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TECHNICAL NOTE

A quick, convenient and economical method for the reliable determination of methylglyoxal in millimolar concentrations: the *N*-acetyl-L-cysteine assay

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Abstract The determination of methylglyoxal (MG) concentrations in vivo is gaining increasing importance as high levels of MG are linked to various health impairments including complications of diabetes. In order to standardize the measurements of MG in body fluids, it is necessary to precisely determine the concentration of MG stock solutions used as analytical standards. The "gold standard" method for the determination of MG concentration in the millimolar range is an enzyme-catalyzed endpoint assay based on the glyoxalase I catalyzed formation of *S*-lactoylglutathione. However, as this assay used purified glyoxalase I enzyme, it is quite expensive. Another method uses a derivation reaction with 2,4-dinitrophenylhydrazine, but this substance is explosive and needs special handling and storage. In addition, precipitation of the product methylglyoxal-bis-2,4-dinitrophenylhydrozone during the reaction limits the reliability of this method. In this study, we have evaluated a new method of MG determination based on the previously published fast reaction between MG and *N*-acetyl-L-cysteine at room temperature which yields an easily detectable condensation product, *N*-α-acetyl-*S*-(1-hydroxy-2-oxo-prop-1-yl)cysteine. When comparing these three different assays for the measurement of MG concentrations, we find that the *N*-acetyl-L-cysteine assay is the most favorable, providing an economical and robust assay without the need for the use of hazardous or expensive reagents.

Keywords Methylglyoxal · Glyoxalase I · 2,4-Dinitrophenylhydrazine · *N*-acetyl-L-cysteine · Spectrophotometric measurements

Abbreviations

2,4-DNPH	2,4-Dinitrophenylhydrazine
MG	Methylglyoxal
ε	Molar absorption coefficient

Introduction

Methylglyoxal (MG) is a dicarbonyl compound formed by the fragmentation of triose phosphates, intermediates of glycolysis [1], by lipid peroxidation, by the metabolism of acetone and aminoacetone, and by the degradation of DNA [2, 3]. In addition, methylglyoxal is ubiquitous in heated and fermented beverages and foods, such as coffee, toast and soy sauce [4]. MG is detoxified by the glyoxalase I and II enzymes via the intermediate product *S*-lactoylglutathione to yield D-lactate [5].

High levels of plasma MG as observed in patients with diabetes are linked to an increased risk of diabetic complications such as diabetic nephropathy, retinopathy, neuropathy and cardiovascular complication [6]. Therefore, the measurement of MG concentrations in tissues and body fluids is an important analytical method with potential future applications in the clinical laboratory. Many methods for the determination of MG concentration in body fluids have been described. In most cases, these methods use HPLC with either detection by UV, fluorescence or mass spectrometry [7–11]. To standardize these measurements, it is necessary

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to use stock solutions of MG with precisely determined concentrations as analytical standards.

The "gold standard" method for the determination of MG concentrations in the millimolar range, e.g. in diluted commercial MG solutions, is an endpoint assay involving the reaction of MG with reduced glutathione to *S*-D-lactoylglutathione catalyzed by glyoxalase I and occurs via a spontaneously formed hemithioacetal intermediate. This hemithioacetal is isomerized to the product *S*-D-lactoylglutathione by the isomerase activity of glyoxalase I. This method, which was firstly described by Racker [12], requires reduced glutathione and recombinant or purified glyoxalase I, which are both quite expensive.

Another method for MG determination uses 2,4-dinitrophenylhydrazine (2,4-DNPH), but this compound is sensitive to shock and friction and can explode, so care must be taken with its use [13]. In addition, occasional precipitation of the product methylglyoxal-bis-2,4-dinitrophenylhydrozone also limits the reliability of this method. A further issue is that many suppliers provide 2,4-DNPH as a wet product (water is added to minimize the danger of explosion) without specifying the 2,4-DNPH content.

Therefore, there is a demand for a more convenient, safe and also reasonably priced method for the determination of MG concentration in stock solutions. Over a decade ago, Lo et al. [14] described a reaction which might serve this purpose. They demonstrated that *N*-acetyl-L-cysteine reacts rapidly at room temperature with MG with a yield >99.3 %.

The purpose of this study was to compare the different spectrophotometric methods for MG measurement. We propose that the *N*-acetyl-L-cysteine reaction introduced by Lo et al. can be used as a precise analytical assay and has multiple advantages compared to the current methods.

Materials and methods

Materials

Methylglyoxal solution 40 % (*w*/*w*, 6.50 M), glyoxalase I from *Saccharomyces cerevisiae* in buffered aqueous glycerol solution (318 U/mL), reduced glutathione, *N*-acetyl-L-cysteine and 2,4-dintirophenylhydrazine were purchased from Sigma-Aldrich. The spectrophotometric measurements were carried out in a Jenway 6505 scanning spectrophotometer.

Methods

Determination of MG using the glyoxalase I assay

The enzyme glyoxalase I catalyzes the isomerization of the hemithioacetal, formed by the condensation of methylglyoxal and reduced glutathione, to *S*-D-lactoylglutathione. A 100-mM KH_2PO_4 buffer (adjusted to pH 6.6 at room temperature (RT) with 1 M KOH) and an aqueous 100 mM reduced glutathione solution (adjusted to pH 6.6 at RT with solid sodium bicarbonate, freshly prepared) were used. The 40 % MG stock solution, as provided by Sigma-Aldrich, was diluted daily to a 10-mM working solution for following experiments. The measurement was carried out in a 1-ml cuvette at 22 °C. The appropriate volume of KH_2PO_4 buffer (to reach a final volume of 1 mL after addition of the MG solution in the next step), 20 μL of the 100 mM reduced glutathione solution (final concentration, 2 mM) and 3.14 μL (1 U) glyoxalase I were combined and the absorption of the spectrophotometer at 240 nm set to zero. Ten or 30 μL of the 10 mM methylglyoxal solution (final concentration, 0.1 or 0.3 mM) was added to yield a total volume of 1 mL. The absorption at a wavelength of 240 nm was recorded after 30 min. The concentration of *S*-D-lactoylglutathione was calculated using a molecular absorption coefficient of 3,370 M^{-1} cm^{-1} at pH 6.6 [12].

Determination of MG using the 2,4-dinitrophenylhydrazine assay

A 10-mM stock solution of 2,4-DNPH in ethanol was prepared by heating the solution carefully to 80 °C with occasional vortexing. This solution can be stored at room temperature [13]. From this stock solution, a 1:50 dilution with an end concentration of 0.2 mM 2,4-DNPH was prepared daily using a HCl/ethanol solvent consisting of 12 mL HCl per 100 mL ethanol. In addition, a 1-mM MG working solution was freshly prepared from the stock solution every day. For the reaction, 950 μL of 0.2 mM 2,4-DNPH and different volumes of 1 mM methylglyoxal solution with final concentrations of 0, 0.5, 1, 2, 4, 6, 8, 10, 20 and 30 μM were pipetted into a cuvette and made up to 1 mL with distilled water. The reaction was performed by heating the reaction mix for 45 min at 42 °C in an Eppendorf thermomixer at 600 rpm. After the incubation, the samples were incubated for 5 min at room temperature. Spectrophotometer measurements were carried out at 432 nm according to the maximum absorbance of MG-bis-2,4-DNP-hydrazone. The molecular absorption coefficient of 33,600± 100 M^{-1} cm^{-1} for MG-bis-2,4-DNP-hydrazone [13] was used to calculate the concentrations.

Determination of MG using the N-acetyl-L-cysteine assay

An aqueous 500-mM *N*-acetyl-L-cysteine solution was freshly prepared. The reaction was carried out in 100 mM sodium dihydrogen phosphate buffer (adjusted to pH 7.0 with 10 M NaOH) at 22 °C. First, the MG solutions (5, 20 and 50 μL) equating to 0.5, 2 and 5 mM were added up to a volume of 980 μL with sodium dihydrogen phosphate

buffer and the spectrophotometer set to zero. The reaction was started by adding 20 μL of the *N*-acetyl-L-cysteine solution (final concentration up to 10 mM), and the formation of the product *N*-α-acetyl-*S*-(1-hydroxy-2-oxo-prop-1-yl)cysteine was recorded for 10 min at a wavelength of 288 nm.

Method validation

Intra-day precision was defined as the relative standard deviation (RSD) calculated from the values measured from at least three samples (of three different concentrations) measured on the same day. Inter-day precision was calculated using the values measured from three different samples determined at three different days.

Results

Determination of MG using the glyoxalase I assay

The reaction catalyzed by glyoxalase I is observed by following the formation of the product *S*-D-lactoylglutathione at 240 nm. In this experiment, the exact MG concentration was determined at two different nominal MG concentrations (0.1 and 0.3 mM). For an endpoint assay, it is important that the reaction is complete. In order to ensure this, reduced glutathione (2 mM) was used in excess.

The time course of *S*-D-lactoylglutathione formation followed at 240 nm shows an increase of absorbance consistent with a reaction following pseudo-first-order kinetics, reaching a plateau phase when all the MG has been derivatized (data not shown). In the described experiment, the half-time was calculated to be 40.3±3.3 s (n=4). The produced *S*-D-lactoylglutathione has a molecular absorption coefficient of 3,370 M^{-1} cm^{-1} at pH 6.6 [12]. Using this value, the MG concentration of the commercial 40 % stock solution was determined to be 6.62±0.15 M, consistent with the concentration of 6.5 M stated by the supplier (n=3, in triplicate). As the measured absorbance is supposed to be in the linear range of the spectrophotometer (0–2 A.U.), the suggested range of MG concentration is 50–500 μM.

The within-assay (intra-day) precision values were 5.8 % (for 0.3 mM MG) and 4.5 % (for 0.1 mM MG) (n=3). The inter-day precision values were 2.8 % (for 0.3 mM MG) and 2.4 % (for 0.1 mM MG, n=3, in triplicate).

Determination of MG by the 2,4-dinitrophenylhydrazine assay

In a second experiment, the MG concentration was measured using the 2,4-dinitrophenylhydrazine method with MG concentrations between 0.5 and 30 μM and 2,4-dinitrophenylhydrazine in excess (200 μM).

For MG concentrations lower than 6 μM, differing ε values were gained due to a lack of precision of the spectrophotometer in this absorbance range. Product concentrations higher than 30 μM led to occasional precipitation and therefore obstructed spectrophotometric measurements. Another issue is the dissolving of the explosive 2,4-dinitrophenylhydrazine which requires heating and vortexing and is therefore a potentially dangerous procedure. The produced 2,4-dinitrophenyl hydrazone of MG has a molecular absorption coefficient of 33,600±100 M^{-1} cm^{-1} [13]. The MG concentration of the original stock solution can be calculated using the values for concentration between 6 and 30 μM and was found to be 6.56±0.13 M (n=3, in triplicate). The obtained concentration was consistent with the 6.5-M concentration supplied by the manufacturer. Based on the obtained results, the suggested range of measurement for this assay is 6–30 μM.

The within-assay (intra-day) precision values were 2.8 % (for 30 μM MG), 7.88 % (for 20 μM MG) and 9.7 % (for 10 μM MG, n=3). The inter-day precision values were 4.0 % (for 30 μM MG), 5.4 % (for 20 μM MG) and 4.9 % (for 10 μM MG, n=3, in triplicate).

Determination of MG using the *N*-acetyl-L-cysteine assay

A relatively unknown method for MG determination which features many advantages compared to the previous two assays is the reaction of MG with *N*-acetyl-L-cysteine which was originally described by Lo et al. in 1994 [14].

The *N*-acetyl-L-cysteine assay is based on the fast reaction between MG and *N*-acetyl-L-cysteine at room temperature which yields the easily detectable condensation product *N*-α-acetyl-S-(1-hydroxy-2-oxo-prop-1-yl)cysteine. In contrast to the *N*-acetyl-L-cysteine assay which only depends on the spontaneous formation of a hemithioacetal, the glyoxalase I assay has a comparable first step, but further includes the glyoxalase I catalyzed isomerization of the hemithioacetal intermediate [15].

To optimize this reaction for an analytical application, the time course of the reaction was recorded in order to find out the optimal incubation time. Then, standard curves were determined and the optimal range of MG concentration and a precise molar absorption coefficient calculated. A time course experiment of the reaction between MG and *N*-acetyl-L-cysteine was performed (Fig. 1). The reaction observes pseudo-first-order kinetics as *N*-acetyl-L-cysteine was used in excess (10 mM). The half-time of the reaction was determined to be 9.3±1.4 s (n=3).

In order to determine the optimal range of MG concentration, in which the assay is linear, various MG concentrations between 0 and 10 mM were incubated with *N*-acetyl-L-cysteine. The samples were incubated for 5 min at

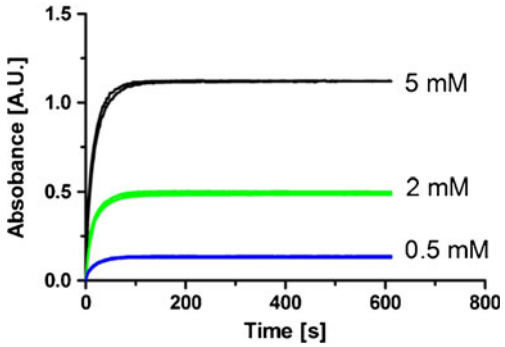

Fig. 1 Spontaneous reaction of *N*-acetyl-L-cysteine (10 mM) with methylglyoxal at room temperature. The time course of the reaction of the different concentrations of MG (0.5, 2, and 5 mM) with *N*-acetyl-L-cysteine was followed by measuring the absorbance of the product *N*-α-acetyl-*S*-(1-hydroxy-2-oxo-prop-1-yl)cysteine at 288 nm. Curves are drawn using the combined data from three independent experiments

room temperature and the change in absorbance at 288 nm measured.

Absorbance was found to be linearly correlated to concentration in the range between 0.25 and 2.5 mM MG (Fig. 2). The within-assay (intra-day) precision values were 2.7 % (for 0.5 mM MG), 1.8 % (for 2.5 mM MG) and 2.2 % (for 5 mM MG). The inter-day precision values were 3.5 % (for 0.5 mM MG), 1.6 % (for 2.5 mM MG) and 1.1 % (for 5 mM MG). According to these results, the optimal range for the determination of MG by the *N*-acetyl-L-cysteine assay is between 0.25 and 2.5 mM.

Since the two previous assays for the determination of MG (glyoxalase I and DNPH) had confirmed that the stated concentration of the commercial MG stock solution was correct, this concentration (using 2.5 mM MG) was used to determine the molar absorption coefficient for the MG adduct *N*-α-acetyl-S-(1-hydroxy-2-oxo-prop-1-yl)cysteine to be 249±8 M^{-1} cm^{-1} (n=14).

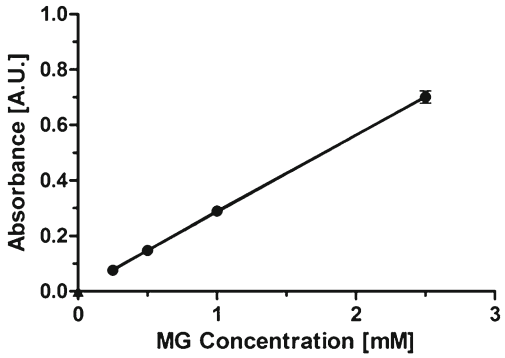

Fig. 2 Standard curves of the reaction between *N*-acetyl-L-cysteine (10 mM) and different concentrations of methylglyoxal. The product *N*-α-acetyl-*S*-(1-hydroxy-2-oxo-prop-1-yl)cysteine was measured at 288 nm after 5 min. Data represent the mean±SD (n=8)

Direct comparison of the *N*-acetyl-L-cysteine assay with the glyoxalase I assay in a dilution experiment

In order to compare the *N*-acetyl-L-cysteine assay with the glyoxalase I assay head-to-head in a dilution experiment, a freshly prepared 1 M MG solution in water was diluted to 2.5 mM (for the *N*-acetyl-L-cysteine assay) and 0.3 mM (for the glyoxalase I assay), the optimal concentrations for each assay. Both assays were performed five times, and the MG concentrations were calculated using the specific extinction coefficients and dilution factors. The MG concentrations determined by the *N*-acetyl-L-cysteine assay and the glyoxalase I assay were 0.98±0.02 and 1.02±0.04 M, respectively (n=5).

Discussion

The results of the glyoxalase I and 2,4-dinitrophenylhydrazine assays were consistent with previous literature. However, both methods have significant disadvantages since the first assay is dependent on an expensive enzymatic reaction and the second requires the handling of a hazardous substance. A comparison of the three methods is summarized in Table 1.

In this paper, the reaction previously described by Lo et al. [14] was optimized for a quantitative application. The molar absorption coefficient was corrected and now corresponds to the total amount of MG in solution. According to previous literature, *N*-α-acetyl-*S*-(1-hydroxy-2-oxo-prop-1-yl)cysteine has a molecular absorption coefficient of 98±6 M^{-1} cm^{-1} [14]. However, this value was obtained using a stopped flow experiment in which the reaction was performed at 37 °C and the extinction was only measured only for the first 2 s. This measurement did not take into account that MG in solution is present not only as a free monomer but also as monohydrate, dihydrate and other oligomerization products [16].

Table 1 Comparison of the three different spectrophotometrical methods of MG determination

Assay	Glyoxylase I	2,4-DNPH	*N*-acetyl-L-cysteine
ε (M^{-1} cm^{-1})	3,430	33,600	249
MG concentration	50–500 μM	6–30 μM	0.1–10 mM
Cost	Expensive	Economical	Economical
Reaction time	10 min	45 min	90 s
Dangerous	No	2,4-DNPH explosive	No
Experimental problems	Loss of enzyme activity upon storage	Occasional precipitations	None

Before being able to react with *N*-acetyl-L-cysteine, the MG oligomers and hydrates need to dissociate to yield free MG [14]. The half-time described by Lo et al. [14] for the reaction of free MG was 0.25 s. The half-time calculated in our experiments of 9.3±1.4 s corresponds to the complete reaction of MG and is predominantly determined by its liberation from the hydrated forms.

It has to be avoided that the buffer, in which the stock solutions are diluted, interferes with the correct determination of MG. The commercially available 40 % MG preparations contain water, and it is recommended that all dilutions are made in distilled water. It is not advisable to use diluents containing nucleophilic reagents such as thiols and amines since they might react with MG [17, 18].

This new assay encourages routine determination of MG solutions especially if they were stored for a long time, frozen or the concentration is uncertain as it is easy, quick and economical. It might also be possible to adapt this assay for other equally reactive dicarbonyl components such as glyoxal.

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References

1. Phillips SA, Thornalley PJ (1993) The formation of methylglyoxal from triose phosphates. Investigation using a specific assay for methylglyoxal. Eur J Biochem 212:101–105
2. Zou Z, Wu L, Ding H, Wang Y, Zhang Y, Chen X, Zhang CY, Zhang Q, Zen K (2012) MicroRNA-30a sensitizes tumor cells to *cis*-platinum via suppressing beclin 1-mediated autophagy. J Biol Chem 287:4148–4156
3. Chaplen FW, Fahl WE, Cameron DC (1996) Detection of methylglyoxal as a degradation product of DNA and nucleic acid components treated with strong acid. Anal Biochem 236:262–269
4. Wang J, Chang T (2010) Methylglyoxal content in drinking coffee as a cytotoxic factor. J Food Sci 75:H167–171
5. Thornalley PJ (1993) The glyoxalase system in health and disease. Mol Aspects Med 14:287–371
6. Brownlee M (1995) Advanced protein glycosylation in diabetes and aging. Annu Rev Med 46:223–234
7. Ohmori S, Mori M, Kawase M, Tsuboi S (1987) Determination of methylglyoxal as 2-methylquinoxaline by high-performance liquid chromatography and its application to biological samples. J Chromatogr 414:149–155
8. Espinosa-Mansilla A, Duran-Meras I, Salinas F (1998) High-performance liquid chromatographic–fluorometric determination of glyoxal, methylglyoxal, and diacetyl in urine by prederivatization to pteridinic rings. Anal Biochem 255:263–273
9. Lapolla A, Flamini R, Tonus T, Fedele D, Senesi A, Reitano R, Marotta E, Pace G, Seraglia R, Traldi P (2003) An effective derivatization method for quantitative determination of glyoxal and methylglyoxal in plasma samples by gas chromatography/mass spectrometry. Rapid Commun Mass Spectrom: RCM 17:876–878
10. Nemet I, Varga-Defterdarovic L, Turk Z (2004) Preparation and quantification of methylglyoxal in human plasma using reverse-phase high-performance liquid chromatography. Clin Biochem 37:875–881
11. Espinosa-Mansilla A, Duran-Meras I, Canada FC, Marquez MP (2007) High-performance liquid chromatographic determination of glyoxal and methylglyoxal in urine by prederivatization to lumazinic rings using in serial fast scan fluorimetric and diode array detectors. Anal Biochem 371:82–91
12. Racker E (1951) The mechanism of action of glyoxalase. J Biol Chem 190:685–696
13. Gilbert RP, Brandt RB (1975) Spectrophotometric determination of methyl glyoxal with 2,4-dinitrophenylhydrazine. Anal Chem 47:2418–2422
14. Lo TW, Westwood ME, McLellan AC, Selwood T, Thornalley PJ (1994) Binding and modification of proteins by methylglyoxal under physiological conditions. A kinetic and mechanistic study with N alpha-acetylarginine, N alpha-acetylcysteine, and N alpha-acetyllysine, and bovine serum albumin. J Biol Chem 269:32299–32305
15. Thornalley PJ (1990) The glyoxalase system: new developments towards functional characterization of a metabolic pathway fundamental to biological life. Biochem J 269:1–11
16. Krizner HE, De Haan DO, Kua J (2009) Thermodynamics and kinetics of methylglyoxal dimer formation: a computational study. J Phys Chem A 113:6994–7001
17. Ferguson GP, VanPatten S, Bucala R, Al-Abed Y (1999) Detoxification of methylglyoxal by the nucleophilic bidentate, phenylacylthiazolium bromide. Chem Res Toxicol 12:617–622
18. Zeng J, Davies MJ (2005) Evidence for the formation of adducts and *S*-(carboxymethyl)cysteine on reaction of alpha-dicarbonyl compounds with thiol groups on amino acids, peptides, and proteins. Chem Res Toxicol 18:1232–1241