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28-day repeated dose response study of diglycolic acid: Renal and hepatic effects☆

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ABSTRACT

The acute oral toxicity of diglycolic acid (DGA) was evaluated. Groups of female rats (n = 8 rats/group) received 28 consecutive daily single doses of 0.3, 1.0, 3.0, 10.0, 30.0, 100.0 or 300.0 mg DGA/kg body weight by gastric intubation. One group of animals served as vehicle control. Tissues and blood serum were collected at necropsy on day 29. Select organs were weighed and fixed in formalin for histopathological analysis. Animals from the 300 mg/kg bw dose group were removed from the study after 5 consecutive days of treatment as a consequence of adverse treatment related effects. The animals in the remaining treatment groups survived the exposure period. No adverse clinical signs were observed throughout the exposure period in the surviving animals. No significant differences from controls were observed for feed and fluid consumption or body weight gain in the surviving animals. Lesions were observed in the kidneys, liver, stomach, intestine, thymus, spleen and bone marrow in rats from the 300 mg/kg dose group and signs of renal tubular regeneration were observed only in the 100 mg/kg dose group. These results suggest that high levels of pure DGA would need to be consumed before renal and other forms of organ toxicity are observed.

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1. Introduction

Diglycolic acid (DGA), a minor metabolite of diethylene glycol (DEG) has been implicated as one of two active agents responsible for human renal toxicity associated with several mass poisonings in which DEG (CAS RN 111-46-6) was illegally substituted for propylene glycol or glycerin in fever reducers (Bowie and McKenzie, 1972; Hanif et al., 1995), topical medications for the treatment of burns (Cantarell et al., 1987), medicinal glycerol products (Pandya, 1988), medications for upper respiratory tract infections (Okuonghae et al., 1992) and in traditional/ethnobotanical uses for inflammation, wound healing and cosmetic purposes (Drut et al., 1994; Ferrari and Giannuzzi, 2005). DGA specifically affects cells of the human proximal tubule (HPT) and rapidly accumulates in these cells proposedly as a consequence of enhanced uptake by dicarboxylate transporters found on the HPT cells (Landry et al., 2011). The accumulation of DGA in the HPT ultimately leads to DGA-induced severe cortical necrosis (Ferrari and Giannuzzi, 2005; Herbert et al., 1978). In all of the above cases, poisoned individuals presented with acute renal failure, metabolic acidosis, neurologic symptoms and other end organ effects.

Recently, it was reported that DGA is an impurity produced during the synthesis of several carboxymethyl carbohydrate preparations (CMPs) including carboxymethyl cellulose and carboxymethyl starches and that it cannot be completely removed from these preparations (Koschella et al., 2011). CMPs are used in the food science and drug industries as thickening agents, emulsion stabilizers, suspending agents and tablet binders. As such, there is a potential for human exposure through the consumption of processed foods like boxed cake mixes and ice cream products as well as tablet formulations.

A literature review indicated that the available information on DGA toxicity was limited. Preliminary information from a 28 day dose response study conducted in our laboratory using nine

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different dose levels suggested that low dose exposure to DGA in nulliparous female Sprague-Dawley rats did not adversely affect kidney or liver function; however, at elevated DGA dosages (300 mg/kg bw) marked kidney and liver effects were observed (Keltner et al., 2015; Mossoba et al., 2015). These findings suggested a sharp dose response curve for DGA. At the time this data was presented a comprehensive pathological examination of the tissues and complete clinical chemistry results were not available and as such this information was not reported. Recently, Robinson et al. (2017) using limited exposure dosages demonstrated similar effects in male Wistar rats. To date a comprehensive dose-response characterization of the effects of DGA on the kidney and liver in the rat has not been published nor have other target organs been identified. This manuscript presents comprehensive toxicological findings on DGA toxicity obtained from an acute rat 28-day repeated dose oral toxicity study highlighting effect observed on the liver and kidney and describing effects on other target organs induced as a consequence of high dose DGA exposure.

2. Materials and methods

2.1. Animals and animal husbandry

The experimental protocol was approved by the Animal Care and Use Committee at the Office of Applied Research and Safety Assessment, Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (2011) and the Animal Welfare Act of 1966 (P.L. 89–544), as amended. Female Sprague Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) were used as experimental animals and placed in acclimation for up to one week during which time they were singly housed in polycarbonate micro isolators. Females were utilized in this study because it has been shown that in cases where gender differences exist, females have been shown to be more sensitive to toxic insult than males. All animals received Harlan 2018 Certified rodent chow (meal) and reverse osmosis deionized water *ad libitum*. After acclimation, the animals were placed into polycarbonate tubs containing sani chips and ear tagged (Monel metal ear tags, size no. 1, Newport, Kentucky). The ear tag number was utilized to track these animals throughout the study. Light in the study room was provided on a 12-h light/dark cycle, the room temperature was maintained at 73–74 °F and the relative humidity was maintained at 48–59%. All dosed animals were observed twice daily for signs of toxicity. Animals found in a moribund condition and animals showing severe pain or enduring signs of severe distress were euthanized using carbon dioxide. Signs of toxicity include lethargy, anorexia, hunched posture, ruffled coat and loose stool. Animals euthanized are considered equivalent to animals that died on test.

2.2. Test material

The test article, diglycolic acid (DGA purity = 99%), was ordered from Sigma-Aldrich and stored according to the MSDS Sheets supplied by the manufacturer (dry, at room temperature). DGA was administered in water by gastric intubation at a volume of 10 ml/kg of body weight. Solutions were prepared by dissolving the appropriate amount of test article in ultra-pure water (18.2 MΩ) from an A10 Advantage water purification system (Millipore, Billerica, MA). Solutions were stored in the refrigerator and allowed to come to room temperature before administration and analysis. The gavage dosing solutions were analyzed in-house using a triple-quadrupole mass spectrometer in MRM mode monitoring the 133 → 75.1 and 133 → 89.1 transitions. Isotopically-labelled d_4-DGA (98% purity, Cambridge Isotopes) was used as an internal standard. Dosing solutions were found to be within ±10% of the target dose. Solutions were re-analyzed after 14 and 28 days to ensure stability.

2.3. Experimental design

Sixty-four nulliparous female Sprague Dawley rats (VAF/Plus) 57–70 days old and weighing 201–225 g were assigned to one of eight experimental groups (n = 8 animal/group) by weight using a stratified random assignment procedure. The dose levels tested were 0, 0.3, 1.0, 3.0, 10.0, 30.0, 100.0 and 300.0 mg/kg of body weight (bw). Each animal cohort was assigned a group number proportional to its dose level such that animals assigned to receive the lowest (0 mg/kg bw) and highest treatment doses (300.0 mg/kg bw) were classified as Group 1 and Group 8, respectively. DGA was administered in water by gastric intubation on a daily basis for 28 consecutive days using a volume of 10 ml/kg bw. Double distilled deionized water served as the vehicle control. All dosing solutions were analyzed prior to administration to ensure that they were within ±10% of the target dose. During the dosing period, feed and fluid consumption were measured every 3 days. Water was provided in 16 oz water bottles and each study animal was supplied with a fresh water bottle every 3 days. Feed was provided in Holtge Study animal feed cups, which were changed every 6 days. Feed cups and water bottles were checked twice per day to ensure that feed and fluid was available to all study animals.

During the course of the study, animals were placed into metabolism cages on exposure days 2, 4, 8, 16, 22 and 26 and urine samples were collected the following morning (20 ± 2 h urine collection). While in metabolism cages, water was provided in water bottles and feed cups were provided with the metabolism cages. Water bottles and feed cups were checked twice per day to ensure that fluids were available to the animals on study. Metabolism cage feeders and water bottles were changed daily.

2.3.1. Urine analysis

Standard urine analysis was conducted on all urine samples. Urine samples were spun at 3000 rpm for 10 min at 4 °C. The urine samples were frozen at −80 °C for subsequent evaluation of early biomarkers of renal toxicity (Mossoba et al., 2017). Urine test strips were used for the determination of Ketones, Glucose, Protein and plus Leukocytes, Nitrites, Bilirubin, Blood, Urobilinogen, and Specific Gravity levels in urine.

2.4. Pathology and clinical chemistry

On day 29, experimental and control animals were euthanized with carbon dioxide. Blood serum was collected and select organs were weighed and preserved in formalin. Serum was submitted for clinical chemistry analysis and tissue samples were submitted for histopathologic analysis.

2.4.1. Pathology

Animals were euthanized by carbon dioxide inhalation and blood was withdrawn via hepatic portal vein until exsanguination. At euthanasia, experimental animals were examined for gross abnormalities. The heart, intestine, kidneys, glandular stomach, liver, thymus, spleen were examined. Organs with gross lesions were preserved in 10% neutral buffered formalin. Additionally, both the sternum and femur were preserved in 10% formalin for evaluation of the bone marrow. Tissues were embedded in paraffin, sectioned at 3–5 μm, stained with hematoxylin and eosin and examined by light microscopy. Histopathological tissue preparation and examination of the fixed tissues was done by Experimental Pathology Laboratories, Inc. (Herndon, VA).