

Regular Article

Oxidative Formation of Methylglyoxal in Glycerol Preparations during Storage

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Methylglyoxal (MGO) is a reactive α -dicarbonyl compound that causes carbonylation of protein and DNA through the pathways of the Maillard reaction. It is known that MGO is physiologically involved in renal dysfunction, vascular disorders, and the acceleration of aging. In this study, we showed for the first time, that a trace amount of MGO was present as an impurity in glycerol preparations used as external medicines and intravenous infusions, when kept unused. The concentration of MGO in the glycerol solutions, diluted to a concentration of 20%, significantly increased after storage for one month when compared to the MGO concentration immediately after opening. Following storage for 6 months at 25°C, MGO concentration increased by about 300 times (approx. 170 μ M), and at 40°C, it increased by about 600 times (approx. 350 μ M). In the case of intravenous infusion preparations containing 10% glycerol, the MGO concentration increased by 4–15 times (approx. 70 μ M) after 2 months of storage at 40°C, and reached over 200 μ M after 6 months. Results from the present study showed that glycerol in pharmaceutical preparations is gradually oxidized to form MGO *via* autoxidation, depending on the temperature and dissolved oxygen content. Thus, we suggest that precautions should be taken when storing glycerol preparations in bottles or plastic containers, with respect to the storage temperature and sealability to prevent MGO formation due to oxidation of glycerol.

Key words methylglyoxal; glycerol preparation; stability; oxidation; carbonyl stress

INTRODUCTION

Methylglyoxal (MGO) is a well-known byproduct of glucose metabolism and polyol pathway. It belongs to the group of extremely reactive α -dicarbonyl compounds that exhibit cytotoxicity¹⁾ and genotoxicity.²⁾ Previous studies have demonstrated the involvement of MGO in the onset of arteriosclerosis,³⁾ chronic kidney diseases,⁴⁾ and neurodegenerative diseases.⁵⁾ It is also reported that MGO modifies skin collagen *in vivo* through the Maillard reaction and produces advanced glycation end products (AGEs), which accumulate and inhibit the function of fibroblasts.⁶⁾ Moreover, recent studies suggest that MGO directly reacts with vascular endothelial cells and leads to vascular disorders.^{7,8)}

Glycerol, also known as glycerin, is a simple three-carbon tri-alcohol. Due to its mild antimicrobial and antiviral properties, it is widely used in wound and burn treatments. It is also used as a humectant in pharmaceutical preparations and as an osmotic agent against cerebral edema. We coincidentally found that MGO was eluted from filter products, such as ultrafiltration membranes that used glycerin as a moisturizer. Glycerol and its preparations are usually stored at room temperature. Since the expiration date of glycerol preparations is usually long, there are chances of formation of byproducts derived from glycerol after opening the containers. However, till now there are no reports on the stability of glycerol in glycerol-containing preparations.

In this study, we examined the formation of MGO by the oxidation of glycerol in 85% glycerin[®], listed in Japanese Pharmacopoeia (Class 2 Pharmaceutical Products), and in 10% glycerol-containing intravenous infusion, such as Glyceol[®], used to regulate intracranial osmotic pressure. Furthermore,

we also investigated the conditions that led to the formation of MGO from glycerol and factors that suppressed its formation.

MATERIALS AND METHODS

Chemicals and Medical Preparations 1,2-Diamino-4,5-methylenedioxymethylene (MDB) was purchased from Dojindo (Kumamoto, Japan). Methylglyoxal 1, 1-dimethyl acetal was purchased from Sigma (St. Louis, MO, U.S.A.). HPLC-grade acetonitrile and ammonium formate, ultrapure water, sodium metabisulfite, trichloroacetic acid, and 2-mercaptoethanol were obtained from Wako Pure Chemical Corporation (Osaka, Japan). Purified MGO was synthesized from methylglyoxal 1, 1-dimethyl acetal, according to a previously described method.⁹⁾ Five glycerol preparations (Glycerin[®] based on Japanese Pharmacopoeia, 84–87%), for medical use, were purchased from five pharmaceutical companies (Kozakai Pharmaceutical Co., Ltd. (Japan), Maruishi Pharmaceutical Co., Ltd. (Japan), Showa Seiyaku Co., Ltd. (Japan), Yakuhan Pharmaceutical Co., Ltd. (Japan), and Iwaki Seiyaku Co., Ltd. (Japan)). Five 10% glycerin-containing intravenous infusions, used for regulating the intracranial osmotic pressure, were obtained from five pharmaceutical companies (Chugai Pharmaceutical Co., Ltd. (Japan), Terumo Co., Ltd. (Japan), Fuso Pharmaceutical Industries Co., Ltd. (Japan), Kyowa CritiCare Co., Ltd. (Japan), and Hikari Pharmaceutical Co., Ltd. (Japan)).

Measurement of MGO Using HPLC with Fluorescent Detection The concentration of MGO was measured by a previously described method¹⁰⁾ using MDB, with minor modifications. After suitable dilution of each sample solution, trichloroacetic acid was added to a final concentration of 4% and centrifuged at 12000 $\times g$ for 10 min at 4°C. The superna-

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tant was diluted with an equal volume of 7mM MDB solution and incubated at 60°C for 40 min in the dark. This MDB derived sample was then analyzed by HPLC with fluorescence detection. A 10 μ L volume of the resultant mixture was injected into a reverse-phased column (ODS-4, GL Science Co., 4.6 \times 150 mm), pre-equilibrated with the mobile phase solution which consisted of water:methanol:acetonitrile (10:7:3). The flow rate and running time were set at 1.0 mL/min and 15 min, respectively. The retention times and peak areas were monitored at excitation and emission frequencies of 355 and 393 nm, respectively.

Determination of MGO Concentration in Glycerol Solutions The concentration of MGO in the five glycerol preparations for medical use was determined immediately after opening the containers and diluting the solution 50 times with ultrapure water. In addition, the MGO concentrations in these glycerol preparations were determined after storage at 40°C for 1–2 months, using the same dilution procedure. The 20% glycerol solutions were prepared from 85% glycerol preparation (Glycerin “Maruishi”[®]) by dilution using ultrapure water. The MGO concentration in the 20% glycerol solutions was determined on the day of the preparation and after storage at 4, 25, and 40°C for 1, 2, and 6 months.

Effect of Antioxidant and Dissolved Oxygen on the Formation of MGO The 20% glycerol solutions were stored at 40°C, in the presence of either ethylenediaminetetraacetic acid (EDTA) or metabisulfite, for 2 months. The dissolved oxygen in these solutions was removed by ultrasonic treatment with N₂ purge for 30 (A), 10 (B) and 0 min (C). The dissolved oxygen concentration was determined by an assay kit (AZ-DO-10, Kyoritsu Chemical-Check Lab., Corp. Tokyo, Japan). The non-treated solution (C: 8.5 mg O₂/L) and the solutions with dissolved oxygen removed (A: < 1 mg O₂/L, B: 2–3 mg O₂/L), were stored at 40°C. The MGO concentration in each sample was determined on the day of the preparation and after 1 month.

MGO Concentration in Intravenous Infusions Containing Glycerol The concentration of MGO in intravenous infusion preparations ($n=4$) containing 10% glycerol, obtained from five companies, was determined before and after storage at 40°C for 1, 2, and 6 months. The samples were prepared by withdrawing a portion of infusion fluid from the drip bag into a syringe and diluting it appropriately using ultrapure water.

Statistical Analysis Values are presented as the mean \pm standard deviation (S.D.). Data were evaluated using the Tukey comparison test. p Value less than 0.05 was considered to be a statistically significant difference.

RESULTS

Determination of MGO Concentration in Glycerol Preparation MGO was detected in all the five glycerol preparations obtained from different pharmaceutical companies, immediately after opening the containers (1.5–3.5 μ M). When the glycerol preparations were stored at 40°C, as per the stability test of the Japanese Pharmacopoeia, significant increase in the MGO concentration was observed in all the products after 1 or 2 months (7.5–42 μ M) (Fig. 1).

Furthermore, as shown in Fig. 2, the MGO concentration in the 20% glycerol solutions, prepared from 85% glycerol as a usual moisturizing agent, was significantly higher after

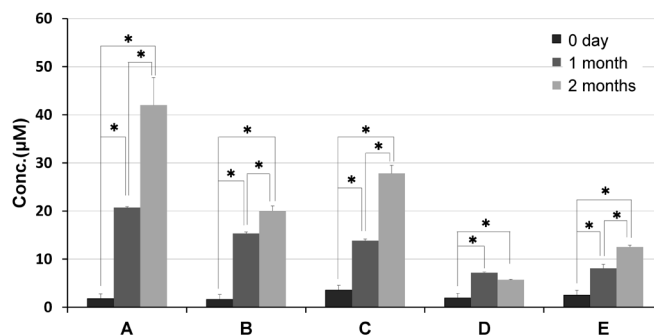


Fig. 1. Increase in MGO Concentration in Various Glycerol Preparations during Storage

An 85% glycerol preparation was stored at 40°C for the different time durations (0, 1, and 2 months) and the MGO content was determined after adequate dilution with ultrapure water. A; Iwaki, B; Yakuhan, C; Showa, D; Kozakai, and E; Maruishi. Values are presented as the mean \pm standard deviation (S.D.) ($n=4$). Error bars represent the S.D. * ($p < 0.05$).

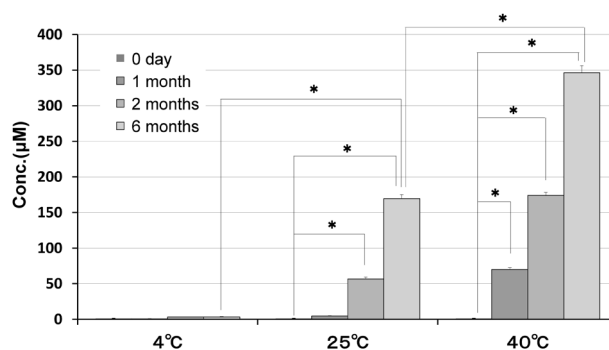


Fig. 2. Effects of Storage Temperature and Duration on the Formation of MGO in Glycerol Solution

The 20% glycerol solutions were stored at the indicated temperature (4, 25, 40°C) for different time durations (0, 1, 2, and 6 months), and the MGO content was determined after adequate dilution with ultrapure water. Values are presented as the mean \pm S.D. ($n=4$). Error bars represent the S.D. * ($p < 0.05$).

storage for 1 month at 40°C and 2 months at 25°C when compared to the MGO concentration just after opening the container. Following 6 months of storage at 25 and 40°C, the MGO concentration increased by about 300 (170 μ M) and 600 times (350 μ M), respectively.

Effects of Antioxidant on the Formation of MGO in Glycerol Solution To evaluate the inhibitory effects of antioxidants on MGO formation, we added two type of antioxidants, namely EDTA and metabisulfite, in the 20% glycerol solutions during storage for 2 months. After storage at 40°C for 1–2 months, the increase in MGO concentration was significantly inhibited by the addition of metabisulfite, as shown in Fig. 3. In contrast, EDTA did not have any effect on MGO formation (data not shown). These data imply that a hydrophilic antioxidant, and not a metal chelating agent, is useful to prevent the formation of MGO in glycerol.

Effects of Dissolved Oxygen Concentration on the Formation of MGO in Glycerol Solution It was expected that dissolved oxygen might be involved in the autoxidation process of glycerol to MGO. Therefore, we investigated the influence of changes in the dissolved oxygen concentration on the oxidative formation of MGO from glycerol. As shown in Fig. 4, lowering the dissolved oxygen concentration affected the formation of MGO in the glycerol solutions. Although the MGO concentration in 20% glycerol solution increased after storage at 40°C for 1 month, the concentration reduced signifi-

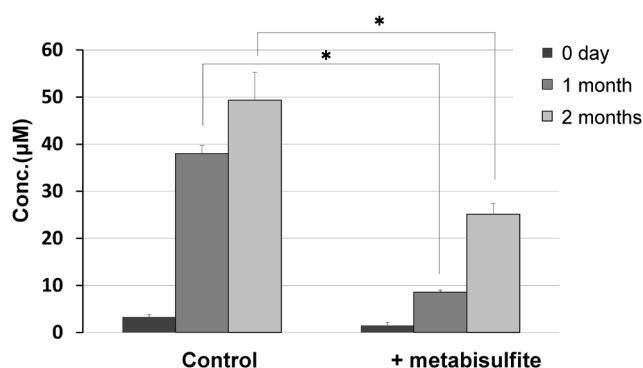


Fig. 3. Effect of Metabisulfite on MGO Formation in Glycerol Solution

The 20% glycerol solutions were stored at 40°C, with or without metabisulfite, for different time durations (0, 1, and 2 months) and the MGO content was determined after adequate dilution with ultrapure water. Values are presented as the mean \pm S.D. ($n=4$). Error bars represent the S.D. * ($p<0.05$).

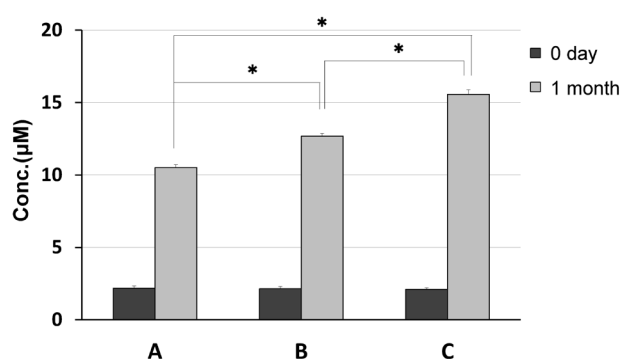


Fig. 4. Effect of Dissolved Oxygen Concentration on MGO Formation in Glycerol Solution

The 20% glycerol solutions were degassed by N_2 purge under ultrasonic treatment for 30 (A: <1 mg O_2/L), 10 (B: $2-3$ mg O_2/L) and 0 min (C: 8.5 mg O_2/L) and stored at 40°C for 1 month. The MGO content was determined after adequate dilution with ultrapure water. Values are presented as the mean \pm S.D. ($n=4$). Error bars represent the S.D. * ($p<0.05$).

cantly based on the concentration of dissolved oxygen in the solution before storage.

MGO Formation in Intravenous Infusion Containing Glycerol We measured the concentration of MGO in intravenous infusion, used for regulating the intracranial osmotic pressure, that contained 10% glycerol. Results showed that micromolar levels of MGO ($4.2-6.5\mu M$) were found in all the five samples obtained from different pharmaceutical companies (Fig. 5). Moreover, following storage for 2 months at 40°C, the MGO concentration in the intravenous infusions increased by 4–15 times compared to the concentration before storage. After 6 months of storage, the MGO concentration in the intravenous infusions reached levels of $70-220\mu M$ (Fig. 6).

DISCUSSION

Riddle and Lorenz reported that MGO is formed in glycerol by non-enzymatic reactions.¹¹⁾ A recent study showed that carbonyl compounds, including MGO, were produced from glycerol in e-cigarettes subjected to heat.¹²⁾ However, there are no reports yet, regarding detailed investigation on the oxidative changes to glycerol in medical preparations. Our HPLC method previously developed for MGO determination has quantitative ranges between 0.05 and $2.0\mu M$.¹⁰⁾ Thus, this

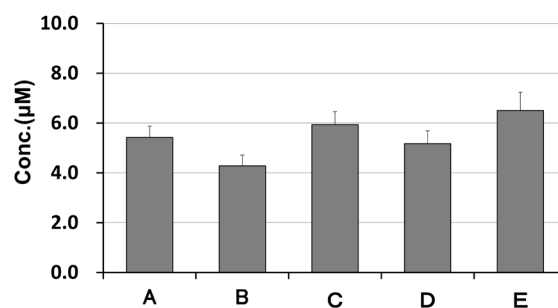


Fig. 5. Concentration of MGO in Intravenous Infusion Containing 10% Glycerol

The MGO content in intravenous infusion was determined immediately after opening the package and adequately diluting with ultrapure water. A; Chugai, B; Kyowa, C; Terumo, D; Hikari E; Fuso. Values are presented as the mean \pm S.D. ($n=4$). Error bars represent the S.D.

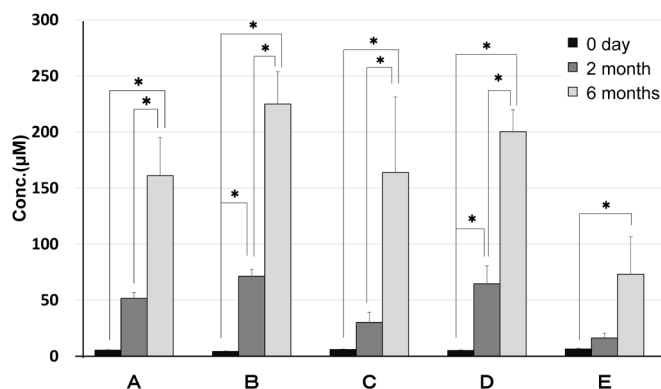


Fig. 6. Increase in MGO Concentration in Intravenous Infusion, Containing 10% Glycerol, during Storage at 40°C

Each infusion bag, containing glycerol, was stored at 40°C for different time durations (0, 1, 2, and 6 months) and the MGO content was determined after adequate dilution with ultrapure water. A; Chugai, B; Kyowa, C; Terumo, D; Hikari E; Fuso. Values are presented as the mean \pm S.D. ($n=4$). Error bars represent the S.D. * ($p<0.05$).

method has sufficiently applicable for the measurement of MGO in glycerol preparation samples.

Glycerol is used in medical, pharmaceutical, and personal care preparations; often to improve smoothness, provide lubrication, and act as a humectant. From the results of our study, it was found that MGO was initially present in a very small quantity in the glycerol preparations. However, its concentration increased during storage at either 25 or 40°C, regardless of whether the containers with the glycerol preparations were opened or not. Medicinal glycerol is produced through purification, concentration, and distillation of aqueous solutions obtained by hydrolyzing natural fats and oils. Thus, we found that the initial concentrations of MGO in products from different companies varied owing to the variation in the raw materials (production lot). Results from the assessment of the temperature effect on MGO formation in Japanese pharmacopoeia grade glycerol (84–87%) showed that the MGO concentration did not change much during the period of refrigerated storage (4°C). However, when stored at room temperature (25°C), a significant increase in the MGO concentration was noticed after one month. A more remarkable increase in MGO concentration was observed in 85% glycerol preparations for medical use, as demonstrated in the stability acceleration test at 40°C for 6 months. This test is generally followed for medical preparations listed in the Japanese Pharmacopoeia (17th

edition).

We showed representative results using 20% glycerol solution prepared from “Glycerin Maruishi®” (Fig. 2) because this product is commercially available in 500 mL size (expiration data: 3 years), which is usually used for longer period and stored at room temperature compared to small size products. When comparing the data shown in Figs. 1 and 2, it is clear that the rate of MGO formation in 20% glycerol solution was significantly higher than that in 85% glycerol preparation. Although the dissolved oxygen concentration in 85% glycerol is low, it is anticipated that this concentration increased after dilution with water at the same temperature. Therefore, it was considered that the content of dissolved oxygen in the solution was involved in the oxidative formation of MGO. In fact, we observed that the MGO formation rate at 40°C was reduced with a reduction in the initial dissolved oxygen concentration (Fig. 4). In addition, we found the rate of MGO formation was higher when the head space of the medication bottle was wider. In the present study, we did not perform specific treatments, such as N₂ replacement, for head space of each bottle after opening. Thus, the present study indicated that the MGO formation rate might depend mainly on the dissolved oxygen concentration in 85% glycerol preparations.

As shown in Fig. 3, it was clear that the addition of a water-soluble antioxidant could effectively suppress the oxidative formation of MGO in 20% glycerol solution. This result indicates that autooxidation of glycerol may be a key factor involved in MGO formation. Since glycerol is generally obtained from plant and animal sources through purification processes and has been used as a component in a variety of medical preparations, such as humectant for skin, it is possible that various trace contaminants may be present in glycerol-based medical devices and products.¹³⁾ Accordingly, when various trace oxidants, such as lipid peroxide, are present as impurities from raw materials of glycerol, the formation of MGO during the storage varies in each product dependent on the oxidative reactions between glycerol and the trace impurities. In the present study, the addition of EDTA was ineffective to prevent MGO formation. As EDTA is a typical chelating agent used to scavenge metal ions which may accelerate the oxidative reaction, our result suggests that trace metal contamination does not act as an oxidation catalyst for forming MGO in glycerol solution.

Based on these results, it was clear that MGO is gradually formed in the glycerol preparations during storage, even at room temperature after opening, and its formation is accelerated depending on the temperature and dissolved oxygen concentration. It was also revealed that water-soluble antioxidant is effective in inhibiting the MGO production due to the oxidation of glycerol. Therefore, we have estimated that the amount of MGO generated differs depending on the dissolved oxygen concentration and the trace oxidants contained in each glycerol product.

When glycerol that is within the expiration date and stored at a temperature less than 25°C, is used as an external medicine, the concentration of MGO formed from glycerol is considered not to have any detrimental effect on human health. However, when the MGO content in glycerol preparations exceeds a threshold concentration of over 10 µM, following storage at high temperature, its application may induce protein carbonylation in the tissues.

Hence, it is feared that the repetitive application of glycerol preparations, containing MGO, promotes carbonylation of the skin. Glycation of collagen is thought to cause skin sclerosis and wrinkles, and it has been reported that lysine and arginine residues in collagen protein of the skin are susceptible to glycation by MGO.¹⁴⁾

Glyceol® is an intravenous infusion commonly used as a therapeutic agent for cerebral edema.^{15–17)} In the present study, it was shown that MGO is present in the intravenous infusion solution at much higher concentration than that in normal human blood plasma.¹⁰⁾ In addition, since the drip bag is usually stored at room temperature, the concentration of MGO can increase overtime. Administration of these MGO-contaminated infusions would thus allow direct entry of MGO into the blood vessels and tissues. Specifically, MGO contained in intravenous infusion may cause damage to the vascular endothelium,^{7,8)} and when taken by the organs, such as kidney and brain, there is concern that MGO may modify proteins and DNA to form AGEs.^{18,19)}

Although the reaction mechanism of MGO formation from glycerol is still uncertain, our results clearly indicate that MGO is formed from glycerol at high temperature under aerobic conditions. Thus, in the future, it is necessary to control the quality of glycerol preparations, considering the purity of the raw materials and the storage methods. Also, it is desirable to prevent the formation of MGO in glycerol preparations by using effective water-soluble antioxidants, such as metabisulfite.

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Conflict of Interest The authors declare no conflict of interest.

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