



Letter

Improvement of a fish holding device for oral administration to zebrafish

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ABSTRACT — Gavage has been used for many years in mammalian toxicology and experimental animal biology, as a direct oral administration method. It has the advantage of providing an exact dosage per body weight; however, gavage is not considered applicable to small fish like zebrafish (*Danio rerio*), owing to the difficulty associated with handling. In this study, a new gavage method for zebrafish was developed to minimize stress and damage to their digestive tracts, without the need for anesthesia. This device allows us to hold the zebrafish firmly in a predetermined position without directly touching them. This method involves holding the fish with a novel device and uses a specific syringe for dosing the test substance into the digestive organs. An acute oral toxicity test of potassium dichromate was conducted in adult zebrafish to demonstrate the new gavage method. No mortality was observed in the vehicle (water) control group. Furthermore, the median lethal dose (LD₅₀) values of potassium dichromate in male and female zebrafish were 103 and 195 mg/kg body weight, respectively. These values are directly comparable to the values in rats.

Key words: Gavage, Zebrafish, Holding device

INTRODUCTION

Zebrafish (*Danio rerio*) are used in the early development of pharmaceuticals as an alternative to experimental rodents, such as mice and rats. Zebrafish can be bred in a limited space, and their organs and tissues are similar to those of humans, in terms of both structure and function (Dodd *et al.*, 2000; Lieschke and Currie, 2007). In many studies using zebrafish, oral administration with a mixture of test substance and fish feed has been used to avoid damage by gavage (Zang *et al.*, 2011; Lin *et al.*, 2016). However, there are issues associated with the estimation of precise individual food consumption, despite the use of individual housing, which requires many test tanks and a

large space.

Mammalian toxicology and experimental animal biology studies use gavage as a direct oral administration method as it provides an exact intake of a dosed test substance, per body weight (Hull, 1995). However, the application of gavage is generally not extended to small model fish, such as zebrafish, due to the difficulty associated with handling without damage or stress. A method to anesthetize, fix, and administer the test compound directly to zebrafish has been developed (Dang *et al.*, 2016); however, there are concerns that anesthesia might change or influence physiological conditions in zebrafish, even though a precise dosage is obtained. Previous studies have introduced fish holding devices, but there are no

special devices or solutions for the process of holding fish (Collymore *et al.*, 2013; Dang *et al.*, 2016; Dayal *et al.*, 2016). Therefore, in the past, fish were scooped up with nets or other devices, and the scooped fish were grabbed by hand and placed on the fish holding device. Here, there is a high possibility that the process of holding the fish with the device could cause considerable damage to the fish.

To overcome these issues, this study has developed a new gavage method, with a specific holding device, to reduce damage or stress in zebrafish and facilitate direct oral administration without anesthesia. Our developed device allows the zebrafish to maintain a constant posture so that the test substance can be inserted into the digestive tract. This method does not require the zebrafish to be anesthetized; hence, the time required for gavage is shorter than that with current methods. An acute oral toxicity test of potassium dichromate ($K_2Cr_2O_7$), as a reference compound, in a fish acute toxicity test (OECD, 2011, 2019) via gavage was performed on adult male and female zebrafish to confirm the effectiveness of the new method. Furthermore, the methodology used and the results obtained in zebrafish were compared to those in previously published mammalian experimental studies based on the exact dosage, in mg/kg body weight.

MATERIALS AND METHODS

Animals

An original population of zebrafish (*D. rerio*; NIES-R strain) was obtained from the National Institute for Environmental Studies (Tsukuba City, Ibaraki, Japan) and was maintained in our laboratory. Fish were housed in plastic tanks (five individuals/5 L tank for each sex) and maintained in a flow-through or semi-static condition with dechlorinated tap water (treated with charcoal and a 0.2 μ m membrane filter) at a temperature of $25 \pm 1^\circ C$, under a 13-hr light and 11-hr dark photoperiod cycle. Throughout the culture periods, the fish were fed brine shrimp nauplius larvae (< 24 hr old) and commercial fish feed (HIKARI Labo450, KYORIN Co. Ltd., Hyogo, Japan) two or three times daily. Adult (6–18-month-old) male and female zebrafish were used.

Fish holding device

The fish holding device developed in this study mainly consisted of a zebrafish feed inlet, a guide tube, and a holding device (Fig. 1). The zebrafish scooped up by the net were put into the input port and arrived at the holding fixture via the induction tube together with the appropriate amount of rearing water. There was a notch at the

bottom of the end of the induction tube, and the zebrafish were introduced into the fish holding device (Fig. 1). The retainer had a posture maintenance groove to hold the zebrafish, and the zebrafish reached that position (Fig. 2). The retainer consisted of a main body, a forward holding site, and a backward holding site (Fig. 3).

This structure guided the zebrafish into the posture-maintenance grooves of the retainer body. After confirming that the mouth of the zebrafish was in the position of the anterior or posterior holding site, the main body of the holding device to which the zebrafish was fixed was removed. The mouth of the zebrafish was located at the tip of the retainer body, ensuring a posture that allowed for direct oral administration. To reduce the stress caused by holding the zebrafish, the retainer was made of a flexible and permeable synthetic resin, which can gently hold the zebrafish. The zebrafish was held in one hand and the test substance was administered with a dosing syringe (Fig. 4). The dosing syringe was a commercially available 50 μ L micro syringe equipped with a plastic sonde developed for neonatal mice.

Acute toxicity test

Potassium dichromate (99% pure; CAS no. 7778-50-9; Kanto Chemical Co. Ltd. Tokyo, Japan) was dissolved in deionized water and diluted to make dosing solutions of 31.2, 62.5, 125, 250, 500, and 1000 mg/kg for both sexes (females were also dosed up to 2000 mg/kg). A vehicle control group was included for both sexes, wherein fish were dosed with only deionized water. The dosing solutions were prepared based on an estimated body weight of zebrafish of 1 g.

The zebrafish were starved overnight (~ 18 hr) prior to dosing, dosed by gavage, and observed for 7 days. The dosing volume was 20 μ L/fish for all individuals. The same volume of deionized water was dosed to the fish in the vehicle control group. Seventy males (10 males/group) and 50 females (4–5 females/group) were used in the test. Each fish was observed 2 hr after the dosing and at least once per day from the next day until the end of the observation period. Fish were fed twice on the evening of dosing (approximately 5 hr after dosing) and twice per day thereafter.

After chemical exposure, the zebrafish were maintained in 4.5 L plastic vessels (20 \times 15 \times 16 cm) filled with 3 L dechlorinated tap water. The test vessels were loosely covered with plastic sheets to reduce evaporative water loss and to avoid the entry of dust into the solutions. The test was performed in a temperature-controlled room where the test water temperature was maintained at approximately $25 \pm 1^\circ C$ under a 13-hr light and 11-hr

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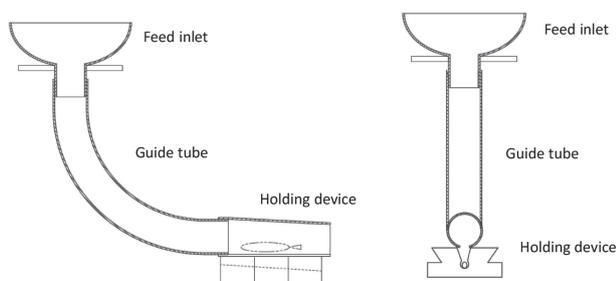


Fig. 1. Fish holding device of zebrafish during gavage.

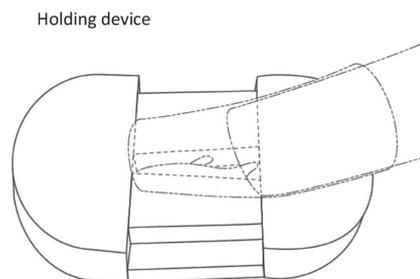


Fig. 2. Zebrafish holding section.

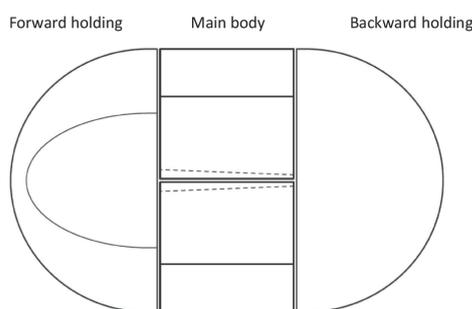


Fig. 3. Structure of the retained portion.

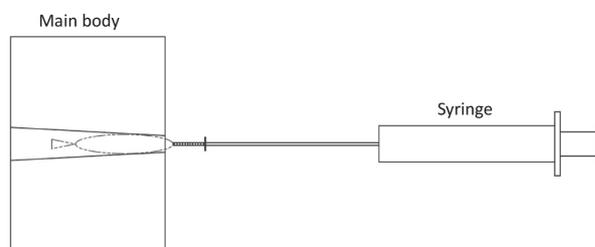


Fig. 4. Forced oral administration using a retainer device.

dark photoperiod cycle. Light intensity during the light period was approximately 1000 lux. Water temperature, pH, dissolved oxygen concentration, conductivity, and salinity at the start and end of the exposure in the control group were 25.0–25.1°C, 7.8–7.1, 8.19–7.61 mg/L, 28.1–30.0 mS/m, and < 0.01%, respectively.

Calculation of LD₅₀

The body weight and body length (standard length) of the zebrafish were measured for all fish that survived at the end of the observation period. Similarly, body weight and length were measured for dead fish. Based on the body weight measured, the actual dose for each individual was calculated. Based on the individual actual dose in each group, the 50% lethal dose (LD₅₀) was calculated using the Probit method for each sex (Hamilton *et al.*, 1977).

RESULTS AND DISCUSSION

Fish holding device

Previous studies have detailed how anesthetized zebrafish can be grabbed by hand and held in a sponge for oral administration (Collymore *et al.*, 2013). However, anesthesia might affect the biological responses of

fish species to the test substance. In addition, this method requires grasping the zebrafish by hand and is therefore affected by human body temperature. In this study, to facilitate direct oral administration without anesthesia, we developed a fish holding device that can securely hold zebrafish in a predetermined posture without direct contact.

For a method involving oral administration via gavage, it is crucial to hold the animals tightly and precisely to dose the exact amount of the test substance. Further, the training for using the device can be completed in a short time. If the zebrafish can spend less time out of the water, it can potentially minimize the stress faced by them. In our preliminary experiment using this device, no mortality was observed in 100 zebrafish (data not shown). Moreover, this study clearly demonstrated that no deaths were observed in any control groups in the acute oral toxicity test, suggesting minimal stress to fish using this device.

This fish holding device can be used to administer the test substance without anesthetizing the fish. Using this fish holding device, a methylene blue solution dye was applied to zebrafish, and it was confirmed that the zebrafish swam immediately into the tank without vomiting the dye solution after administration (data not shown). However, there is one limitation associated with this method, with regards to the dosing volume. Consid-

Table 1. Acute oral toxicity of potassium dichromate in male and female zebrafish (*Danio rerio*) after treatment with a single dose.

Dose Nominal (mg/kg)	Male					Female				
	Actual (mg/kg)	N ^a	Body length ^b (mm)	Body weight ^b (g)	Mortality (%)	Actual (mg/kg)	N ^a	Body length ^b (mm)	Body weight ^b (g)	Mortality (%)
0	0	10	36.3 ± 1.2	0.62 ± 0.07	0	0	5	42.2 ± 1.7	1.34 ± 0.18	0
31.2	50.3	10	35.0 ± 1.9	0.62 ± 0.12	10	23.5	4	41.3 ± 3.5	1.33 ± 0.29	0
62.5	101	10	35.5 ± 1.5	0.62 ± 0.07	60	53.9	4	39.5 ± 3.3	1.16 ± 0.33	0
125	208	10	34.2 ± 0.9 ^d	0.60 ± 0.08	80	107	4	39.5 ± 1.8	1.17 ± 0.21	20
250	403	10	33.1 ± 1.1 ^d	0.62 ± 0.03	100	162	4	41.5 ± 1.3	1.54 ± 0.33	40
500	847	10	33.5 ± 1.5 ^d	0.59 ± 0.08	100	391	5	36.1 ± 6.0	1.28 ± 0.26	83
1000	1298	10	36.2 ± 1.0	0.77 ± 0.08 ^d	100	680	5	41.5 ± 1.7	1.47 ± 0.14	100
2000	— ^c	—	—	—	—	1428	5	42.1 ± 2.1	1.40 ± 0.32	100

^aNumber of fish.^bData represented as mean ± standard deviation.^c—: None tested.^dSignificantly difference compared to vehicle control (nominal dose 0 mg/kg) groups (*: $P < 0.05$)**Table 2.** Comparison of acute oral toxicity of potassium dichromate between zebrafish and rats.

Test conditions and results	Zebrafish	Rats
Strain	NIES-R	Sprague-Dawley
Age	6–18 months Adult	7–9 weeks Young adult
Body weight (g)		
	Male	200–300 ^b
	Female	190–230 ^b
Dosing method	Oral gavage	Oral gavage
Vehicle	Water	Water
Dosing volume (mL/kg bw)	20	0.5–6.0
Dose (mg/kg bw)	23.5–1428	25–300
Fasting period	Overnight prior to dosing	18 hr before dosing
Deaths occurred over:	>= ca 50 mg/kg bw	>= 100 mg/kg bw
LD ₅₀ (mg/kg bw)		
	Male	168 (155–183) ^c
	Female	91 (82–101) ^c

^aMinimum – maximum body weights (bw) in the present study.^bTACONIC BIOSCIENCES, SPRAGUE DAWLEY® RAT MODEL, <https://www.taconic.com/pdfs/sprague-dawley-rat.pdf>^cParentheses indicate 95% confidence limits.

ering the structure and volume of the digestive organs in zebrafish (Wang *et al.*, 2010), 20 µL is the maximum volume for zebrafish. The appropriate dosing volume should be approximately 10 µL, as in a previous study (Collymore *et al.*, 2013).

Acute oral toxicity of potassium dichromate

The mean body length and body weight in each group were 33.1–36.3 mm and 0.59–0.77 g in males, respectively, and 36.1–42.2 mm and 1.16–1.54 g in females, respectively (Table 1). The mean dose was less than the estimated body weight of 1 g in males. Moreover, the

estimated body weight was greater in females, and thus, the actual dose in each group for each sex was recalculated (Table 1). Whereas no deaths were observed in either sex in the vehicle control groups during the observation period, deaths were observed within 24 hr for both sexes with an actual dose of ≥ 101 mg/kg for males and ≥ 107 mg/kg for females. During the experimental period, the 2 hr, 4 hr, 6 hr, 1 day, 2 days, and 6 days LD₅₀ values of potassium dichromate were 153, 111, 93, 112, 112, and 112 mg/kg for males and 430, 430, 430, 239, 195, and 195 mg/kg for females, respectively. These results indicated that there was almost no change in the

LD₅₀ value after the first day of administration in males and after the second day of administration in females. The 7 d LD₅₀ values with 95% confidence limits were estimated to be 103 (75–145) mg/kg for males and 195 (119–319) mg/kg for females, based on the actual dose in each sex of each group (Table 2).

Table 2 shows the comparison of the procedure and results of acute oral toxicity studies on potassium dichromate between zebrafish (present study) and rats (European Chemical Agency, 2011). There were some similarities and differences in the methodologies and results. Regarding the dosing method, gavage was used in both studies. Furthermore, the conditions for dosing, for example starvation before dosing, were almost identical, and thus, the results could be compared directly. The position of zebrafish concerning their systematic classification and their body size is very different from that of rats. Although zebrafish are fish (the lowest class of vertebrates) with a smaller body size than rats, which are mammals (the highest group of vertebrates; Komoike and Matsuoka, 2016), their sensitivity to potassium dichromate was found to be similar at the lowest dose (50–100 mg/kg body weight), with LD₅₀ values of approximately 100–200 mg/kg body weight.

In rats, females were found to be slightly more sensitive than males. In contrast, males were slightly more sensitive than females in zebrafish. The reason for the difference is not clear; however, it might be attributed to their body size. Male rats are bigger than females; however, zebrafish females are bigger than males. Body size/body weight generally affects acute toxicity, with smaller animals being more sensitive than larger animals (Vesela and Vijverberg, 2007). This difference in the response based on sex in rats and zebrafish could also be caused by differences in metabolism, the activity of drug-metabolizing enzymes, and/or metabolic pathways of potassium dichromate, as reported by some studies (Kato, 1974; Förlin, 1980).

This study showed that direct oral administration to zebrafish using the fish holding device resulted in the same trend as that with direct oral administration to rats. The oral administration method to zebrafish developed in this study requires a smaller amount of test substance than that for rats, and hence, they can be utilized for testing poorly soluble and volatile substances. By accumulating data using this device, we would like to expand the range of application of forced oral administration to small fishes.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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