Journal of Chromatography B, 1029–1030 (2016) 102–105

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Determination of methylglyoxal in human blood plasma using fluorescence high performance liquid chromatography after derivatization with 1,2-diamino-4,5-methylenedioxybenzene

Yuki Ogasawara[a,*], Ryo Tanaka[a], Shin Koike[a], Yasue Horiuchi[b], Mitsuhiro Miyashita[b], Makoto Arai[b]

[a] Department of Analytical Biochemistry, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo, 204-8588, Japan
[b] Department of Psychiatry and Behavioral Sciences, Tokyo Metropolitan Institute of Medical Science, Kamikitazawa, Setagaya, Tokyo, 156-8506, Japan

ARTICLE INFO

Article history:
Received 3 June 2016
Received in revised form 6 July 2016
Accepted 8 July 2016
Available online 9 July 2016

Keywords:
Methylglyoxal
Biomarker
HPLC method
Carbonyl stress
Fluorescence detection

ABSTRACT

Methylglyoxal (MG) is a highly reactive dicarbonyl compound that promotes the non-enzymatic glycation of proteins to yield irreversible advanced glycated end products, leading to the cross-linking or degradation of proteins. The physiological relevance of MG currently remains unclear because its metabolic behavior has not yet been elucidated in detail. Although several labeling methods that require a HPLC system have been developed and used to measure MG, a standard method to analyze the content of MG in biological samples has not been established. We herein present a practical method based on HPLC with fluorescence detection to measure low MG levels. MG concentrations were also measured in human blood plasma using the present method in order to demonstrate its utility.

A calibration curve was produced using freshly purified MG at concentrations ranging between 0.05 and 1.0 μM. The intra-day and inter-day relative standard diviations of the method were 2.55% and 4.03%, respectively. The limit of detection and limit of quantification were 60 fmol and 200 fmol, respectively for MG with a 10-μl injection volume of the derivatized sample solution. When the optimized method was applied to human plasma, the resulting concentrations of MG in the plasma of healthy subjects (n = 23) ranged between 0.024 and 0.258 μM (mean ± SD = 0.098 ± 0.066). Thus, the method developed herein is simple, sensitive, and easy to operate for the measurement of MG in biological samples.

1. Introduction

The concentration of methylglyoxal (MG) needs to be measured in physiological studies on the characterization of various diseases, particularly diabetes mellitus [1]. Previous studies have suggested that MG, a by-product of the glycolytic pathway, is involved in the development of diabetic complications and cardiovascular disease [2,3]. Rabbani and Thornalley demonstrated that MG-derived structures are often the main advanced glycated end products (AGEs) in the proteins of tissues and body fluids, with increased levels being detected in diabetes and its associated vascular complications, renal failure, cirrhosis, Alzheimer's disease, arthritis, Parkinson's disease, and aging [4,5]. MG has been shown to play a major role in vascular damage to endothelial cells [6] as well as in the development of hypertension [7], insulin resistance [8], and nephropathy [9]. Furthermore, recent studies have suggested that MG induces carbonyl stress-type schizophrenia [10–12].

A large number of methods have been reported for the quantification of α-ketoaldehydes in clinical samples; however, a general and standard procedure for the measurement of MG has not yet been established. Brief reviews and comparisons of analytical procedures, the majority of which are based on various precolumn derivatizations followed by chromatographic or electrophoretic separation, have been described in the introductory sections of previous studies [13–16].

1,2-Diamino-4,5-dimethoxybenzene (DDB) [13,17–19] has been employed in many studies as a fluorescence labeling agent. However, the purity of commercially available DDB is often insufficient, and it is unstable in solution, in which it readily undergoes oxidative degradation reactions. Furthermore, the aforementioned

Abbreviations: AGEs, advanced glycated end products; DDB, 1,2-diamino-4,5-dimethoxybenzene; DMB, 1,2-diamino-4,5-methylenedioxy-benzene; GLO1, glyoxalase 1; HPLC, high-performance liquid chromatography; LC–MS/MS, liquid chromatography tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MG, methylglyoxal.

* Corresponding author.
E-mail address: yo@my-pharm.ac.jp (Y. Ogasawara).

http://dx.doi.org/10.1016/j.jchromb.2016.07.019
1570-0232/

procedure is complex and includes neutralization, extraction, and concentration steps, which often decrease the accuracy of data obtained in the absence of suitable handling skills. An analytical method based on the labeling of MG with 1,2-diaminobenzene and subsequent LC–MS/MS has recently been reported [14,15]. Although the method using LC–MS/MS is considered to be the most accurate technique currently available, a disadvantage is that it is not suitable for a large number of quantitative measurements and routine clinical use. Moreover, in addition to a large-scale apparatus, a reference material containing stable isotopes ($^{13}C_3$-MG or d_4-MG) is needed for all samples as an internal standard to achieve quantification.

Although DMB was originally synthesized as a labeling agent for α-ketoacids [20] and sialic acids [21], we are the first to utilize it as a pre-labeling reagent for the measurement of MG in human plasma. The combination of DMB and HPLC separation with fluorometric detection achieved sufficient results for the simple measurement of MG in human plasma at physiological levels.

2. Materials and methods

2.1. Chemicals

Purified MG was synthesized from methylglyoxal diacetate according to a previously described method [14] because authentic MG solution contains impurities. DMB, sodium dithionite, 2-mercaptoethanol (2-ME), HPLC-grade methanol, and acetonitrile were purchased from Wako Pure Chemical Co. (Osaka, Japan). All other reagents were the highest grade that was commercially available.

2.2. Preparation of plasma samples

Human blood were obtained from 23 healthy volunteers (11 men and 12 women; mean age:45.6 years; SD:8.6 years) with their informed consent in the present study. For plasma samples, blood was collected into heparin-coated vacutainer tubes and the tubes were immediately placed on ice. Whole blood was centrifuged at 800 × *g*, 4 °C, for 15 min. Plasma was transferred into cryovials and stored at −80 °C before analysis. All participants signed a written consent form, and all plasma studies were approved by the Institutional Review Board of Meiji Pharmaceutical University.

2.3. Pre-labeling and HPLC conditions

MG was measured by HPLC via DMB derivatization with fluorometric detection. The DMB solution (7.0 mM) was prepared using 1.0 M β-mercaptoethanol and 28 mM sodium dithionite. Fifty microliters of the MG standard (final concentration: 0.05–1.0 μM) or diluted plasma was added to 50 μl DMB solution, and the solution was then incubated at 60 °C for 40 min. After cooling on ice, 10 μl of the derivatized solution was injected onto an ODS column (Inertsil ODS-4; 4.6 × 150 mm; GL Sciences; Tokyo, Japan) pre-equilibrated with the mobile phase solution, which consisted of methanol:acetonitrile:water (35:10:55). The chromatographic system consisted of an L2130-pump (Hitachi, Tokyo, Japan) and RF-20A fluorescence detector (Shimadzu, Kyoto, Japan). Standard curves were generated using a 10 mM stock solution of MG diluted with MilliQ water, which was used to prepare solutions with concentrations ranging between 0.1 μM and 20 μM MG. The flow rate was set to 1.0 ml/min, and the run time was 10 min. Retention times and peak areas were monitored at excitation and emission wavelengths of 355 nm and 393 nm, respectively. MG concentrations were measured by extrapolating the area from each run using calibration curves.

2.4. Identification of MG derivatives by LC/MS/MS spectrometry

Aliquots of the pre-labeled and standard samples were subjected to LC–MS/MS in order to identify the DMB derivative of MG (Fig. 1; Mw = 188.13). The LC–MS/MS system consisted of a Shimadzu instrument (LCMS-8040, Shimadzu, Kyoto, Japan), which was employed in the positive electrospray ionization (ESI) mode. The derivatized sample (10 μl) was loaded onto an ODS column, as described above.

2.5. Measurement of MG concentrations in plasma

MG concentrations in plasma were measured using HPLC with fluorescence detection, as described above. A 0.1-ml sample of plasma was diluted 2-fold with distilled water, followed by filtration through an ultrafilter (cut off size: 10 K; NANOSEP, Pall Life Sciences) to remove proteins. The filter tubes were filled with MilliQ water and gently agitated by rotator at 37 °C for 24 h prior to use in order to thoroughly wash out additives including glycerol for membrane. After labeling the ultrafiltered sample with DMB, 10 μl of each derivatized solution was injected onto HPLC.

3. Results and discussion

3.1. Method development

In the proposed method, DMB was used as a pre-labeling agent for derivatization of the α-dicarbonyl residue present in MG (Fig. 1). Optimal conditions were clarified for the pre-labeling reaction and separation systems. The derivatization reaction was completed at 60 °C within 40 min. The sufficient separation of the DMB-MG derivative was achieved by a commonly used C18 column and simple isocratic mobile phase consisting of methanol, acetonitrile, and H_2O without any salts (in a 35:10:55 ratio). Chromatograms of the MG-DMB derivative in the standard solution (A) and human plasma (B) were shown in Fig. 2.

When an existing method with DDB was used to measure the MG concentration in human plasma, we were unable to obtain reproducible results. Thus, in order to avoid complicated and time-consuming procedures, we developed a novel method based on DMB derivatization and HPLC with isocratic elution conditions in order to separate MG derivatives within 10 min. The chemical fluorescence-derivatization of MG is essential for obtaining adequate sensitivity in physiological systems. In addition, the derivatization procedure using DMB is simpler and quicker than that with the widely used DDB reagent [13,17–19].

3.2. Linearity, sensitivity, accuracy, and recovery

Several essential parameters were estimated under optimized conditions in order to establish the method. The calibration curve for MG was linear over the range of 0.05–1.0 μM, presenting a correlation coefficient of 0.999. The limit of detection (LOD) was defined as the lowest detectable concentration with a signal-to-noise ratio of at least 3, whereas the limit of quantification (LOQ) was defined as the lowest quantifiable concentration with a signal-to-noise ratio of at least 10. The LOD and LOQ were 60 fmol and 200 fmol, respectively for MG with a 10-μl injection volume of reaction mixture. The intra-day (n = 5) and inter-day (n = 5) relative standard deviations (RSD) of the assay for MG in human plasma (0.258 μM) were 2.55% and 4.03%, respectively. Recovery experiments were performed by adding known amounts of MG (n = 5, at 0.1 μM and 1.0 μM), and the corresponding recovery levels from human plasma were 98.55 ± 4.04% and 94.69 ± 3.08%, respectively. These values were within the acceptable range, demonstrating that the method was accurate and precise. Under these conditions, the