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Measurement by reversed-phase high-performance liquid chromatography of malondialdehyde in normal human urine following derivatisation with 2,4-dinitrophenylhydrazine

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Abstract

A selective and sensitive method based on derivatisation with 2,4-dinitrophenylhydrazine (DNPH) and consecutive HPLC gradient separation is described for the determination of malondialdehyde (MDA) in urine. Preparation of urine samples involved a one-step derivatisation/extraction procedure. Separation was achieved using a Waters Symmetry™C_{18} column (3.9×150 mm) and linear gradient of acetonitrile in water (from 30% to 70% in 30 min). The overall detection limit of the method was 56 n*M* of MDA in urine. The recovery of MDA was 94.3±8.6%. MDA in urine of healthy volunteers, measured using the method of standard additions, was 0.019±0.012 μmol/mmol creatinine. MDA in the same samples measured using the 2-thiobarbituric acid (TBA) assay was 0.181±0.063 μmol/mmol creatinine. We demonstrate that the commonly used TBA assay in conjunction with HPLC may overestimate the MDA concentration in human urine by almost 10-fold.

Keywords: Malondialdehyde; 2,4-Dinitrophenylhydrazine; 2-Thiobarbituric acid

1. Introduction

Urinary malondialdehyde (MDA) has been widely used in animal models and in humans as a non-invasive biomarker of lipid peroxidation induced by oxidative stress [1–8]. Following peroxidation of polyunsaturated fatty acids, lipid peroxides are converted by consecutive reactions of oxidation, rearrangement and scission into more stable carbonyl compounds, including MDA. The most common method of measuring MDA is based on the reaction with 2-thiobarbituric acid (TBA) in acidic media and a temperature of 100 °C and measuring absorbance at 533 nm [9]. Unfortunately the TBA assay is intrinsically non-specific for MDA as TBA is also reactive to other compounds that may be present in biological samples [10,11]. Employment of high-performance liquid chromatography (HPLC) with UV or fluorimetric detection has improved the selectivity and increased the sensitivity of the method [12–14]. Elevated temperatures in conjunction with low pH potentially can lead to the formation of MDA as an artifact of the assay [11]. Another method for the determination of MDA, other aldehydes and ketones is based on their reaction with 2,4-dinitrophenylhydrazine (DNPH) at low pH with the formation of DNPH derivatives, which are strongly absorbing in

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the region of 300–380 nm. This method has been used to measure MDA, other aldehydes and ketone bodies in plasma [15–17], urine [4,5,18] and other biological samples [19–21]. As the DNPH reagent is highly reactive towards carbonyl compounds, the derivatisation does not require high temperatures to proceed. Unfortunately the assay is not as quick as the TBA assay, it involves multiple liquid–liquid extractions [16–21], recrystallisation and purification of DNPH reagent on a daily basis [18] and can be very easily contaminated with atmospheric aldehydes or impurities in reagents [17]. A new method of measuring MDA in plasma based on low temperature derivatisation of MDA with diaminonaphtalene was recently described, but due to the presence of numerous interfering compounds it is not convenient for measuring MDA in urine [22].

The purpose of the present experiment was to develop a method of measuring MDA in human urine, based on derivatisation with DNPH, that is sensitive and selective towards MDA, does not involve lengthy preparation of the sample or sample boiling and therefore will avoid the possible artifactual production of MDA. To the best of our knowledge this is the first paper to report the values for MDA in normal human urine measured derivatisation with DNPH at 37 °C. We have also demonstrated that the commonly used TBA assay for MDA overestimates the MDA levels in human urine by almost 10-fold compared to the assay based on derivatisation with DNPH.

2. Experimental

2.1. Chemicals and reagent preparation

Ultra-pure water (conductivity less than 0.065 μS cm^{-1}) was used throughout the study. Acetonitrile, methanol, *n*-pentane, chloroform, acetone, orthophosphoric acid (85%) of HPLC grade (HiPerSolv), KOH, urea and HCl (ARISTAR grade) were purchased from VWR International, Poole, UK. 1,1,3,3-Tetramethoxypropane (99%), propionaldehyde (97%), acetaldehyde (99%), butyraldehyde (99%), paraformaldehyde (95%), creatinine (99%) and picric acid solution (1%) were obtained from Aldrich, Dorset, UK. 2,4-Dinitrophenylhydrazine (DNPH, FW 198.1, containing approximately 30% water) and 4,6-dihydroxypyrimidine-2-thiol (2-thiobarbituric acid; TBA 98%) were obtained from Sigma (Dorset, UK). HPLC-grade potassium dihydrogen orthophosphate was purchased from Fisher Scientific UK (Loughborough, UK).

Derivatising agent (ca. 0.31 m*M*) was prepared by dissolving 0.004 g of DNPH in 50 ml of 4 *N* HCl. The DNPH solution was purified by three successive extractions with chloroform by mixing equal volumes of DNPH solution and chloroform in a separating funnel and discarding the bottom organic layer. DNPH solution was stored at room temperature in the dark with an equal volume of chloroform in an airtight container with a magnetic stirrer. Before use the DNPH solution was stirred for 10 min. This solution was freshly prepared on a weekly basis.

TBA solution (0.6%, w/v) was prepared by dissolving 0.6 g of TBA in approximately 80 ml of water, with stirring on a hot plate (50–55 °C). After cooling down to room temperature the volume was adjusted to 100 ml with water. Orthophosphoric acid solution was prepared by diluting concentrated acid in ultra-pure water at a concentration of 1% (v/v).

Potassium phosphate buffer–methanol mobile phase for HPLC was prepared on the day of use by mixing 600 ml of 50 m*M* solution of KH_2PO_4 (pH 6.8) with 400 ml of methanol and filtering through a 0.2 μm nylon filter using a vacuum pump.

2.2. Standards preparation

1,1,3,3-Tetramethoxypropane (TMP) was used to prepare a malondialdehyde stock solution. A volume of 17 μl of TMP was diluted in 0.1 *N* HCl (10 ml) and incubated at 40 °C for 60 min to hydrolyse TMP into MDA (final concentration ca. 10 m*M*). The concentration of MDA was determined by measuring its absorbance at 245 nm (ϵ=13 700) according to Esterbauer et al. [14]. This stock solution was stored at 4 °C and freshly prepared on a weekly basis. Standard solutions of MDA were prepared on the day of use by further dilution with 0.1 *N* HCl.

One millimolar propionaldehyde was prepared on the day of experiment by diluting 7.44 μl of propionaldehyde with water up to 100 ml in a volumetric flask. A volume of 10 μl of this solution was added to each assay and used to quantify the volume of

organic phase removed after the derivatisation/extraction procedure.

2.3. Sample preparation

2.3.1. DNPH assay

Sample preparation (derivatisation and extraction) was carried out in 50 ml capacity pyrex glass bottles with PTFE-lined screw-on lids. To a solution containing 3 ml of urine, 3 ml of water, 10 μl of 1 m*M* propionaldehyde, 10 μl of MDA standard (0, 0.125, 0.25, 0.375 or 0.5 m*M*), and 0.6 ml of DNPH solution, 10 ml of pentane were added. In experiments to determine the effect of urea as an interfering factor, 3 ml of urea solution (0, 0.1, 0.25, 0.5 and 1.0 *M*) were used instead of urine. In blank samples, urine was replaced by pure water. A one-step derivatisation/extraction procedure was carried out in an orbital incubator at 37 °C for 60 min with continuous shaking. At the end of this procedure, samples were allowed to cool down and most of the organic phase was transferred to clean pyrex bottles. At this step great care was taken not to trap any aqueous phase. The exact volume of organic phase transferred at the end of the derivatisation/extraction step was almost impossible to measure due to the physical properties of pentane. To account for this each sample was spiked with the same amount of propionaldehyde (see above). This allowed us to compare peak areas of MDA–DNPH between different samples. Pentane was evaporated to dryness on a hot plate under a stream of nitrogen and the residue was reconstituted in 250 μl of 50% (v/v) acetonitrile–water solution. Samples were then transferred to HPLC vials and 100 μl were injected onto the column for analysis. A reagent blank assay was run with each batch of urine samples.

2.3.2. TBA assay

Derivatisation with TBA was performed according to Wong et al. [23]. Sample preparation was carried out in 15 ml capacity pyrex glass tubes with PTFE-lined screw-on lids. Three milliliters of orthophosphoric acid solution, 0.4 ml of water and 0.6 ml of urine or MDA standard (0.1 0.5, 1.0, 2.0 μ*M*) were vortex mixed after which 1 ml of TBA solution was added. Tubes were placed in a water bath at 100 °C for 1 h. At the end of the incubation, samples were cooled on ice and centrifuged at 14 000 *g*. The supernatant was transferred to glass HPLC vials and 100 μl were injected onto the column for analysis. In blank samples, urine was replaced by pure water. A reagent blank assay was run with each batch of urine samples.

2.4. Chromatographic equipment and conditions

A Waters HPLC system (Waters, MA, USA), incorporating an Alliance 2690 separations module and a 996 photodiode array detector and operated by Millennium[32] software, was used in this study. Separation of DNPH derivatives of MDA and other aldehydes (hydrazones) was achieved using a Waters Symmetry™ C_{18} column (3.9×150 mm). A guard column, Waters Symmetry™ C_{18} (3.9×20 mm), was placed in front of the analytical column for protection. Samples were kept in an autosampler at 18 °C and the column heater temperature was set to 40 °C. A linear gradient of acetonitrile in water (from 30% to 70% in 30 min) was used for the elution of hydrazones. Flow-rate was 1 ml/min. Run time per sample was 30 min. After each sample run, the column was equilibrated by pumping 30% acetonitrile for 10 min at 1 ml/min. The range of wavelengths scanned was 250–500 nm. Peaks were integrated at their maximum absorbance wavelengths (307 nm for MDA–DNPH, 365 nm for propionaldehyde–DNPH). Retention times were 9.555±0.063, 11.105±0.091, 14.407±0.087, 17.717±0.092, 19.049±0.078 and 22.608±0.093 min for MDA–DNPH, formaldehyde–DNPH, acetaldehyde–DNPH, acetone–DNPH, propionaldehyde–DNPH and butyraldehyde–DNPH, respectively. For $S/N>3$ the overall detection limit of the method (including sample derivatisation/extraction and HPLC analysis) was 56 n*M* of MDA in urine.

The same Waters Symmetry™ C_{18} column (3.9× 150 mm) and a guard column, Waters Symmetry™ C_{18} (3.9×20 mm) were used for elution of MDA–TBA adduct. Samples were kept in an autosampler at 10 °C and the column heater temperature was set to 40 °C. The same samples were analysed using two different HPLC conditions: isocratic according to Wong et al. [23] (40% methanol in potassium phosphate buffer) and by the application of a linear gradient of acetonitrile in water (from 15% to 35%

in 20 min). In the case of gradient elution, after each run the column was equilibrated by pumping 15% acetonitrile for 7 min at 1 ml/min. The range of wavelengths scanned was 450–650 nm. Peaks were integrated at 533 nm. The flow-rate for both sets of conditions was 1 ml/min. For $S/N > 3$ the overall detection limit of the method (including sample derivatisation and HPLC analysis) was 70 n*M* of MDA in the standard solution.

2.5. Synthetic hydrazones and identification of peaks

Synthetic hydrazones of formaldehyde, acetaldehyde, acetone, propionaldehyde and butyraldehyde were prepared as described previously [7] by reacting concentrated solutions of the above with concentrated derivatising agent. Hydrazone of MDA was prepared by firstly hydrolysing 1 ml of TMP in 10 ml of 0.1 *N* HCl at 40 °C for 60 min and then reacting with 10 ml of concentrated (ca. 18 m*M*) DNPH solution in 4 *N* HCl. Reactions proceeded rapidly at room temperature with the formation of crystals of hydrazones, which were not soluble in aqueous solution. Crystals of hydrazones were filtered out, flushed with 4 *N* HCl, desiccated and used to prepare 10 μg/ml solutions in acetonitrile. These solutions were used for spiking samples for the identification of DNPH derivatives in urine specimens and blanks. The identification of the DNPH derivatives in urine, or blank, was done in two ways; firstly, by spiking the specimen with MDA, formaldehyde, acetaldehyde, acetone or butyraldehyde solution and comparing its HPLC profile after derivatisation with the non-spiked specimen; and secondly, by spiking the derivatised samples with solutions of synthetic hydrazones in acetonitrile and their coelution.

2.6. Collection of urine

Spot samples of urine were donated by healthy male ($n=5$) and female ($n=5$) volunteers after obtaining informed consent. Samples were collected into sterile containers with airtight lids on the day of the experiment and kept on ice until measurements. All volunteers were on no medication, non-smokers, on mixed diets and of average age 31±4.4 years (from 25 to 39 years).

2.7. Calculations

Due to the physical properties of pentane it is impossible to remove exactly the same volume of it with the pipette from individual assay bottles. Spiking all assays with the same amount of propionaldehyde was used to correct for variations in the volume of organic extract removed. The sample which gave the largest integrated area for the propionaldehyde spike (corresponding to the largest volume of organic phase removed) was assigned 100% of the removal. The remaining samples in the batch were corrected accordingly to the equation for the $PROP_{NORM}$:

$$PROP_{NORM} = (PROP_{area@365}/PROP_{max@365}) \times 100$$

where $PROP_{area@365}$ is the area of the propionaldehyde–DNPH peak in the sample integrated at 365 nm and $PROP_{max@365}$ is the area of the largest propionaldehyde–DNPH peak in the group of samples from the same urine specimen.

MDA in urine was measured using the method of standard additions [24], spiking each assay with 10 μl of standard MDA solution (0.125, 0.25, 0.375 and 0.5 m*M*) and resulting in 1.25, 2.5, 3.75 and 5.0 nmol of MDA per assay correspondingly. For each urine specimen, a linear regression was applied between the concentration of MDA in the assay and the normalised (corrected for the removed volume) area of the MDA (MDA_{norm}) peak at 307 nm. The latter was calculated as:

$$MDA_{norm} = MDA_{area\ @307} + MDA_{area\ @\ 307} \times (100 - PROP_{NORM})/100$$

where $MDA_{area\ @307}$ is the area of the MDA–DNPH peak in the sample integrated at 307 nm. The absolute value of the intercept of the regression line with the *x*-axis representing the concentration of MDA in the assay (in nmol) divided by the 3 ml of urine in the assay gives the concentration of MDA in the urine specimen (in μ*M*).

In recovery experiments a urine specimen was divided in two aliquots, one of them was spiked with 0.5 μ*M* MDA. After measuring MDA in both

aliquots using the method of standard additions described above, the recovery of MDA was calculated as the percentage difference between the concentration of MDA measured in the spiked and non-spiked aliquot of the same specimen and the known concentration of the spike.

Standard curves for the determination of MDA assayed by the TBA method were created by linear regression of the MDA–TBA peak area recovered at 533 nm versus known concentrations of MDA in a water-based standard solution. MDA in urine samples was measured in triplicate.

The concentration of creatinine in urine specimens was used to normalise the measured concentrations of MDA. Creatinine in urine samples was measured in triplicate using the Jaffe reaction as described by Falco et al. [25]. Standard curves for creatinine determination were created by linear regression of the change in absorbance at 485 nm versus known concentrations of creatinine.

The results are expressed as mean value ± SD.

3. Results and discussion

3.1. Chromatography, interferences and matrix effects

Typical chromatograms showing the elution profile of DNPH derivatives of aldehydes and ketones normally present in human urine and water blanks are shown in Fig. 1. Despite the purification of the derivatising reagent and all of the precautions which were taken to avoid contamination, significant peaks corresponding to DNPH derivatives with the volatile formaldehyde, acetaldehyde and acetone were observed in the blanks (see Fig. 1A). There was no MDA–DNPH adduct observed in the blanks (Fig. 2A). Propionaldehyde was used previously [4,5] as an internal standard in animal studies and it was not found in human urine [18]. We derivatised all urine specimens used in this study without a propionaldehyde spike. There was no significant amount of propionaldehyde–DNPH adduct present in any of the analysed specimens (Fig. 1C). We, therefore, used it to spike human urine.

Urea is the main organic component in urine, the concentration of which may vary from one specimen to another due to differences in urinalysis. Along with other components in urine, urea may contribute to a significant matrix effects. To establish where such effects exist, we conducted an experiment in which urine in the assay was replaced with urea solutions of different concentrations. All assays were spiked with the same amount of MDA (10 μl of 0.5 m*M* MDA). The area of MDA–DNPH recovered after derivatisation/extraction appeared to depend significantly on the concentration of urea in the assay mixture (Fig. 3). The mechanism whereby urea influenced the derivatisation was unknown but we found no evidence that it bound DNPH. Certainly its presence in the assay at ca. 600 times the concentration of the MDA spike showed that if it does bind DNPH then it does so only very weakly by comparison with MDA. The highest values for MDA–DNPH areas were observed when no urea was present in the assay. Therefore, it was not valid to create any external calibration (either in water or in a standard solution of urea) for measuring MDA, due to the existing matrix effects. The way to overcome this problem was to use the method of standard additions [24]. Matrix effects are also demonstrated in Fig. 4 that shows that the same standard addition of MDA to the assay mixture produced different areas of MDA–DNPH in four different urine specimens. The linearity of response (Fig. 4) proves that the low DNPH concentration used for derivatisation of samples was not a problem, as there was no saturation of the response at any of the used additions of MDA. The observed matrix effect of urine has also been recently described in human plasma [26], despite the fact that the final concentration of DNPH in the assay was six times higher than used in our experiments.

3.2. Recovery, intra-/inter-day coefficient of variation and stability of MDA–DNPH

In these experiments different specimens of urine ($n = 4$) obtained on four different days were divided in two aliquots, one of them was spiked with 0.5 μ*M* of MDA. Each specimen was analysed using the method of standard additions in triplicate. The recovery of MDA was 94.3 ± 8.6% ($n = 12$) with an intra-day coefficient of variation of 7.1% and an

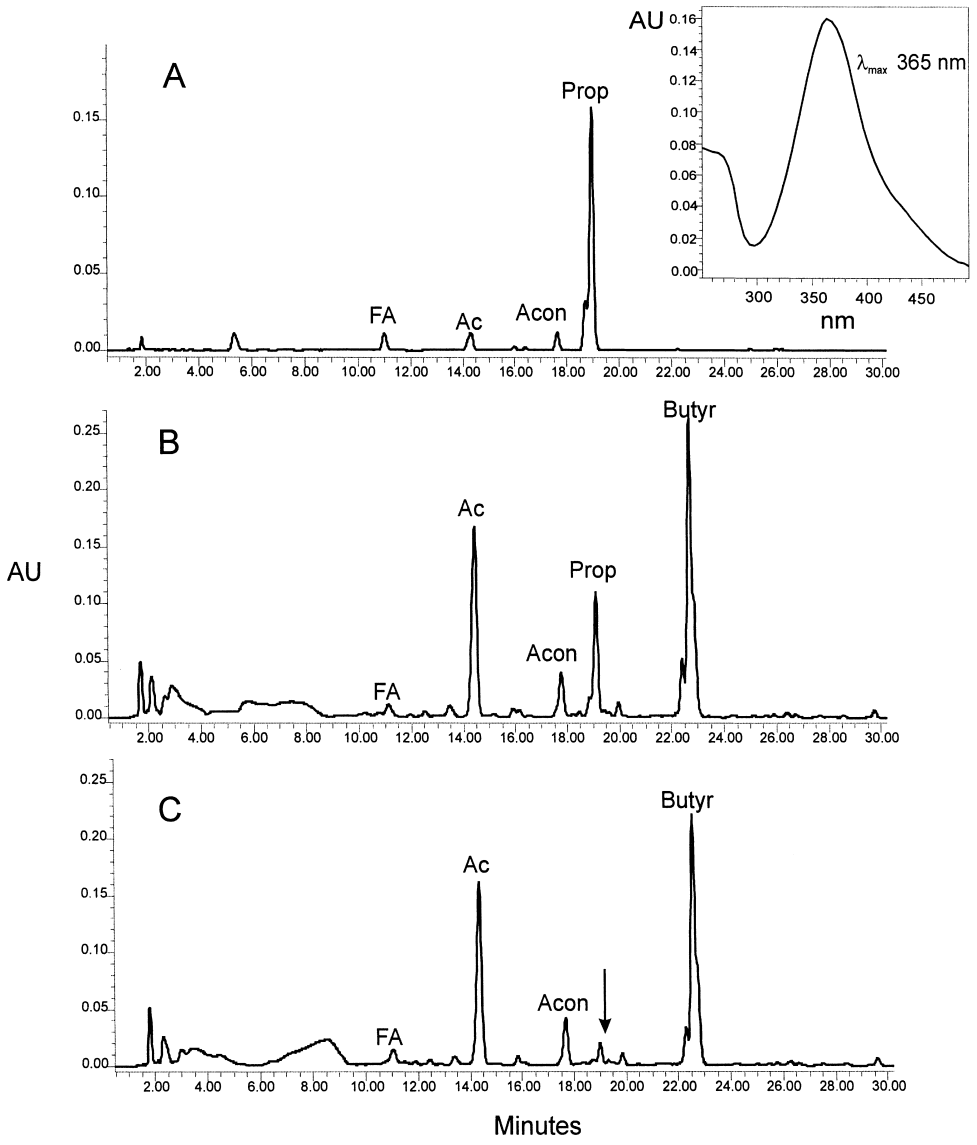

Fig. 1. Typical chromatograms at 365 nm of blank and urine specimen after derivatisation with DNPH. Conditions of derivatisation are as described in the Experimental section. (A) Blank spiked with propionaldehyde, (B) urine specimen spiked with propionaldehyde, (C) the same urine specimen without any spike. Abbreviations of DNPH derivatives with: formaldehyde, FA; acetaldehyde, Ac; acetone, Acon; propionaldehyde, Prop; butyraldehyde, Butyr. (Insert) Spectrum of propionaldehyde–DNPH adduct, λ_{max} = 365 nm. Arrow indicates the elution time for propionaldehyde–DNPH adduct.

inter-day coefficient of variation of 6.0%. The stability of the MDA–DNPH adduct was tested by reinjecting the same samples 24 h later after they had been stored in the dark at room temperature. There were no significant changes in the peak areas, which indicates that MDA–DNPH reconstituted in 50% acetonitrile is stable for at least 24 h when stored at room temperature in the dark.

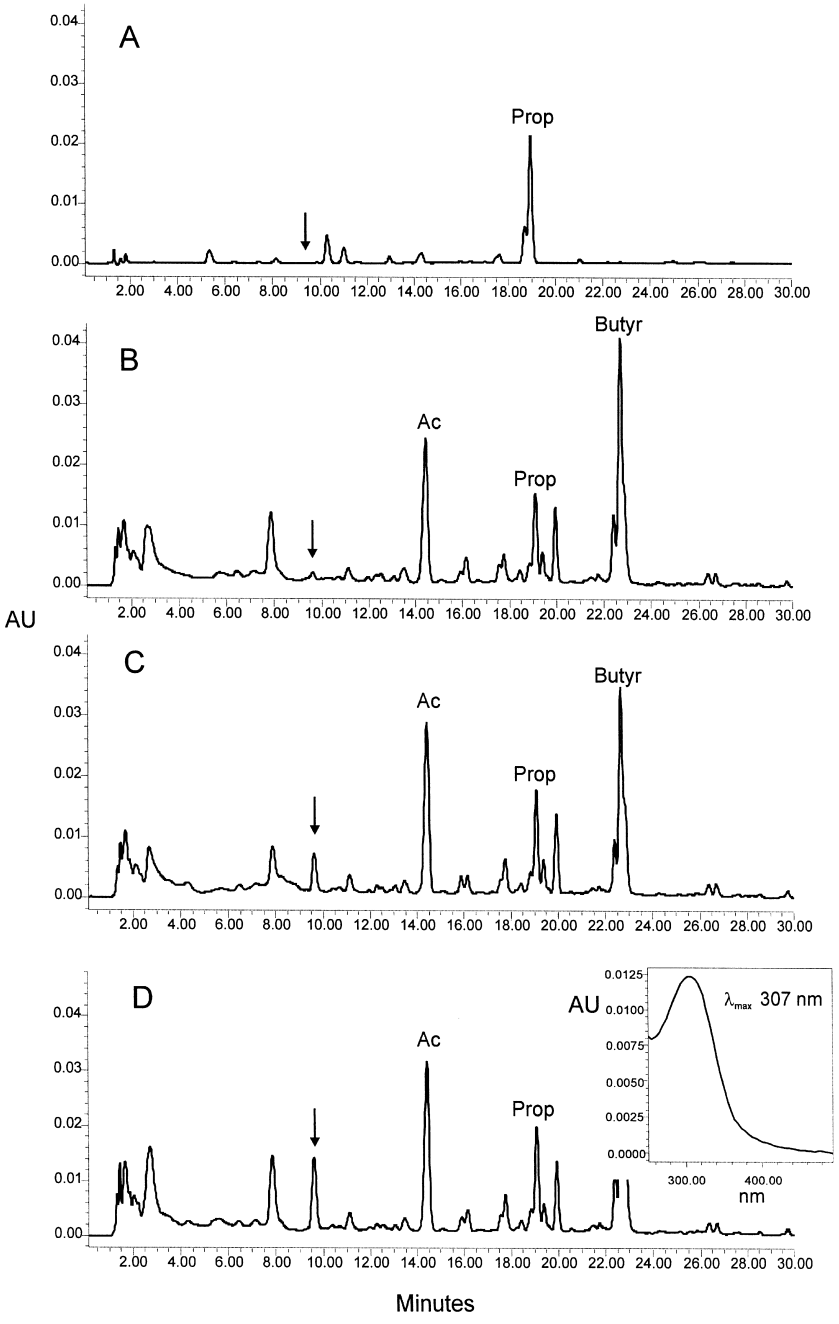

Fig. 2. Typical chromatograms at 307 nm of blank and urine specimen after derivatisation with DNPH. Conditions of derivatisation are as described in the Experimental section. (A) Blank spiked with propionaldehyde, (B) Urine specimen spiked with propionaldehyde only, (C) the same urine specimen spiked with propionaldehyde and 2.5 nmol MDA, (D) the same urine specimen spiked with propionaldehyde and 5.0 nmol MDA. Abbreviations of DNPH derivatives with: acetaldehyde, Ac; propionaldehyde, Prop; butyraldehyde, Butyr. (Insert) Spectrum of MDA–DNPH adduct, $\lambda_{max} = 307$ nm. Arrow indicates the elution of MDA–DNPH adduct.

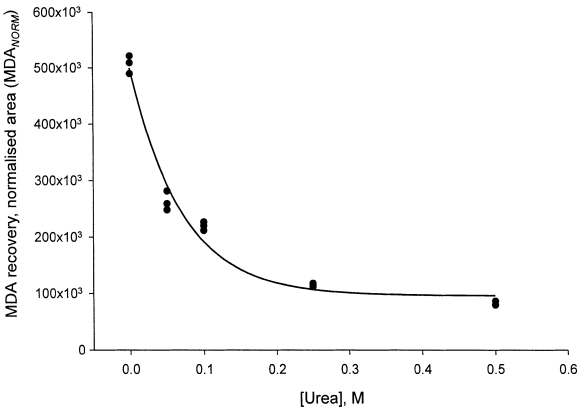

Fig. 3. The effect of concentration of urea in the assay on the derivatisation of MDA. The regression equation is $y(x) = y_0 + a \exp(-bx)$, where $y_0 = 95970$, $a = 403500$, $b = 14.38$ and $r^2 = 0.989$. Conditions of derivatisation are as described in the Experimental section except 3 ml of urea solution (0, 0.1, 0.25, 0.5 and 1.0 *M*) were used instead of urine. MDA_{NORM} is calculated as described in the Experimental section. All assays were spiked with 10 μl of 0.5 m*M* MDA (5 nmol MDA per assay). Three assays were performed for each concentration of urea.

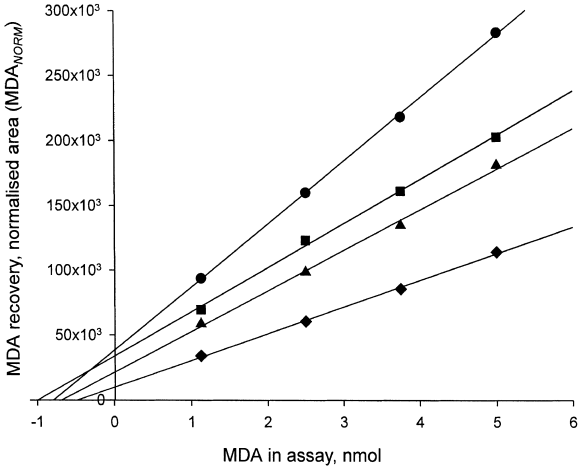

Fig. 4. Example of the measurement of MDA in four different urine specimens using the method of standard additions. Assays were spiked with 10 μl of standard MDA solutions 0.125, 0.25, 0.375 and 0.5 m*M* (corresponding to 1.25, 2.5, 3.75 and 5.0 nmol of MDA in the assay). Different symbols represent different urine specimens. Regression lines are $y(x) = kx + y_0$, where $y_0 = 38\,317.9$, $k = 48\,579.4$, $r^2 = 0.9995$ for specimen 1 (circles); $y_0 = 33\,823.9$, $k = 34\,007.5$, $r^2 = 0.9973$ for specimen 2 (squares); $y_0 = 21\,391.9$, $k = 31\,266.5$, $r^2 = 0.9961$ for specimen 3 (triangles); $y_0 = 9893.8$, $k = 20\,540$, $r^2 = 0.9985$ for specimen 4 (diamonds). MDA_{NORM} is calculated as described in the Experimental section.

3.3. Effects of storage of urine

Urine left over from the recovery experiments spiked with MDA was frozen in airtight containers and stored at −20 °C for 3 weeks. When thawed and reanalysed, the concentration of MDA had reduced by 43±15.7%. Storage in airtight containers on ice for 24 h did not result in any significant reduction in the concentration of MDA. Therefore, MDA in urine should be analysed within 24 h of collection and stored in an airtight container at 0 °C until analysis.

3.4. Human studies

In these experiments, urine specimens were stored on ice and analysed using the DNPH method within 2 h of collection. The concentration of MDA in urine of healthy volunteers was measured to be 0.019±0.012 μmol/mmol creatinine (0.169±0.111 μmol/g creatinine). These values are 5–10 times smaller than previously reported values of 0.89±0.35 nmol/mg creatinine [27], 0.23±0.02 μmol/mmol creatinine [8] and 1.94±0.79 μmol/g creatinine [13] and 1.5±1.5 μmol/mg creatinine [28]. In all the above cited papers, MDA in urine was measured as the MDA–TBA adduct, the determination of which involved incubation of urine with TBA at 100 °C for 1 h at low pH. In our method, urine was incubated at a significantly lower temperature (37 °C). We decided to additionally analyse our samples using TBA. The samples were analysed after they had been in storage at −20 °C for 3 weeks. Typical chromatograms at 533 nm for standard solutions of MDA and urine after incubation with TBA are shown on Fig. 5A, B. Previously we demonstrated that when spiked urine was stored for 3 weeks at −20 °C, the affect of storage was to reduce the level of MDA measured as the MDA–DNPH adduct by 43%. Thus, we might have expected that MDA measured in our stored samples as the MDA–TBA adduct would be significantly lower than that measured as MDA–DNPH in fresh samples. In fact the MDA levels measured as the MDA–TBA adduct were 0.181±0.063 μmol/mmol creatinine (1.60±0.55 μmol/g creatinine). As such, even allowing for an expected reduction in MDA concentration, the actual value measured as the MDA–TBA adduct was of the order of 10 times the

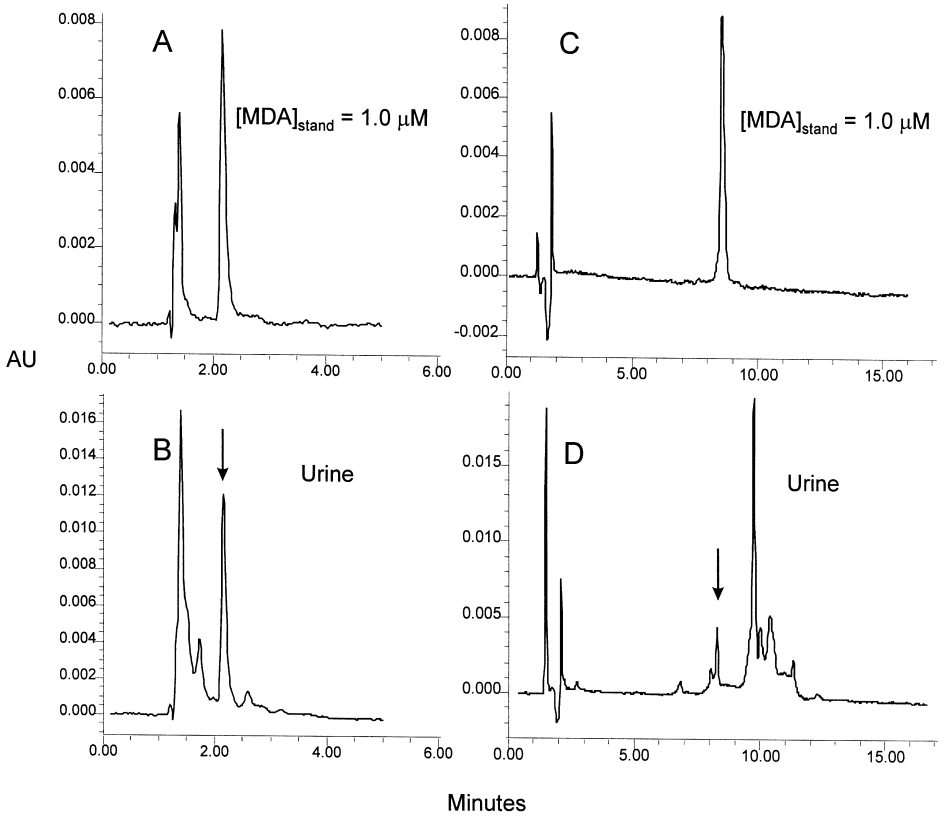

Fig. 5. Typical chromatograms at 533 nm of 1.0 m*M* solution of MDA and urine specimen after derivatisation with TBA. Volume injected on the column was 100 μl. (A, B) isocratic elution; (C, D) gradient elution. Elution conditions are as described in the Experimental section. Arrow indicates the retention time of MDA–TBA adduct according to standard (A, C).

value measured using DNPH. One possible reason for this large difference could be that during incubation of urine at high temperature and under acidic conditions MDA was formed from bladder/urethra cell debris that could be present in the samples. This possibility has yet to be addressed, for example, by prior filtration of samples, in the published literature. Whether incubation at high temperature coincident with low pH in the presence [8,13] or absence ([27] and present paper) of an antioxidant (BHT) can lead to the formation of MDA during the assay remains to be proved. Another possible reason is that TBA is not selective towards MDA, but also reacts with other compounds present in urine [2,10,11,29]. The use of HPLC is supposed to overcome this problem as well as to increase the sensitivity of the assay compared to the UV–VIS spectrophotometric detection. Unfortunately, the widely used isocratic method of elution [13,14,23,30] appears not to be effective enough to separate MDA–TBA from adducts of TBA with other TBA-reactive compounds, which then leads to an overestimation of the MDA concentration (Fig. 5A, B). The employment of a linear gradient of acetonitrile in water allows the separation of MDA–TBA from other TBA-adducts (Fig. 5C, D) that are responsible for the majority of the absorbance at 533 nm.

MDA is bound to proteins [29] and some amino acids (lysine, serine) [2]. Urine was not ultra filtered or pretreated in any other way in our experiments. Samples were derivatised with DNPH in acidic pH (final concentration of HCl in the assay 0.364 *M*) and simultaneously extracted with *n*-pentane. Therefore, the method described in this paper determines the total amount of MDA present in urine. Whether this incubation at 37 °C can still lead to the forma-

tion of MDA as an artefact will be the subject of future experiments.

4. Conclusions

In this paper we present a selective and sensitive method for the determination of MDA in human urine based on a derivatisation with DNPH at 37 °C. HPLC with UV detection and gradient elution is used for the separation of the MDA–DNPH adduct from DNPH adducts with other aldehydes present in urine. The concentration of MDA in normal human urine measured with the described method is 0.019 ± 0.012 μmol/mmol creatinine. We demonstrate that the widely used TBA method for the determination of MDA incorporating isocratic separation conditions probably overestimates the concentration of MDA present in urine by almost 10-fold.

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