatinine levels ($r^2=0.05$, $n=80$), which suggests that MDA does not correlate with the degree of renal impairment. We matched CRF patients with glomerular and non-glomerular causes of renal failure for creatinine levels and found that MDA levels were higher in patients with glomerulonephritis (0.16 ± 0.06 μM) than in those with CRF from non-glomerular causes (0.12 ± 0.04 μM, $P=0.002$).

Conclusions. We have introduced a reliable and sensitive HPLC technique to enhance the specificity of MDA-$(TBA)_2$ measurement, with a significant improvement in HPLC column life. Using this method, picomole quantities of MDA can be detected in plasma. We have shown that MDA levels are significantly raised in patients with CRF due to glomerulonephritis, regardless of serum creatinine, which suggests that there is oxidative injury independent of any possible MDA retention due to renal impairment.

Key words: glomerulonephritis; high performance liquid chromatography; malondialdehyde; reactive oxygen species; thiobarbituric acid

Introduction

Reactive oxygen species (ROS) are mediators of vascular and tissue damage in various diseases, including diabetes, rheumatoid arthritis, and ischaemic-reperfusion injury [1]. They have been implicated in the pathogenesis of renal disease in various animal models of renal failure, including glomerulonephritis [2]. There is also evidence to suggest increased free radical activity during haemodialysis [3] and after renal transplantation [4].

The identification and quantification of ROS by direct methods using electron spin resonance (ESR) spectroscopy has been carried out in many cellular systems. However, it is not a routine method of analysis, because of the expensive nature of the equipment and a lack of data regarding ESR spectra of *in vivo* biological systems. Alternatively, oxygen-free radicals can be detected indirectly, by measurement of their oxidative attack on lipids and proteins, resulting in products such as conjugated dienes, hydroperoxides and aldehydes. This type of analysis is nonetheless limited by the lack of selectivity and

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sensitivity of the various assays and the possibility of *ex-vivo* oxidation.

Malondialdehyde (MDA) is a three-carbon, low-molecular weight aldehyde that can be produced from free radical attack on polyunsaturated fatty acids, such as arachidonic acid. The analysis of MDA by the thiobarbituric acid assay has been widely employed over many years, initially in food as a measure of rancidity, and then in biological systems for assessment of lipid peroxidation [5]. It is a spectrophotometric assay, based upon heating of the sample under acidic conditions to form an adduct MDA-$(TBA)_2$, which can be detected at 532 nm. The specificity of the assay can be questioned, since aldehydes, other than MDA, can react with TBA and various other pigments may absorb at 532 nm, giving an over-estimation of MDA concentrations [6].

High-performance liquid chromatography (HPLC) of the MDA-$(TBA)_2$ complex has been introduced to improve the specificity of the reaction [7]. The separation of the complex from other interfering compounds by reverse phase HPLC techniques has led to a reduction in observed MDA levels in various biological fluids. Ranges in control plasma are now reported as low as 1–2 μM MDA [8], which is more than a fivefold decrease from previous estimations where HPLC was not employed [9].

We have focused upon spectrophotometry rather than the less widely used fluorimetry as the method of detection, to avoid detection of compounds that fluoresce at similar wavelengths to MDA, which may interfere with the assay [10]. Here we report the modification of an existing HPLC–spectrophotometric method [11], with an evaluation of sensitivity, precision, recovery, and column life. We have found that the addition of a deproteinization step prior to TBA incubation led to significant improvements in column life and gives similar sensitivity to methods using post-column visible detection, without the need for complex sample preparation or organic extractions.

To date, there are few data on HPLC analysis of MDA in biological fluids applied to samples from patients with renal diseases. We report the validation and comparison of plasma samples obtained from healthy controls and various renal patients, including transplants, end-stage renal failure (ESRF) and chronic renal failure (CRF) patients, with particular emphasis on patients suffering from glomerulonephritis where ROS have been implicated as mediators of glomerular damage [12].

Subjects and methods

Chemicals and reagents

Chemicals and reagents were of the highest analytical grade available and were as follows: 1,1,3,3-tetraethoxypropane (TEP), trichloroacetic acid (TCA), phosphoric acid, 2-thiobarbituric acid (TBA), butylated hydroxytoluene (BHT) and potassium phosphate all obtained from Sigma–Aldrich Co. Ltd (Poole, Dorset, England). Methanol (HPLC grade) and absolute ethanol were purchased from Rathburn Chemicals Ltd (Walkerburn, Scotland) and Hayman Ltd (Witham, Essex, England) respectively.

Standard and sample preparation

TEP standards were freshly prepared daily. The solvent blank was absolute ethanol (400 ml/l) in distilled water. The stock standards were 10 mM and 100 μM TEP and the working calibrants were 0.0, 0.25, 0.50, 0.75 and 1.0 μM TEP, prepared from dilution of the 100 μM TEP with absolute ethanol (400 ml/l). The HPLC calibration was performed for each run. Samples were calculated as μM MDA equivalent from the TEP standard calibration (1:1 conversion under acidic conditions).

Blood samples were obtained from 40 healthy individuals with normal renal function, 40 kidney transplant recipients, 40 CRF patients and 40 ESRF patients on haemodialysis (HD). The CRF group was further sub-divided into biopsy-proven glomerular disease (group 1, $n=20$), including diabetic nephropathy, ANCA-positive glomerulonephritis, lupus nephritis, mesangiocapillary glomerulonephritis, and IgA nephropathy. This was matched with a group of patients with similar creatinine levels (group 2, $n=20$) with non-glomerular causes of renal failure, including renovascular disease, acute tubular necrosis (ATN), urinary-tract infection, interstititial nephritis, polycystic kidney, and obstruction. Diagnosis of ATN and interstitial nephritis was made on biopsy, the rest were based on the history, imaging and other appropriate investigations. Bloods from the HD patients were taken prior to the dialysis session, and all patients were dialysed using modified cellulose acetate membranes (Haemophan). This group was also subdivided into patients with ESRF due to diabetic nephropathy (14 NIDDM, 6 IDDM) or other causes of ESRF.

Whole blood was collected by venepuncture into EDTA-lined Vacutainer tubes. Plasma was obtained by centrifugation at 1000 *g* for 15 min at 4°C, separated and stored at −70°C.

Analytical procedure

The TBA assay was adapted from Chirico [11]. A deproteinization step was developed and optimized in terms of protein recovery and sensitivity of calibration, using the following acids: phosphoric, trichloroacetic, or perchloric, and different extraction procedures and sample volumes. The TBA incubation was carried out using various sample supernatant and TBA reagent volumes. The optimized assay was as follows: 250 μl plasma or standard was treated with 25 μl 0.2% BHT (in absolute ethanol), followed by addition of 1 ml 5% TCA (aqueous), which was vortexed and centrifuged at 4000 *g* for 10 min; 500 μl 0.6% TBA (aqueous) was added to the deproteinized supernatant (700 μl), vortexed and then reacted for 45 min at 90°C. After cooling at 4°C and a final centrifuge at 4000 *g* for 10 min, an aliquot (120 μl) was directly injected onto the HPLC.

HPLC separation

The separation of the MDA-$(TBA)_2$ adduct was performed using the automated Waters™ HPLC system (510 Pump, 717 Autosampler and 486 Detector (at 532 nm), driven by

the Millenium 2010 Chromatography Manager software (Waters Ltd, Watford, Hertfordshire, England).

The analytical column was a reverse phase silica based C_{18} column, (Spherisorb 5ODS 2, HPLC Technology Ltd (Warrington, Cheshire, England)), with column dimensions of 25 cm × 4.6 mm and a C_8 guard column (HPLC Technology Ltd). The elution buffer was determined to be 65% 50 mM KH_2PO_4–KOH, pH 7.0, 35% MeOH, which was degassed under vacuum through a 0.45 μM filter (Millipore, type HAWP). The sample run was 10 min, with a flow rate of 1.2 ml/min, an injection volume of 120 μl, and visible detection at 532 nm.

All standards and samples were run and data analysed automatically *via* the Millenium software package. Mean peak area was determined for each sample ran in duplicate and the levels of MDA were calculated directly from the calibration of the TEP standards.

Statistical analysis was performed using the non-parametric Mann–Whitney U test. A P value of <0.05 was considered to indicate a significant difference.

Results

Method optimization

The existing method [11,13] was limited by the necessary but time consuming column washing procedures after each run and the short column life, i.e. only 60 and 300 runs quoted for the guard column and analytical column respectively. This may be attributed to protein contamination of the columns, which is irreversible and will lead to loss of sensitivity, resolution, and drifting retention times. We have investigated the effect of acid extraction (a simple method for protein removal) on the TBA reaction and HPLC separation.

There were no significant differences in the calibration sensitivity using phosphoric, perchloric and TCA at 5% strength. TCA was therefore chosen because of its more common usage in protein removal, as a result of its lower toxicity. MDA recovery of TEP spiked control plasma samples were similar using hot (90°C) and cold (room temperature) extraction, unlike a previous study where MDA levels were higher in tissue samples using hot TCA incubation [14].

Calibration and detection limit

The assay was calibrated from 0.25 to 1.0 μM TEP. A typical calibration plot is shown in Figure 1, with a regression line corresponding to $y = 99496 \times -708$, $r^2 = 0.998$. The calibration was linear to at least 10.0 μM TEP.

A typical chromatogram of a plasma sample is shown in Figure 2. The average retention time of the MDA-$(TBA)_2$ adduct was 7.0 min at a flow rate of 1.2 ml/min. The initial peak (at about 3 min), may be attributed to non-retained plasma components, which were unreactive to TBA. The second peak (4.2 min) was TBA complexed with a variety of components, including aldehydes other than MDA.

The detection limit was defined as two times the standard deviation of the blank response, and was estimated by analysis of 15 replicates of the blank solvent in triplicate. The detection limit was determined as 0.055 μM MDA in a plasma sample, which equates to 6.60 pmol MDA for a 120 μl injection.

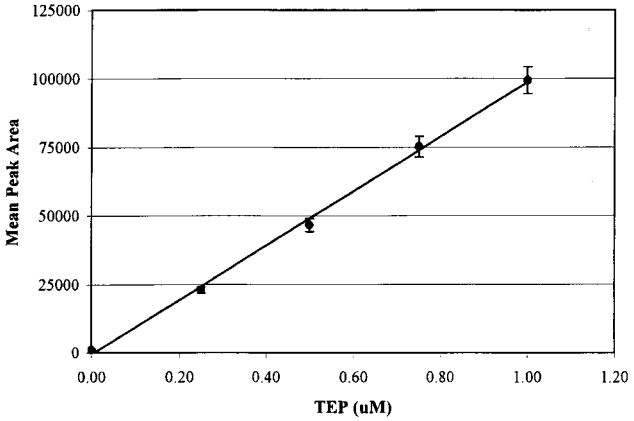

Fig. 1. A typical TEP calibration line, with regression line y = 99496x–708, $r^2 = 0.998$.

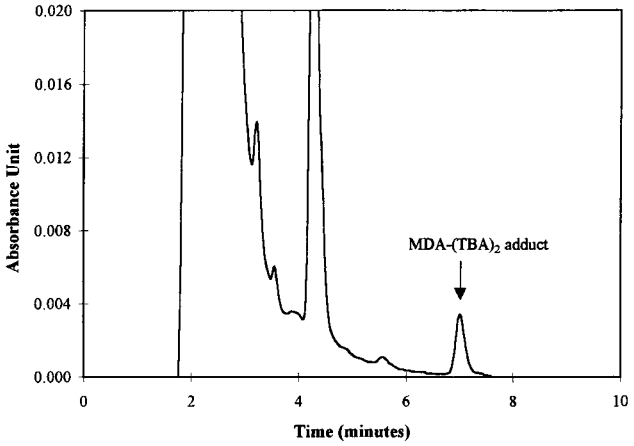

Fig. 2. A typical sample chromatogram, detailing the elution of the MDA-$(TBA)_2$ adduct at 7.0 min.

Precision, interferences, sample stability, and column life

Aliquots taken from pooled plasma of healthy controls were spiked with 75, 150 or 225 μM TEP standards to give a low, medium, and high range of MDA concentrations. Within-run precision was assessed by analysing 10 replicates of the three pools. Between-run precision was determined by carrying out duplicate measurements of the three pools on 10 different occasions. The within-run and between-run precision was less than 4.5% and 10.0% respectively (Table 1).

We investigated potential interference from a range of substances, including haemoglobin, triglyceride, bilirubin, urea, and creatinine. The mean analytical recovery was 101% (range: 97–120%), despite increasing additions of interferent up to final concentrations of haemoglobin (6 g/l), triglyceride (16 mM), bilirubin

Table 1. Precision data on MDA-spiked control plasma pools using three MDA concentrations

MDA concentration	Mean ± SD (μM)	CV (%)	*n*
Within run			
high	0.874 ± 0.028	3.2	10
medium	0.710 ± 0.030	4.2	10
low	0.369 ± 0.013	3.5	10
Between runs			
high	0.854 ± 0.054	6.3	10
medium	0.673 ± 0.065	9.7	10
low	0.373 ± 0.052	7.5	10

(400 μM), creatinine (1000 μM), and urea (50 mM). Similar recovery was obtained from MDA spiked to a uraemic and healthy control plasma pool, suggesting little interferences from the uraemic milieu.

BHT, an antioxidant, is added to the plasma sample before the TBA incubation to minimize lipid peroxidation during the heating stage. Therefore MDA levels should reflect *in vivo* oxidation and not enhanced production during the sample pre-treatment. It is possible that the stability of the sample may be improved by adding BHT to the fresh plasma before storage at −70°C. This was investigated by adding BHT to low, medium, and high plasma TEP spikes in fresh control plasma pools and analysing for MDA levels after storage for 1, 8, 15 and 60 days. The storage of samples for up to 14 days had no significant effect on MDA levels, but the average intra-assay precision of the untreated plasma samples was lower (6.8%) than the BHT treated samples (10.7%). There was a slight reduction in oxidation, which occurred on storage for 60 days at −70°C, with an average increase of 9% and 17% to MDA levels for the BHT and untreated samples respectively. It was not possible to batch samples with BHT, because of the loss in precision.

Column life was significantly improved compared to the existing method, in which protein contamination of the analytical HPLC column resulted in irreversible peak broadening and drifting retention times after approximately 200 injections. This compares with an average of 800 runs over each of three columns with our modified method.

Sample analysis

The serum creatinine concentrations were less than 120 μmol/l in the healthy controls and greater than 500 μmol/l in the ESRF group. The creatinine concentrations (mean ± SEM (μmol/l)) were also 182 ± 16 (transplant), 300 ± 35 (CRF), 294 ± 46 (CRF, group 1) and 307 ± 54 (CRF, group 2).

Figure 3 shows that MDA levels (mean ± SD) were significantly higher in plasma from the group of CRF patients (0.14 ± 0.06 μM) and the two groups of ESRF patients (0.32 ± 0.14 μM and 0.32 ± 0.09 μM), when compared to healthy controls (0.11 ± 0.03 μM). There were no significant differences between patients with ESRF due to diabetic nephropathy compared to those with other causes of ESRF. Levels in healthy controls and transplanted patients (0.12 ± 0.03 μM MDA) were similar.

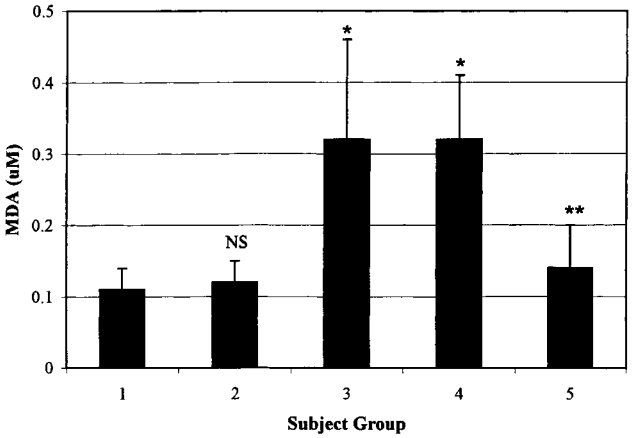

Fig. 3. MDA levels in various subject groups. 1, Healthy controls ($n=40$); 2, transplants ($n=40$); 3, diabetic ESRF ($n=20$); 4, non-diabetic ESRF ($n=20$); 5, CRF ($n=40$). Results are expressed as mean ± SD. $*P<0.001$, $**P=0.008$ *vs* healthy controls.

There was a poor degree of correlation between MDA and creatinine levels in the CRF group and transplant group ($r^2=0.05$, $n=80$), as shown in Figure 4. MDA levels were significantly higher in the glomerular disease group (0.16 ± 0.06 μM) compared with the non-glomerular disease group (0.12 ± 0.04 μM), $P=0.002$.

Discussion

The determination of MDA is one of the most commonly used methods for monitoring lipid peroxidation in biological samples. The frequently used TBARS method is fairly sensitive but not specific.

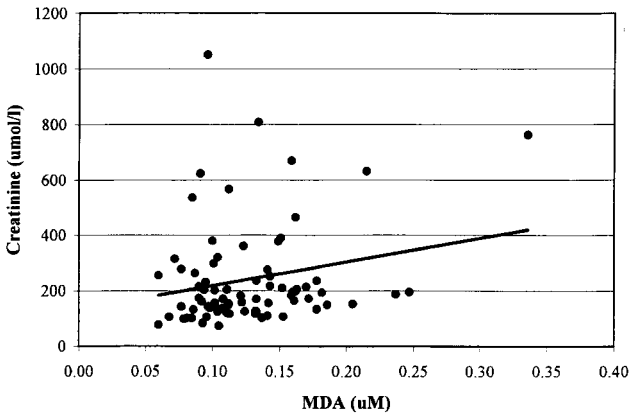

Fig. 4. A correlation of MDA *vs* creatinine in the CRF and transplant groups ($r^2=0.05$, $n=80$).

Recently, various HPLC methods have been applied to improve the specificity of the TBA reaction. We have adapted an existing HPLC method [11,13], by introducing an acid deproteinisation step, using TCA prior to TBA incubation. This has resulted in more than a three-fold improvement in column life, with similar sensitivity and without the need for extensive pre-treatment.

Using this method, we have found that patients on HD and CRF patients had significantly higher MDA levels than healthy controls. Well-functioning renal transplant patients were no different from controls. Previous research has concentrated upon the HD group, where enhanced lipid peroxidation may result from free radical activity generated by complement activation and release of cytokines during the dialysis procedure, caused by exposure of blood to bio-incompatible dialysis membranes. We have found high MDA levels in the ESRF group, which maybe attributed to their exposure to the Haemophan membrane.

There is a wide variation in the published levels of MDA in the plasma of HD patients as measured by the TBARS method, with the majority showing an increase compared to controls [15], whilst others showed no difference [16]. These findings reflect the irreproducibility and inaccuracy of the TBARS method. A recent analysis using a lengthy and complex HPLC method has shown levels of 0.11–0.50 μM MDA in paediatric HD patients [17], which supports our findings.

It has been postulated that there are higher levels of oxidative injury in diabetic nephropathy than in other nephropathies. We have shown that there are no differences in MDA levels in patients with ESRF due to diabetic nephropathy compared to other causes of ESRF. These results are in agreement with Gotoh *et al.* [18], who found no differences in MDA levels in subcutaneous fat in these two groups using the TBARS method. ROS have been implicated in the pathogenesis of diabetes induced tissue injury, with hyperglycaemia causing ROS production from glucose auto-oxidation and non-enzymatic protein glycation. It is also possible that glucose, with its known antioxidant properties, may reduce MDA formation in the circulation. However, a preliminary analysis indicated no inverse correlation between MDA and serum glucose or glycated haemoglobin levels ($r^2 = 0.02$ and 0.06 respectively, data not shown).

MDA is a low molecular weight, water soluble compound. It is therefore expected that there would be some filtration by the glomerulus and excretion by the kidneys, and recently the MDA–TBARS complex has been characterized in urine from healthy controls [19]. The increased MDA levels in the HD and CRF groups may therefore reflect reduced plasma clearance of the compound rather than increased oxidation due to the primary renal lesion or the uraemic state. Our findings of a very poor correlation between creatinine and MDA levels in the CRF and transplant group do not however support this hypothesis. We measured MDA levels in two groups of CRF patients, one of which had renal impairment due to ongoing glomerulonephritis and the other whose renal failure was due to non-glomerular and non-inflammatory causes. Both groups were matched for serum creatinine. There was a significant increase in MDA levels in the glomerulonephritis group, which suggests that there is oxidative injury independent of the degree of renal impairment and this is supported by the demonstration, using other methodologies, of the involvement of reactive oxygen species in experimental nephritis [2].

In conclusion, we have developed and validated an analytical HPLC method for determination of malondialdehyde in plasma, which is simple to perform, has high sensitivity and specificity and results in substantial improvement in HPLC column life. This simple and reproducible technique offers an opportunity to further characterize the role of oxidative injury in the pathogenesis of both experimental and clinical glomerular inflammation.

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