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Short communication

Methyl malondialdehyde is not suitable as an internal standard for malondialdehyde detection in urine after derivatisation with 2,4-dinitrophenylhydrazine

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Abstract

A previously described method of measurement of malondialdehyde (MDA) in human urine after derivatisation with 2,4-dinitrophenylhydrazine (DNPH) was tested for a possibility of using methyl malondialdehyde (MeMDA) as an internal standard. Despite structural similarity, those compounds were found to produce different yields of derivatisation under the same conditions depending on urine matrix. We conclude, that MeMDA is not suitable as an internal standard for the measurement of MDA in urine under previously reported conditions when DNPH is used as a deriviatising agent.

Keywords: Derivatisation, LC; Methyl malondialdehyde; 2,4-Dinitrophenylhydrazine

1. Introduction

Urinary malondialdehyde (MDA) has been widely used to monitor oxidative stress in a variety of models [1–7]. However, the use of different analytical techniques and the lack of a reliable internal standard have led to significant variability in the results achieved with this method. Methyl malondialdehyde (MeMDA) appears to satisfy the requirements for an appropriate internal standard in that it is structurally similar to MDA and it is naturally absent from biological matrices. Its use as an internal standard for measuring MDA after derivatisation with phenylhydrazine has been recently validated for rat liver microsomes and human plasma [8]. The authors defined strict reaction conditions in order to obtain comparable yields of phenylhydrazine derivatives of MDA and MeMDA. Deviation from these conditions led to irreproducible results that were attributed to incomplete derivatisation of MDA or MeMDA. The direct quantification of MDA (without the derivatisation step) using MeMDA as an internal standard has recently been reported in rat liver microsomes, plasma [9] and in rat brain homogenates [10,11] by capillary zone electrophoresis. MeMDA was also used as an internal standard for measurements of MDA after derivatisation with 2,4-dinitrophenylhydrazine (DNPH) in human plasma. Samples had to be diluted to 10% to take account of significant matrix effects on the derivatised yields of MDA and MeMDA [12].

We recently reported a method for the determination of MDA in human urine using the method of standard additions after derivatisation with DNPH [13]. Strong matrix effects of urine on the derivatised yields of MDA-DNPH were observed for different specimens. Here we describe an attempt to use MeMDA as an internal standard for measuring MDA in urine after derivatisation with DNPH.

2. Experimental

2.1. Chemicals

All organic solvents were of HPLC grade (HiPerSolv), NaOH and HCl (ARISTAR grade) were purchased from VWR International, Poole, UK. 2,4-Dinitrophenylhydrazine (FW 198.1, containing approximately 30% water), 1,1,3,3-tetramethoxypropane (99%), 3-dimethylamino-2-methyl-2-propenal (99%) and propionaldehyde (97%), were obtained

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from Aldrich, Dorset, UK. HPLC grade KH_2PO_4 was purchased from Fisher Scientific UK (Loughborough, UK). Ultra-pure water was used throughout the study.

2.2. Preparation of standards

MeMDA was synthesized by modifying a reported procedure [11]. Briefly, 0.5 g 3-dimethylamino-2-methyl-2-propenal, 0.2 g sodium hydroxide and 0.7 ml water were incubated at 70 °C with continuous vigorous stirring until the initial phases merged into one. Liquid was evaporated under reduced pressure and white powdery crystals were washed with a mix of acetone:isopropanol (four times) and then acetone:ethanol (three times) (both mixtures were 50:50% (v/v)). The powder was then lyophilised. The MDA solution was prepared by hydrolysing 1,1,3,3-tetramethoxypropane in 0.1 mM HCl at 40 °C for 60 min as described previously [13]. Stock solutions of MeMDA and MDA were prepared in 50 mM KH_2PO_4 buffer at pH 7.0. Concentrations of stock solutions of MeMDA and MDA were calculated based on the molar absorbance of MeMDA at $\lambda = 274$ nm ($\varepsilon = 29900\,\mathrm{mol\,l^{-1}\,cm^{-1}}$) and MDA at $\lambda = 267$ nm ($\varepsilon = 31800\,\mathrm{mol\,l^{-1}\,cm^{-1}}$) [8].

2.3. Preparation of samples

Spot samples of urine donated by healthy individuals ($n = 6$) were pooled on three different occasions. An informed consent was obtained. Sample preparation (derivatisation and extraction) was carried out according to [13] except a higher concentration of DNPH reagent was used (0.05 g in 50 ml of 4 M HCl, ca. 3.87 mM). Briefly, 3 ml urine, 3 ml water, 10 μl of 1mM propionaldehyde, 10 μl MDA standard and 10 μl MeMDA standard (0, 0.05, 0.1, 0.2, 0.3, or 0.5 mM each), 0.6 ml of DNPH solution, 10 ml of pentane were incubated at 37 °C for 60 min with continuous shaking. In blank samples, urine was replaced with water. After cooling the pentane phase was removed and evaporated under nitrogen. The residue was reconstituted in the mobile phase and injected on the HPLC column. Peak areas were integrated at their $\lambda_{max} = 307$ nm for MDA-DNPH and $\lambda_{max} = 322$ nm for MeMDA-DNPH. Synthetic hydrazones of MDA and MeMDA were prepared by reacting concentrated solutions of the above with concentrated derivatising agent [13]. The identification of MDA-DNPH and MeMDA-DNPH in the HPLC spectra was done by spiking the derivatised samples with solutions of synthetic hydrazones in acetonitrile and their coelution. Derivatisation of MeMDA and MDA was performed in triplicate in each pool of urine and compared with derivatisation in water (blank samples, no matrix effects). The latter were performed in triplicate on three occasions.

2.4. Chromatographic system 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 MeMDA was achieved using a Waters Symmetry^{TM} C_{18} column (3.9 × 150 mm) and a guard column, Waters Symmetry^{TM} C_{18} (3.9 × 20 mm). Gradient conditions were as described previously [13]. Briefly, a linear gradient of acetonitrile in water (from 30 to 70% in 30 min) at a flow rate of 1 ml/min was used for the elution. The column temperature was set to 40 °C. The range of wavelengths scanned was 250–400 nm.

Table 1
Comparison of derivatisation yields for MDA and MeMDA in pooled urine and water

Matrix		k_{MDA}/k_{MeMDA}	P-value	r^2_{MDA}	r^2_{MeMDA}
Urine	Pool 1	1.309	<0.001	0.9999	0.9998
	Pool 2	1.198	0.004	0.9989	0.9969
	Pool 3	1.446	0.021	0.9993	0.9804
Water	Exp. 1	0.968	0.499	0.9999	0.9996
	Exp. 2	0.972	0.428	0.9999	0.9964
	Exp. 3	0.993	0.722	0.9999	0.9912

Regression analysis ($y(x) = kx + b$, r^2) was performed by plotting the integrated peak areas of MDA-DNPH and MeMDA-DNPH against the known added amounts of MDA and MeMDA (see section 2.5). k_{MDA}, r^2_{MDA} and k_{MeMDA}, r^2_{MeMDA} are the slopes of regression lines and values r^2 for MDA and MeMDA, respectively.

2.5. Calibration curves and calculations

Method of standard additions [14] was used to create calibration curves for MDA and MeMDA. Each assay, containing 3 ml of pooled urine specimen (or water), was spiked in triplicate with 10 μl MDA standard and 10 μl MeMDA standard (0, 0.05, 0.1, 0.2, 0.3 or 0.5 mM each). This corresponded to standard additions of 0, 0.5, 1.0, 2.0, 3.0 or 5.0 nmol of MDA and MeMDA per assay. Regression analysis ($y(x) = kx + b$, r^2) was performed by plotting the integrated peak areas of MDA-DNPH and MeMDA-DNPH against the known added amounts of MDA and MeMDA. For each experiment, the ratio of slopes of the regression lines (k_{MDA}/k_{MeMDA}) for MDA and MeMDA was calculated.

Two-way ANOVA was performed using Minitab (version 13) software. The P-values for the interaction term between compound (MDA and MeMDA) and concentration (standard additions) for each experiment are given in Table 1. The slopes of regression lines were considered significantly different when $P < 0.05$.

3. Results and discussion

Typical chromatograms showing the elution profile of DNPH derivatives of MDA and MeMDA in non-spiked, spiked human urine, and water blank are shown in Fig. 1.

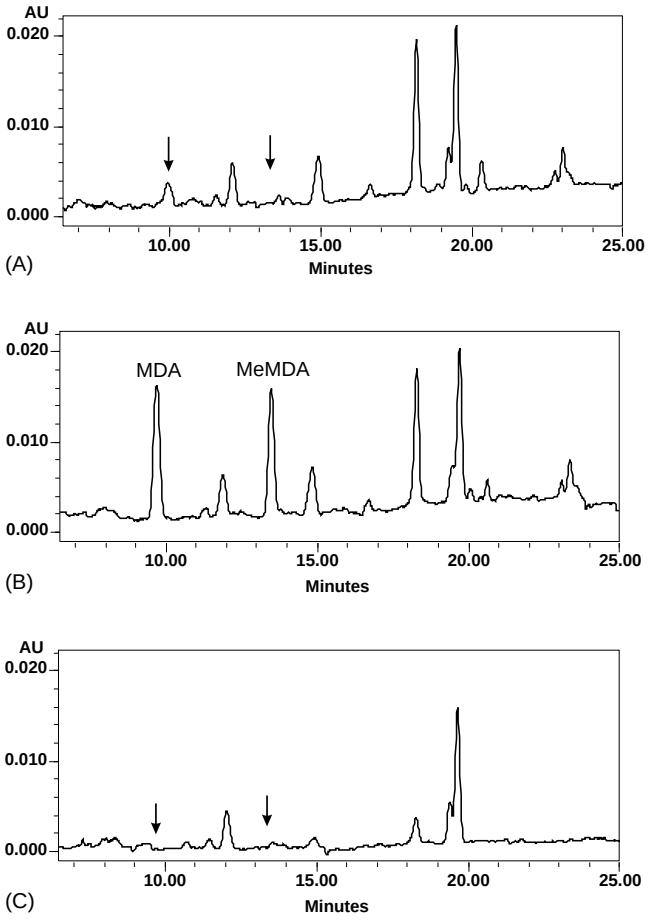

Fig. 1. Typical chromatograms at 307 nm of urine specimen and blank after derivatization with DNPH. Conditions of derivatisation are as described in Experimental section. A—non-spiked urine specimen, B—the same urine specimen spiked with 5 nmoles of MDA and MeMDA and C—water blank. (↓) indicate the elution times for MDA-DNPH and MeMDA-DNPH.

In water derivatisation yields for MeMDA and MDA were found to be consistent and very similar, with the ratio of their slopes (k_{MDA}/k_{MeMDA}) close to 1 (Table 1). Small differences observed could probably be due to small differences in the molar absorbance coefficients of the derivatised products. For example, the presence of the methyl group may affect the molar absorbance of derivatives as it does influences the λ_{max} for MDA-DNPH and MeMDA-DNPH (307 versus 322 nm) as it is shown on Fig. 2.

In urine derivatisation yields for MDA and MeMDA were different with a significant variation in the slopes of their regression lines and in the ratio of k_{MDA}/k_{MeMDA} (Table 1). Derivatisation yield of MeMDA was always less in urine compared to MDA. An increase in the reaction time up to 2 h did not significantly alter the k_{MDA}/k_{MeMDA} ratio (not shown), suggesting that MeMDA was more sensitive to matrix effects of urine than MDA. It was reported previously that the optimal derivatisation yield for both compounds was found at pH 4.0 (citrate buffer) using phenylhydrazine (not DNPH) as the deriviatising agent [8]. In the present study, increasing the pH to 4.0 by including 1 M citrate buffer in the assay was found to have reduced the yield of MDA-DNPH by approximately 25% and MeMDA-DNPH by approximately 80%. Our results suggested that derivatisation of MDA and especially MeMDA with DNPH required a lower pH (pH of all assay mixtures, including all three pools of urine after an addition of an appropriate amount of DNPH, were within the range of 1.8–2.0; final concentration of HCl in our assay mixture was approximately 0.363 M).

In conclusion, we have shown that under the conditions of our study MeMDA was not suitable as an internal standard for the measurement of MDA in urine using DNPH as the deriviatising agent.

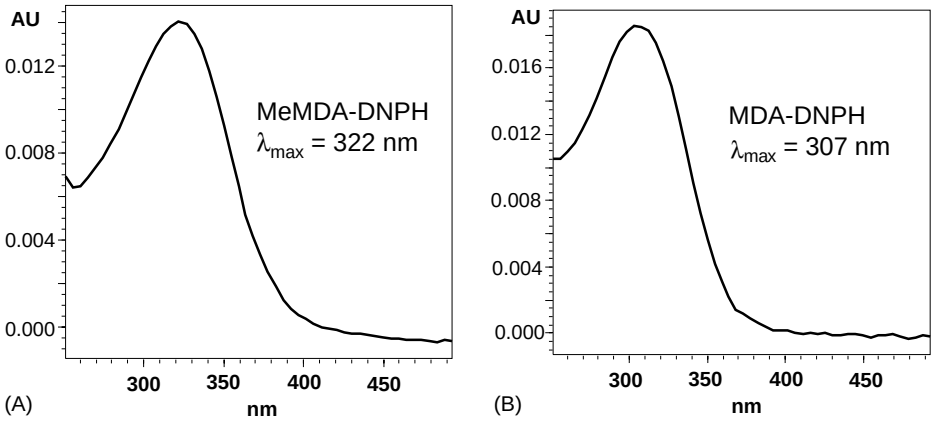

Fig. 2. Typical UV spectra of MeMDA-DNPH and MDA-DNPH from urine spiked with MeMDA and MDA. Conditions of derivatisation are as described in Experimental section. A—MeMDA-DNPH derivative, λ_{max} = 322 nm and B—MDA-DNPH, λ_{max} = 307 nm.

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References

[1] H.H. Draper, L. Polensek, M. Hadley, L.G. McGirr, Lipids 19 (1984) 836.
[2] H. Mahmoodi, M. Hadley, Y.X. Chang, H.H. Draper, Lipids 30 (1995) 963.
[3] S.N. Dhanakoti, H.H. Draper, Lipids 22 (1987) 644.
[4] J.A. Drury, J.A. Nycyk, R.W.I. Cooke, Clin. Chim. Acta 263 (1997) 177.
[5] M.A. Shara, P.H. Dickson, D. Bagchi, S.J. Stohs, J. Chromatogr. 576 (1992) 221.
[6] Y.L. Deng, P.H. Yu, J. Chromatogr. Sci. 37 (1999) 317.
[7] Y.L. Deng, F. Boomsma, P.H. Yu, Life Sci. 63 (1998) 2049.
[8] G. Cighetti, P. Allevi, L. Anastasia, L. Bortone, R. Paroni, Clin. Chem. 48 (2002) 2266.
[9] R. Paroni, I. Fermo, G. Cighetti, Anal. Biochem. 311 (2002) 101.
[10] K. Claeson, F. Aberg, B. Karlberg, J. Chromatogr. B 740 (2000) 87.
[11] K. Claeson, G. Thorsen, B. Karlberg, J. Chromatogr. B 751 (2001) 315.
[12] A.S. Sim, C. Salonikas, D. Naidoo, D.E.L. Wilcken, J. Chromatogr. B 785 (2003) 337.
[13] O. Korchazhkina, C. Exley, A.S. Spencer, J. Chromatogr. B 794 (2003) 353.
[14] M. Bader, J. Chem. Edu. 57 (1980) 703.