Fish Haus

A Novel Protocol for the Oral Administration of Test Chemicals to Adult Zebrafish

Liqing Zang,[1] Daizo Morikane,[1] Yasuhito Shimada,[2–4] Toshio Tanaka,[2–4]
and Norihiro Nishimura[1]

Abstract

A novel protocol using gluten as a carrier material was developed to administer chemicals to adult zebrafish, *per os* (p.o.). To evaluate the capacity of gluten to retain chemicals, we prepared gluten granules containing eight types of chemicals with different Log P_{ow} values and immersed them in water. Less than 5% of chemicals were eluted from gluten granules within 5 min, a standard feeding time for zebrafish. Although retention capability was dependent on the hydrophilicity and hydrophobicity of the chemicals, the gluten granules retained 62%–99% of the total amount of chemical, even after immersion in water for 60 min. Vital staining dyes, such as 4-Di-2-Asp and Nile red, administered p.o., were delivered into the gastrointestinal tract where they were digested and secreted. Subsequently, we conducted a pharmacokinetic study of oral administration of felbinac and confirmed that it was successfully delivered into the blood of zebrafish. This indicates that chemicals administered using gluten granules are satisfactorily absorbed from the digestive tract and delivered into the metabolic system. The absorption, distribution, and pharmacokinetics of chemicals given by oral administration were also compared with those of chemicals given by alternative administration routes such as intraperitoneal injection and exposure to chemical solution.

Introduction

O VER THE PAST 20 YEARS, the zebrafish (*Danio rerio*) has emerged as a pre-eminent vertebrate biological model for studying developmental biology and embryogenesis,[1,2] and more recently, for human disease and the screening of therapeutic drugs.[3–5] A number of favorable characteristics, including its high fecundity, small size, rapid development and generation time, optical transparency during early development, tractability in forward genetic screens, and genetic similarity to humans,[6] have increased its popularity as a model of human disease and development. Zebrafish respond to chemicals, such as small molecules, drugs, and environmental toxicants, similarly to mammals. For example, recent studies revealed that zebrafish exhibit mechanisms of general defense against xenobiotic chemicals that are equivalent to those in mammals. These included enzyme induction and oxidative stress, and validate the use of zebrafish in toxicological testing and drug discovery.[7]

Methods have been developed for chemical administration to zebrafish. Embryos are exposed to a chemical bath or are microinjected with chemical solutions directly into the yolk sac.[8] Larval zebrafish are commonly used for chemical screening, and are often placed into multi-well dishes to swim in water containing various dissolved chemical compounds.[9–13] Chemical screening can also be performed in adult zebrafish using water-borne exposure or intraperitoneal injection (i.p.). For water-borne exposure, fish are immersed in chemical solutions at a density of one fish per 100–200 ml, with chemical solutions changed daily where long-term exposure is required.[14–18] For intraperitoneal injection, fish are anesthetized and chemical solutions are injected into their peritoneum in a small volume (generally $< 10 \mu l$).[19–21] However, almost all of the methods described above involve water-borne exposure; methods for treatment with chemicals possessing little or no solubility in water need to be established.

Oral administration methods for zebrafish have also been reported. Toxicology studies have developed a purified casein-based diet formulated for oral administration of chemicals to medaka (*Oryzias latipes*),[22] and these have been modified for zebrafish.[8] Additionally, an oral perfusion system was developed to permit continuous electrocardiographic

Departments of [1]Translational Medical Science and [2]Molecular and Cellular Pharmacology, Pharmacogenomics, and Pharmacoinformatics, Mie University Graduate School of Medicine, Tsu, Mie, Japan.
[3]Department of Bioinformatics, Mie University Life Science Research Center, Tsu, Mie, Japan.
[4]Department of Omics Medicine, Mie University Industrial Technology Innovation Institute, Tsu, Mie, Japan.

recording outside of the normal aqueous environment.[23] However, these methods can be difficult to manipulate and are labor-intensive.

Here we describe a simple and novel protocol for oral administration of test chemicals to adult zebrafish using gluten as a binding agent. We confirmed the capacity of gluten granules to retain chemicals of different solubility when immersed in water, and compared this method with other methods such as i.p. or exposure administration.

Materials and Methods

Animals and maintenance

Zebrafish were supplied by the Zebrafish International Resource Center (University of Oregon, Eugene, OR) and maintained in our facility according to established protocols.[24] "Adult zebrafish" used in this study were 4–6-month-old males of AB line and *rose* mutant line which were bred in our facility. Fish were fed twice daily with commercial flake food (Hikari Tropical Fancy Guppy, Kyorin, Hyogo, Japan) and once daily with live *Artemia nauplia*. During the chemical screening experimental period, fish were fed the untreated or chemically treated gluten granules in addition to the two diets described above.

Preparation of gluten granules

Water was added to gluten powder (Wako, Osaka, Japan) and this mixture was kneaded together using a medicine spoon. The dough was stored overnight at $-80°C$. The frozen dough was then lyophilized (DC-400, Yamato Scientific Co., Ltd., Japan). After complete drying, gluten was transferred to a mortar and carefully ground to granules (not to powder). To adjust the granule size as suitable for consumption by adult zebrafish, the grinding process was repeated until all of the granules could pass through a $700\,\mu m$ mesh sieve. Granules were stored at $4°C$ in an air-tight container prior to use.

Preparation of chemical-containing gluten granules

The relevant physiochemical properties of the chemicals used in this elution study are given in Table 1. Pyranine was obtained from Alfa Aesar (Heysham, UK). Toluidine blue O, C.I. Food Yellow 3 free acid and Coumarin 7 were obtained from Sigma Aldrich (St. Louis, MO). Phloxine B was obtained from Acros Organics (Geel, Belgium). Hematoporphyrin was obtained from ChromaDex (Irvine, CA). Solvent Red 197 and Solvent Yellow 43 were obtained from Canon Inc. (Tokyo, Japan). Stock solutions of Pyranine, Toluidine blue O, and C.I. Food Yellow 3 free acid were prepared by dissolution in sterile distilled water; the remaining five chemicals were dissolved in dimethyl sulfoxide (DMSO; Nacalai Tesque, Kyoto, Japan) at 50 mg/ml. Working solutions were prepared from each stock solution by adding sterile distilled water to provide a final concentration of 10 mg/ml.

For fluorescent dye-containing gluten preparation, stock solutions of 4-Di-2-Asp (4-(4-diethylaminostyryl)-N-methylpyridinium iodide; Sigma Aldrich) and Nile red (9-(diethylamino)-5H-benzo[a]phenoxazin-5-one; Wako) were prepared respectively in DMSO and acetone at 10 mg/ml and diluted with water to make a 1 mg/ml working solution.

BPAA (Biphenyl-4-ylacetic acid), also known as felbinac (Xunda Pharmaceutical Co., Ltd., Hubei, China), was

TABLE 1. CHEMICAL CHARACTERISTICS OF THE EIGHT TEST CHEMICALS USED IN THE ELUTION STUDY

Common name	IUPAC name	CAS no.	Formula	Mol. weight (G/mol)	$Log\ P_{ow}^{*}$	Peak wavelength (NM)
Pyranine	Trisodium 8-hydroxypyrene-1,3,6-trisulfonate	6358-69-6	C16H7Na3O10S3	524.385	-3.97	408
Toluidine blue O	3-Amino-7-(dimethylamino)-2-methylphenothiazin-5-ium chloride	92-31-9	C15H16ClN3S	305.83	0.16	593
C.I Food Yellow 3 Free Acid	8-Hydroxy-5-(4-sulfophenylazo)-2-naphthalenesulfonic acid	5859-11-0	C16H12N2O7S2	408.406	1.4	484
Solvent Red 197	3-(Diethylamino)-7-imino-7H-(1)benzopyrano(3',2':3,4)pyrido(1,2-a)benzimidazole-6-carbonitrile	52372-39-1	C23H19N5O	381.43	2.08	579
Coumarin 7	3-(1H-Benzimidazol-2-yl)-7-(diethylamino)2H-chromen-2-one	27424-55-4	C20H19N3O2	333.384	3.68	470
Solvent Yellow 43	2-Butyl-6-(butylamino)-1H-benz(de)isoquinoline-1.3(2H)-dione	19125-99-6	C20H24NsO2	324.417	4.78	485
Phloxine B	Disodium 2,3,4,5-tetrachloro-6-(2,4,5,7-tetrabromo-6-oxido-3-oxo-3H-xanthen-9-yl)benzoate	18472-87-2	C20H2Br4C14Na2O5	829.634	5.47	539
Hematoporphyrin	3,3'[7,12-bis(1-hydroxyethyl)-3,8,13,17-tetramethylporphyrin-2,18-diyl]dipropanoic acid	14459-29-1	C34H38N4O6	598.689	6.45	399

*Estimated values obtained from EPI Suite 4.10 software, provided by the U.S. Environmental Protection Agency and available from http://www.epi.gov/opptintr/exposure/pub/episuite.htm CAS, Chemical Abstracts Service.

dissolved in methanol to produce a 15 mg/ml stock solution which was then diluted with water to make a 2.625 mg/ml working solution.

Each chemical working solution was added to gluten powder at a 1:1 (ml:g) ratio, respectively. The wet dough was kneaded, freeze-dried, and ground to granules as described above.

Confirmation of elution from gluten granules

To determine the peak absorption wavelength, the chemical working solutions were further diluted with water to 0.05 mg/ml and the absorption spectrum between 300 and 1000 nm was scanned using a Varioskan (Thermo Fisher Scientific, Waltham, MA). The optimum absorbance wavelength (λ) was selected for each chemical (Table 1), for use in determining the concentration of chemicals eluted from the gluten granules. Gluten granules containing 0.5 mg of chemical were immersed in 100 ml of water with gentle stirring, and a 1 ml water sample was collected at 5, 10, 20, 30, and 60 min after immersion. The absorbance of water samples at the predetermined wavelength ($n = 5$) and of standard solutions were analyzed. A calibration curve was constructed by plotting the absorbance of a series of standard solutions (concentration range 0.156–10 μg/ml) and used to estimate the concentration of each chemical.

Fluorescent dye administration

Adult male zebrafish of the *rose* mutant line at 6 months of age (3.5–4 cm body length; average weight: 0.38 g) were used and fasted overnight before experiments.

On the day before an oral administration test, fish were divided randomly (one fish per 0.5 L water tank). Gluten granules containing 1 μg/mg of 4-Di-2-Asp or Nile red (2 mg per fish, $n = 5$) were added to each tank and fish consumed them freely within 5 min. Untreated gluten granules were also administered as a control. For intraperitoneal (i.p.) administration, fish were anesthetized with 0.16 mg/ml Tricaine (Wako) and injected with 5 μl of 4-Di-2-Asp or Nile red solutions (0.4 mg/ml, diluted with saline). Fish in the control group were treated with the vehicle only (4% DMSO or acetone). For exposure treatment, five fish were transferred into 500 ml of 4-Di-2-Asp (10 μg/ml) or Nile red (0.1 μg/ml) solution, and incubated in the dark for 1 h. After incubation, the fish were rinsed three times in 500 ml of fresh water for 5 min.

Fish were anesthetized either immediately or 6 h after being treated (as described above) and observed under a fluorescence microscope (MZ 16F, Leica, Wetzlar, Germany) with GFP3 (4-Di-2-Asp) or GFP2 (Nile red) filters (Leica). Part of the zebrafish abdominal skin was removed for observation of visceral organ morphogenesis.

Felbinac administration

Adult male zebrafish of AB strain at 5–6 months of age (2.9–3.5 cm body length, average weight: 0.26 g) were fasted overnight before experiments. Fish were randomly divided into three groups, each containing 30 fish. On the day before p.o. administration, fish were placed into 0.5 L water tanks (one fish per tank). Gluten granules containing 2.625 μg/mg of felbinac were fed to the 'p.o.' group at 2 mg per fish, approximately equivalent to 20 mg/kg body weight. In the ' i.p.' group, each fish was weighed to calculate the required dose.

Felbinac solution (1.05 mg/ml) was administered intraperitoneally at a dose of 20 mg/kg. Injection volumes ranged from 3.5 to 5.5 μl. In the exposure treatment group, fish were immersed in 1 μg/ml felbinac solution at a density of 1 fish per 100 ml. One hour later, fish were rinsed several times in 1 L of fresh water; this point was determined as 0 h after exposure treatment.

At 0.5, 2, 5, 12, and 24 h ($n = 5$ at each point) after felbinac treatment, 5 μl of blood was collected through dorsal artery puncture from each fish by using a heparinized glass capillary needle. Ethanol was added to blood samples to adjust the final volume to 250 μl. This sample was then vortexed and centrifuged at 7700 g for 10 min. Blood from fish treated with either untreated gluten granules or vehicle were also collected as controls and processed in the same manner.

High performance liquid chromatography (HPLC) analysis

Felbinac was dissolved in ethanol to produce a 5 mg/ml stock solution for calibration standards preparation. The stock solution was diluted with ethanol to produce 0.2, 0.4, 0.6, 0.8, and 1.0 μg/ml of calibration standards.

A 20 μl aliquot of each sample supernatant was analyzed by an HPLC system consisting of a L-7100 pump, L-7200 autosampler, L-7300 column oven, L-7400 UV detector (Hitachi High-Technologies, Tokyo, Japan), and a Shimadzu CR-7A computing integrator (Shimadzu, Kyoto, Japan). The system was equipped with a 4.6 mm × 250 mm C18 column (6.5 μm particle size, Wako) maintained at 40°C. Elution was carried out isocratically with a mobile phase consisting of acetonitrile/phosphoric acid (0.01 M) (1:1, v/v) at a flow rate of 1 ml/min. The eluate was monitored at a wavelength of 254 nm. Blank data were obtained from the blood of the control fish described above. Drug concentrations were determined by measuring the peak area and comparing it to the peak area of known standards.
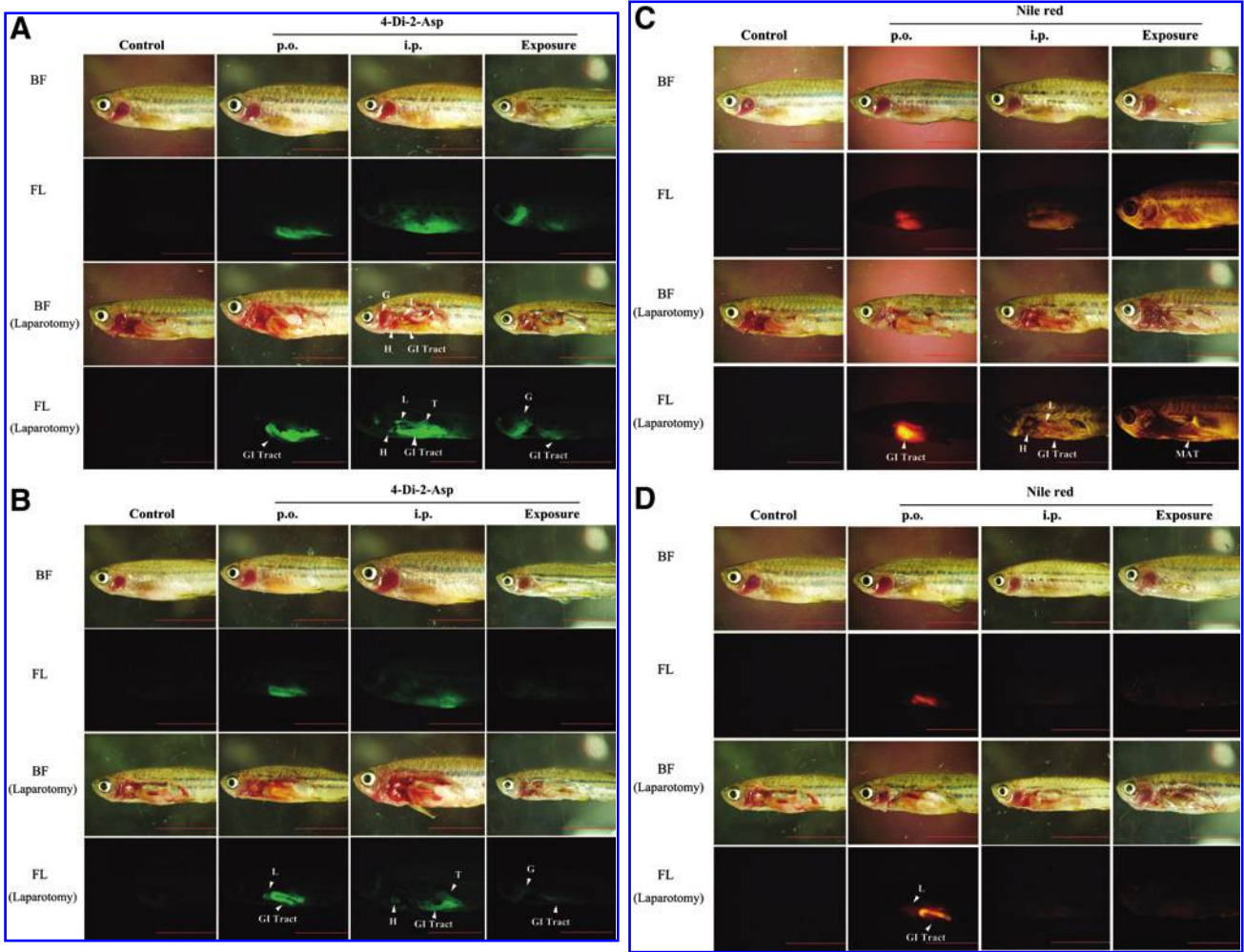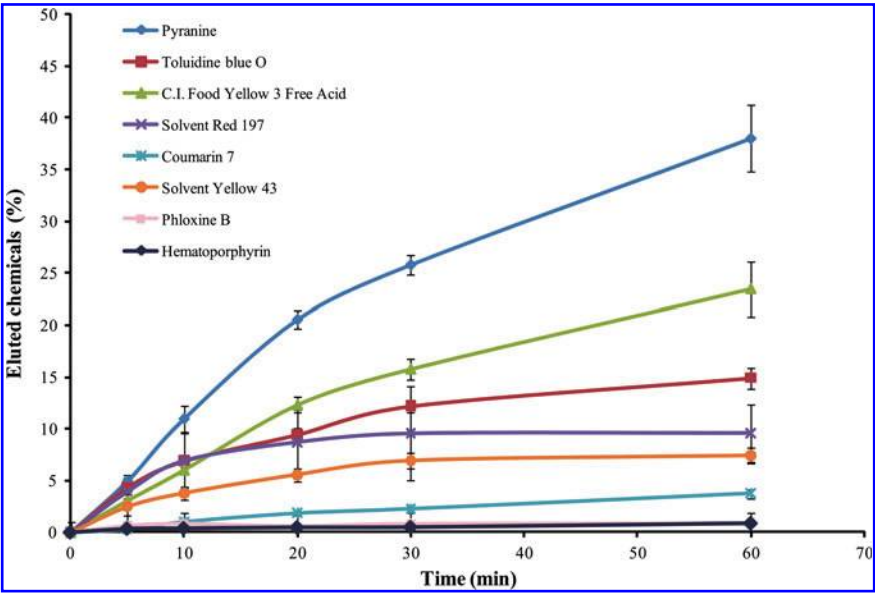
Results

Properties of chemical-containing gluten granules

Wheat gluten is easily obtained as a by-product of wheat flour. Purified gluten powder is white and odorless and can absorb approximately its own weight of water. We confirmed that 1 g of gluten could absorb approximately 1 ml of water. Gluten granules were prepared as described in the Materials and Methods section. These granules are insoluble, float on the surface of water, and can retain their form and remain afloat for up to 30 min. To test whether gluten is preferred by zebrafish, the adult AB line zebrafish were fed gluten granules and their feeding behavior was observed. The average time needed for a single fish to completely consume 2 mg of gluten granules was 79 seconds ($n = 40$). Furthermore, the fish consumed 4 mg of gluten granules within 4 min, and approximately 80% of the 5 mg feeding was consumed within 5 min. Since it is recommended that all food should be eaten within 5 minutes,[24] we determined that the optimal amount of gluten granules for administration of a test chemical to zebrafish should be below 5 mg per fish and should be set at 2–4 mg per fish.

To investigate the amount of chemicals eluted from gluten granules, we selected eight chemicals (fluorescent dyes)

FIG. 1. Elution of test chemicals from gluten granules. Time-dependent change in the ratio of eluted test chemicals to the original amount of test chemical contained in the gluten granules (mean±S.D., $n=5$). Water samples containing eluted chemicals from gluten granules were collected at 5, 10, 20, 30, and 60 min after immersion in water. The concentration of each chemical was determined by measuring the absorption at the respective peak wavelengths given in Table 1.

FIG. 2. The uptake and distribution of 4-Di-2-Asp and Nile red after p.o., i.p., and exposure administrations. Fluorescence images of 4-Di-2-Asp **(A, B)** and Nile red **(C, D)** in *rose* mutant zebrafish immediately **(A, C)** and 6 h **(B, D)** after treatment. BF, bright field; FL, fluorescence; GI Tract, gastrointestinal tract; H, heart; L, liver; T, testis; G, gills; MAT, mesenteric adipose tissue.

covering a range of log octanol-water partition coefficients (Log P_{ow}) from -3.97 to $+6.45$ (Table 1). Gluten granules containing each chemical were prepared and immersed in water. Water samples containing chemicals eluted from these gluten granules were collected and their absorbance at a predetermined wavelength was measured ($n = 5$). The concentration of each chemical in the water samples was calculated from the regression equation of the calibration curve. The time courses for elution of each chemical from the gluten granules are shown in Figure 1. Up to 5 minutes, all chemicals were eluted less than 5% or not at all. Pyranine, toluidine blue O, and C.I. Food Yellow 3 free acid were soluble in water, and the elution curves exhibited an increased modal average over time. Pyranine showed a maximum elution rate of 38%, and the elution rate of chemicals with Log P_{ow} values of -3.97 to $+1.4$ ranged from 15%–38% when gluten granules when immersed in water for up to 60 min. For chemicals with Log P_{ow} values of 2–5 (Solvent Red 197, Coumarin 7, and Solvent Yellow 43), the elution curves showed a slow increase over time and less than 10% of the chemical was eluted from the granules within 60 min. Chemicals with Log P_{ow} values above 5.0 (e.g., Phloxine B (5.47) and Hematoporphyrin (6.45)) were only minimally eluted from gluten granules.

Absorption and distribution of test chemicals

We used two fluorescent dyes, 4-Di-2-Asp and Nile red, to observe the absorption and distribution of test chemicals contained in gluten granules. The *rose* mutant zebrafish, derived from the AB strain and carrying a mutation in the endothelin receptor B gene (*ednrb1*) that leads to a pigment deficiency phenotype,[25] were treated with both dyes *via* p.o., i.p., and exposure administration routes (Fig. 2). Since the adult *rose* mutant zebrafish have no pigmentation in the ventral skin, a sensitive fluorescence image can be derived.

4-Di-2-Asp is a green fluorescent vital staining dye used for labeling hair cells,[26] has a log P_{ow} value of 0.38, and was used here as a representative hydrophilic chemical. Zebrafish treated orally with gluten granules containing 4-Di-2-Asp showed strong, localized fluorescence in the gastrointestinal tract (Fig. 2A, p.o.). After 6 h of digestion, the signal weakened and was relocated to the posterior intestine and the anus. A low-intensity label was observed in the liver, indicating that the 4-Di-2-Asp absorbed from the intestinal wall was metabolized in the liver (Fig. 2B, p.o.). The intraperitoneal injection of 4-Di-2-Asp resulted in widespread vital staining in the peritoneal cavity, including in the gastrointestinal tract, heart, liver, and testis (Fig. 2A, i.p.). After 6 h, the fluorescence signal became weak and irregularly distributed (Fig. 2B, i.p.). Water exposure of zebrafish to 4-Di-2-Asp solution caused a remarkable concentration of fluorescence at the gills and a relatively weak fluorescence in the gastrointestinal tract (Fig. 2A, exposure), each of which had mostly disappeared after 6 h (Fig. 2B, exposure).

Nile red has a strong yellow-gold emission and is commonly used as a vital staining dye for neutral lipid droplets *in vivo*.[27,28] In this study, we used Nile red as a representative hydrophobic chemical having a Log P_{ow} value of 4.38. The fluorescence morphologies after p.o. and i.p. administration were similar to that shown at 0 h and 6 h after 4-Di-2-Asp vital stain. Fish exposed to Nile red solution exhibited intense fluorescence across the whole body, because Nile red is a li-

pophilic dye and can stain the intracellular lipid droplets at the body surface. The laparotomy photograph revealed that the fluorescence was not localized in either the gastrointestinal tract or the other internal organs. In contrast, the mesenteric adipose tissue was strongly stained by Nile red (Fig. 2C, exposure). After 6 h of metabolism, only low fluorescence was detected (Fig. 2D, exposure). We also attempted to detect fluorescence in the blood collected from 4-Di-2-Asp- or Nile red-stained fish but could find no signal, suggesting that concentrations in blood were low and/or undetectable (data not shown).

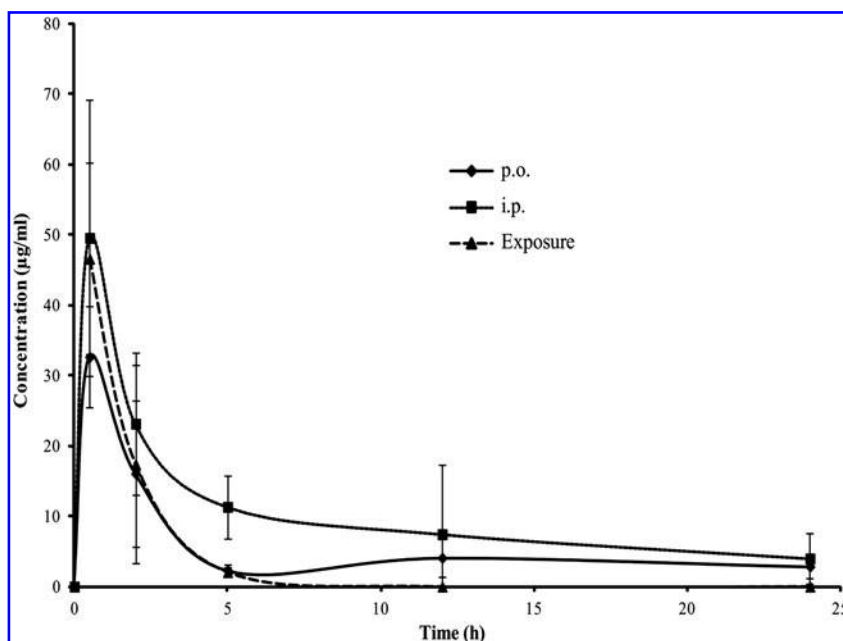### Felbinac pharmacokinetics in zebrafish

The oral administration protocol using gluten granules was applied to administer felbinac to adult zebrafish. Felbinac is a nonsteroidal anti-inflammatory agent and a hydrophobic chemical (Log $P_{ow} = 3.19$). According to the elution properties of Coumarin 7 (Log $P_{ow} = 3.68$) from gluten granules (which showed no elution at 5 min), we inferred that no felbinac could have been eluted into water because the gluten granules containing felbinac were entirely consumed by the fish within 5 min. No interfering peak was detected from the chromatogram of the blank sample (untreated control) at the retention time that corresponds to felbinac by HPLC analysis (data not shown). Figure 3 shows the time-dependent change in the average concentration of felbinac in blood plasma collected from zebrafish after p.o., i.p., or exposure administration. For all three methods, the maximum concentration (C_{max}) value of felbinac in blood plasma was detected at 0.5 h (T_{max}) after administration. This result demonstrates the rapid absorption of felbinac by zebrafish. The observed felbinac C_{max} value was 32.6 ± 7.1, 49.6 ± 19.6, and $46.6 \pm 13.6\,\mu g/ml$ after p.o., i.p., and exposure administration, respectively (Table 2). The area under the plasma concentration-time curve (AUC) corresponding to the above was 128 ± 16.6, 240.5 ± 45.9, and $84.4 \pm 15\,\mu g\,h/ml$, respectively. The plasma concentrations of felbinac after p.o., i.p., and exposure administration decreased rapidly with a half-life of approximately 2 h, with the drug being almost completely metabolized after 5 h.

Discussion

Gluten is a fraction isolated from wheat, which is an important source of nutritional protein, both in foods prepared directly from sources containing it and as an additive to foods otherwise low in protein. For zebrafish reared in the laboratory, fish may be fed living prey, an artificial diet, or a mixture of both. Gluten or wheat flour is an ingredient of the commonly used Zeigler adult zebrafish artificial diet (Zeigler Bros, Inc., Gardners, PA) and the Hikari Tropical Fancy Guppy food that we used daily. We tested whether the fish would eat gluten only as a food and found that they preferred gluten granules.

Gluten is composed mainly of two proteins: gliadin and glutenin. Gluten proteins are water insoluble, so can be purified by washing away the associated starch.[29] When gluten powder is mixed with water, glutenin molecules cross-link to form a submicroscopic network that associates with gliadin, increasing the viscosity and extensibility of gluten.[30] A three-dimensional sponge-like structure can be observed under a scanning electron microscope.[31] We hypothesized that a chemical could be enclosed within this reticulate network and would be retained even when immersed in water. Thus, we

FIG. 3. Mean plasma concentration-time profiles of felbinac after p.o., i.p. (20 mg/kg), and exposure (1 μg/ml) administration. Each *point* and *vertical bar* indicates the mean and S.D., respectively, of five zebrafish. *Solid and dotted lines* represent the fitted mono-exponential curve for the mean data.

developed and optimized this simple protocol based on gluten to administer chemicals to adult zebrafish.

We analyzed the retention capability of gluten for several chemicals possessing different Log P_{ow} values (Fig. 1). The Log P_{ow}, defined as the logarithm of the octanol–water partition coefficient of a solvent, is closely correlated to water solubility.[32,33] Chemicals can therefore be divided into different categories based on their hydrophobicity (Log P_{ow} value): chemicals with Log P_{ow} values between -3 and $+1$ are hydrophilic, $+1$ to $+2$ are intermediate hydrophobic, and above $+3$ are very hydrophobic.[34] Our results indicated that the chemicals analyzed in this study were not (or only minimally) eluted from gluten granules within 5 min, regardless of their hydrophilicity or hydrophobicity. When considering the 'five-minute rule' in feeding, this implies that more than 95% of the total amount of chemical in the granules would be consumed by an adult zebrafish. We demonstrated that 2–4 mg of the gluten could be consumed completely by one fish in 5 min, so the dosage of a test chemical can be controlled by changing the concentration of the chemical in the gluten granules or changing the amount of gluten granules fed to the zebrafish. Additionally, even when gluten granules were immersed in water for 60 min, gluten granules retained 62%–85% of the total amount of hydrophilic or intermediate hydrophobic chemicals (Log $P_{ow} < 2.0$) and 90%–99% of highly hydrophobic chemicals (Log $P_{ow} > 3.0$). These results show that increasing hydrophobicity of a chemical (i.e., high Log P_{ow} values) is correlated with its retention by gluten granules.

TABLE 2. PHARMACOKINETIC PARAMETERS (MEANS ± S.D., $N = 5$) OF FELBINAC FOLLOWING P.O., I.P., AND EXPOSURE ADMINISTRATION

Administration route	AUC_{0-24h} (μg h/ml)	C_{max} ((μg/ml)
p.o. (20 mg/kg)	128 ± 16.6	32.6 ± 7.1
i.p. (20 mg/kg)	240.5 ± 45.9	49.6 ± 19.6
Exposure	84.4 ± 15	46.6 ± 13.6

We also investigated how the test chemicals were absorbed and distributed in zebrafish, using oral, i.p., and exposure administration methods. Hydrophilic and hydrophobic chemicals administered by oral or i.p. administration were localized in the gastrointestinal tract and peritoneal cavity, respectively. In contrast, when administering chemicals by exposure, fluorescence from the hydrophilic vital staining dye, 4-Di-2-Asp, was detected in gill and digestive tract, whereas the hydrophobic vital staining chemical, Nile red, dyed the entire body surface nonspecifically, but with no evidence of fluorescence in the gastrointestinal tract. Following oral administration, it appeared that the chemicals were absorbed by the digestive system, entering the hepatic portal system and progressing thereon into the liver through the portal vein before reaching the systemic circulation. Conversely, chemicals injected by i.p. administration could be absorbed by one of two pathways. The first of these is through the portal system, connecting to the circulation *via* the visceral peritoneum and the mesentery, whereas the second is the direct emission of chemicals into the systemic circulation *via* the parietal peritoneum and the lymphatics.[35] Where zebrafish are exposed into chemicals in solution, their gills are considered to play a central role in the absorption of dissolved chemicals. Physicochemical and biochemical factors including electric charge, molecular weight, octanol-water partition coefficient, and concentration would influence the transport of chemicals through the gill.[36] Chemicals administered by exposure administration can also be absorbed by the gastrointestinal tract because, although freshwater fish do not ingest water for osmoregulation, the ingestion by zebrafish of water containing dissolved chemicals has been reported.[37] Indeed, in our experiments, after exposure to 4-Di-2-Asp dissolved in water, fluorescence was observed in the zebrafish gastrointestinal tract. The absorption of chemicals by dermal uptake *via* a diffusion-controlled process[38] is also possible in exposure administration. However, the strategy used in this study of orally administering gluten granules impregnated with chemicals is designed to target these chemicals to the

gastrointestinal tract, where the gluten granules are digested and the chemicals subsequently absorbed.

In our studies of the pharmacokinetics of felbinac in zebrafish, the AUC values for felbinac following i.p. administration were ~1.8 times higher than that after the p.o. route. This could be due to hepatic 'first-pass' effects. After oral administration of gluten granules containing felbinac at a dose of 20 mg/kg, the observed felbinac C_{max} value was almost half that reported in the literature,[39] in which the pharmacokinetic parameters were determined from rats after oral administration of felbinac as a suspension in distilled water at a dose of 10 mg/kg. Although the absolute applied dose of felbinac administered to zebrafish in exposure administration could not be calculated, the AUC value was about 0.66 and 0.35 times lower than the AUC values after p.o. and i.p. (20 mg/kg) administration routes, which would be a useful reference to further studies.

In this study, we have identified the characteristics of three different routes of chemical administration: p.o., i.p., and exposure. These methods have different advantages and disadvantages. The advantages of i.p. administration include ease of manipulation, reduced use of chemicals (i.e., money is saved, in the case of expensive drugs), and high drug bioavailability, but this technique also causes elevated infection risk (due to its invasivity) and is therefore unsuitable for long-term and/or repeated administration. Exposure administration is also easy to manipulate, but it requires large amounts of chemical (which can elevate costs) and is limited to water-soluble test chemicals. There is also considerable difficulty in calculating the precise chemical intake by a complex absorption system that is vastly different from human physiology.

Here we developed a novel protocol for oral administration, the most common and convenient method for drug delivery in humans. Our method is based on the use of gluten as an ideal carrier material, and offers the advantages of noninvasivity, minimized chemical consumption, and convenience. The disadvantage of our method is that fish have to be fed gluten granules individually to determine total consumption effectively. On the other hand, the individual feeding procedure is more advantageous for avoiding competition and a hierarchy among the fish than the group feeding. This novel protocol should be widely applicable for toxicological testing and drug discovery, and especially efficient for the screening of hydrophobic and/or insoluble chemicals.

Acknowledgments

Disclosure Statement

No competing financial interests exist.

References

1. Fishman MC. Genomics. Zebrafish—The canonical vertebrate. Science 2001;294:1290–1291.
2. Barros T, Alderton W, Reynolds H, Roach A, Berghmans S. Zebrafish: An emerging technology for *in vivo* pharmacological assessment to identify potential safety liabilities in early drug discovery. Br J Pharmacol 2008;154:1400–1413.
3. Penberthy WT, Shafizadeh E, Lin S. The zebrafish as a model for human disease. Front Biosci 2002;7:d1439–1453.
4. Lieschke G, Currie P. Animal models of human disease: Zebrafish swim into view. Nat Rev Genet 2007;8:353–367.
5. Stern H, Zon L. Cancer genetics and drug discovery in the zebrafish. Nat Rev Cancer 2003;3:533–539.
6. Lamason RL, Mohideen MA, Mest JR, Wong AC, Norton HL, Aros MC, et al. SLC24A5, a putative cation exchanger, affects pigmentation in zebrafish and humans. Science 2005;310:1782–1786.
7. Jones HS, Panter GH, Hutchinson TH, Chipman JK. Oxidative and conjugative xenobiotic metabolism in zebrafish larvae *in vivo*. Zebrafish 2010;7:23–30.
8. Spitsbergen JM, Tsai HW, Reddy A, Miller T, Arbogast D, Hendricks JD, et al. Neoplasia in zebrafish (*Danio rerio*) treated with N-methyl-N′-nitro-N-nitrosoguanidine by three exposure routes at different developmental stages. Toxicol Pathol 2000;28:716–725.
9. Chiu L, Cunningham L, Raible D, Rubel E, Ou H. Using the zebrafish lateral line to screen for ototoxicity. J Assoc Res Otolaryngol 2008;9:178–190.
10. Molina G, Vogt A, Bakan A, Dai W, Queiroz de Oliveira P, Znosko W, et al. Zebrafish chemical screening reveals an inhibitor of Dusp6 that expands cardiac cell lineages. Nat Chem Biol 2009;5:680–687.
11. Tiefenbach J, Moll P, Nelson M, Hu C, Baev L, Kislinger T, et al. A live zebrafish-based screening system for human nuclear receptor ligand and cofactor discovery. PLoS One 2010;5:e9797.
12. Rihel J, Prober D, Arvanites A, Lam K, Zimmerman S, Jang S, et al. Zebrafish behavioral profiling links drugs to biological targets and rest/wake regulation. Science 2010;327:348–351.
13. Ou H, Cunningham L, Francis S, Brandon C, Simon J, Raible D, et al. Identification of FDA-approved drugs and bioactives that protect hair cells in the zebrafish (*Danio rerio*) lateral line and mouse (*Mus musculus*) utricle. J Assoc Res Otolaryngol 2009;10:191–203.
14. Lam S, Mathavan S, Tong Y, Li H, Karuturi R, Wu Y, et al. Zebrafish whole-adult-organism chemogenomics for large-scale predictive and discovery chemical biology. PLoS Genet 2008;4:e1000121.
15. Kily L, Cowe Y, Hussain O, Patel S, McElwaine S, Cotter F, et al. Gene expression changes in a zebrafish model of drug dependency suggest conservation of neuro-adaptation pathways. J Exp Biol 2008;211:1623–1634.
16. Lien C, Schebesta M, Makino S, Weber G, Keating M: Gene expression analysis of zebrafish heart regeneration. PLoS Biol 2006;4:e260.
17. Oppedal D, Goldsmith M. A chemical screen to identify novel inhibitors of fin regeneration in zebrafish. Zebrafish 2010;7:53–60.
18. Sun L, Wen L, Shao X, Qian H, Jin Y, Liu W, et al. Screening of chemicals with anti-estrogenic activity using *in vitro* and *in vivo* vitellogenin induction responses in zebrafish (*Danio rerio*). Chemosphere 2010;78:793–799.
19. Zodrow J, Tanguay R. 2,3,7,8-tetrachlorodibenzo-p-dioxin inhibits zebrafish caudal fin regeneration. Toxicol Sci 2003;76:151–161.
20. Liu Y, Wang J, Wei Y, Zhang H, Xu M, Dai J. Induction of time-dependent oxidative stress and related transcriptional effects of perfluorododecanoic acid in zebrafish liver. Aquat Toxicol 2008;89:242–250.
21. Esbaugh A, Perry S, Gilmour K. Hypoxia-inducible carbonic anhydrase IX expression is insufficient to alleviate intracellular

metabolic acidosis in the muscle of zebrafish, *Danio rerio*. Am J Physiol Regul Integr Comp Physiol 2009;296:R150–160.

22. DeKoven DL, Núñez JM, Lester SM, Conklin DE, Marty GD, Parker LM, et al. A purified diet for medaka (*Oryzias latipes*): Refining a fish model for toxicological research. Lab Anim Sci 1992;42:180–189.

23. Milan DJ, Jones IL, Ellinor PT, MacRae CA. *In vivo* recording of adult zebrafish electrocardiogram and assessment of drug-induced QT prolongation. Am J Physiol Heart Circ Physiol 2006;291:H269–273.

24. Westerfield M. The zebrafish book: A guide for the laboratory use of zebrafish (*Danio rerio*). Ed. 4. ed. Eugene, OR; M. Westerfield; 2000.

25. Parichy DM, Mellgren EM, Rawls JF, Lopes SS, Kelsh RN, Johnson SL. Mutational analysis of endothelin receptor b1 (rose) during neural crest and pigment pattern development in the zebrafish *Danio rerio*. Dev Biol 2000;227:294–306.

26. Hernández P, Moreno V, Olivari F, Allende M. Sub-lethal concentrations of waterborne copper are toxic to lateral line neuromasts in zebrafish (*Danio rerio*). Hear Res 2006;213:1–10.

27. Jones K, Alimov A, Rilo H, Jandacek R, Woollett L, Penberthy W. A high throughput live transparent animal bioassay to identify non-toxic small molecules or genes that regulate vertebrate fat metabolism for obesity drug development. Nutr Metab (Lond) 2008;5:23.

28. Flynn Er, Trent C, Rawls J. Ontogeny and nutritional control of adipogenesis in zebrafish (*Danio rerio*). J Lipid Res 2009;50:1641–1652.

29. Van Der Borght A, Goesaert H, Veraverbeke WS, Delcour JA. Fractionation of wheat and wheat flour into starch and gluten: Overview of the main processes and the factors involved. J Cereal Sci 2005;41:221–237.

30. Shewry P, Halford N, Belton P, Tatham A. The structure and properties of gluten: An elastic protein from wheat grain. Philos Trans R Soc Lond B Biol Sci 2002;357:133–142.

31. Freeman TP, Shelton DR, Bjerke JM, Skierkowski K. The ultrastructure of wheat gluten: Variations related to sample preparation. Cereal Chem 1991;68:492–498.

32. Chiou CT, Freed VH, Schmedding DW, Kohnert RL. Partition coefficient and bioaccumulation of selected organic chemicals. *Environ Sci Techol* 1977;11:475–478.

33. Mackay D. Partition coefficient and bioaccumulation of selected organic chemicals. Environ Sci Technol 1977;11:1219.

34. Verliefde A, Van Vliet N, Amy G, Van der Bruggen B, van Dijk JC. A Semi-quantitative method for prediction of the rejection of uncharged organic micropollutants with nanofiltration. Water Practice Technol 2006;1:10.

35. Lukas G, Brindle SD, Greengard P. The route of absorption of intraperitoneally administered compounds. J Pharmacol Exp Ther 1971;178:562–564.

36. Spacie A, Hamelink JL. Alternative models for describing the bioconcentration of organics in fish. Environ Toxicol Chem 1982;1:309–320.

37. Jagadeeswaran P, Sheehan JP. Analysis of blood coagulation in the zebrafish. Blood Cells Mol Dis 1999;25:239–249.

38. Konrádsdóttir F, Loftsson T, Sigfússon SD. Fish skin as a model membrane: Structure and characteristics. J Pharm Pharmacol 2009;61:121–124.

39. Puglisi G, Ventura C, Spadaro A, Campana G, Spampinato S. Differential effects of modified beta-cyclodextrins on pharmacological activity and bioavailability of 4-biphenylacetic acid in rats after oral administration. J Pharm Pharmacol 1995;47:120–123.

Address correspondence to:
Liqing Zang, M.D.
Department of Translational Medical Science
Graduate School of Medicine
Mie University
2-174 Edobashi
Tsu, Mie 514-8507
Japan

E-mail: liqing@doc.medic.mie-u.ac.jp