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Letter

Comparing time-series of chemical concentrations in zebrafish (*Danio rerio*) embryos/larvae exposed to teratogens with different hydrophobicity; caffeine, sodium valproate, and diethylstilbestrol

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ABSTRACT — Developmental toxicity is an adverse developmental outcome, i.e., death, malformation, growth retardation, or functional deficiency. Recently, alternative methods of assessing developmental toxicity using zebrafish (*Danio rerio*) as a preliminary screening have attracted attention because of their low cost and high throughput. However, most toxicity evaluations have been based on a chemical concentration in an aqueous solution, and the chemical concentrations in embryos/larvae and their temporal behavior have in most cases been unclear, regardless of differences of chemical hydrophobicity. In the present study, we selected three teratogens with different hydrophobicities (caffeine, CA, log K_{ow} -0.07; sodium valproate, VA, log K_{ow} 0.26 (pH 7.4); and diethylstilbestrol, DES, log K_{ow} 5.07), and we measured their concentrations in embryos/larvae exposed to these chemicals every 24 hr post-fertilization (hpf) until 144 hpf. Kinetic analysis based on a one-compartment fish model that yields first order kinetics for CA and VA revealed that concentrations of both CA and VA in embryos/larvae increased gradually and became saturated by around 100 hpf. In contrast, DES concentrations in embryos/larvae reached a maximum at 48 or 72 hpf and then decreased gradually. The present study suggests that the temporal pattern of chemical concentrations is a function of the hydrophobicity of the chemicals.

Key words: Zebrafish, Developmental toxicity, Concentration in embryos/larvae, Hydrophobicity, Lipid concentration

INTRODUCTION

Developmental toxicity is an adverse developmental outcome, i.e., death, malformation, growth retardation, or functional deficiency (Hood, 2016). In drug development, the presence of developmental toxicity in drug candidates has usually been tested using rodent and non-rodent mammalians, e.g., rats and rabbits (International Conference on Harmonisation, 1994), but such testing has proven to be time-consuming and very expensive (Piersma, 2004). Recently, alternative methods using zebrafish (*Danio rerio*) as a preliminary screening have attracted attention because of their low cost and high throughput (Gibert *et al*., 2013).

In published reports, embryos have usually been exposed from two hours post fertilization (hpf) until as long as 144 hpf, and indicators of developmental abnormalities have been monitored microscopically (Brannen *et al.*, 2010; Selderslaghs *et al*., 2012; Yamashita *et al.*, 2014). Evaluation of developmental toxicity via these methods has been based on the concentration of each compound in the water (C_w). However, C_w does not reflect the actual amount to which the embryos/larvae is exposed *in vivo*, the amount of the toxicant that is directly associated with developmental toxicity.

Only a few papers have reported the toxicant con-

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centrations in zebrafish embryos/larvae. Some of those papers have reported the chemical concentration of each compound in zebrafish embryos/larvae (C_e) at only one point in time, or they have reported the time-series of the concentration in the embryos/larvae up to at most 120 hpf (Ball *et al.*, 2014; Diekmann and Hill, 2013; Huang *et al.*, 2010).

Bioaccumulation is the accumulation of chemicals in an organism through any route (Sanz-Landaluze *et al.,* 2015). In general, bioaccumulation in a typical fish, including zebrafish, can be described in terms of two first-order kinetic processes, i.e., uptake and depuration, based on a one-compartment fish model (OECD, Organization for Economic Cooperation and Development, 2012). The kinetic parameters and bioconcentration factors (BCFs) calculated on the basis of the first-order model using zebrafish embryos/larvae have previously been reported by Sanz-Landaluze *et al.* (2015). However, it has been presumed that compounds with different values of the *n*-octanol/water partition coefficient (K_{ow}) would be differentially permeable into the zebrafish body and that the temporal patterns of C_e during the tests would differ because of differences in hydrophobicity, but no experimental data evidencing such differences have been reported.

The purpose of this study was to analyze the C_e of teratogens representing different K_{ow} values and to compare the characteristics of the C_e time series. We exposed zebrafish embryos to three chemicals, caffeine (CA), sodium valproate (VA), and diethylstilbestrol (DES), which are known to be human and/or rodent teratogens. Their log K_{ow} values are -0.07 for CA, 0.26 (pH 7.4) for VA, and 5.07 for DES (Lombardo *et al*., 2001; Kyowa Hakko Kirin, 2017; Hansch *et al*., 1995). We measured the C_e of teratogens every 24 hpf up to 144 hpf and performed the appropriate kinetic analyses.

MATERIALS AND METHODS

Test organisms

Wild-type zebrafish (*Danio rerio*, NIES-R strain, National Institute for Environmental Studies, Japan) were used for all experiments. The adult fish were maintained with dechlorinated tap water (26 ± 1°C) under flow-through conditions on a 16-hr light/8-hr dark cycle and were fed with recently hatched (< 24 hr old) brine shrimp (*Artemia*) from Great Salt Lake (EGGS-90, Kitamura, Kyoto, Japan). Fertilized eggs were obtained by placing two male fish into breeding tanks, each of which contained one female fish, in the morning on the day of the experiment.

Conditions of exposure to test compounds

CA and VA were purchased from Wako Pure Chemicals (Osaka, Japan). DES was obtained from Tokyo Chemical Industry (Tokyo, Japan).

Developmental toxicity tests for these test chemicals were performed to determine exposure concentrations that would be used to calculate C_e. Details of the tests are shown in the Supplemental data and Supplemental Table 1. Supplemental Table 2 shows the concentrations of each compound in the water (C_ws) measured during the developmental toxicity tests. Supplemental Table 3 shows the percentages of developmental abnormality and mortality in the tests and observed representative effects. To calculate the chemical concentrations of each compound in zebrafish embryos/larvae (C_es), we first determined the test solution concentrations that caused developmental abnormalities or mortality during exposure. Those test solution concentrations were 155 and 621 mg/L for CA, 41.5 and 166 mg/L for VA, and 0.537 and 1.07 mg/L for DES. In addition, we tested a DES concentration of 0.268 mg/L, which caused minor developmental abnormalities, to obtain more information about the temporal behavior of C_es for teratogens with high solubility in fat. Each test solution was prepared with dilution water only (reconstituted water: ISO 6341-1982) (OECD, 1992) for CA and VA and with dilution water containing 0.01% dimethyl sulfoxide for DES. Normal embryos by 5 hpf were exposed to the test solutions (about 2 mL/embryo) in glass beakers. The beakers were sealed and maintained for up to 144 hpf at 28 ± 1°C on a 14-hr light/10-hr dark cycle. The test solutions of CA and VA were not renewed, whereas the DES solution was renewed every 24 hr to maintain the actual C_w.

Measuring concentrations of test compounds in test solutions

We measured the C_ws of the three test compounds in all tests. For CA and VA, the C_ws in the test solutions were measured at the start and end of the exposure period. For DES, the test solutions were analyzed in two sets: (1) duplicate solutions freshly prepared and (2) duplicate solutions before renewal or at the time of 100% mortality. The solutions sampled were appropriately diluted with acetonitrile to produce the same composition as the eluent for each compound. The treated samples were analyzed by high performance liquid chromatography (HPLC) using a LC-2010AHT instrument (Shimadzu, Kyoto, Japan) equipped with an L-column2 ODS (octadecyl silica) column (length, 150 mm; inner diameter, 2.1 mm; particle size, 5 μm; Chemicals Evaluation and Research Institute, Tokyo, Japan). Each 20-μL sample was eluted

in a mobile phase of acetonitrile: water (1:9 v/v for CA, 4:6 v/v for VA, and 5:5 v/v for DES), and each toxicant was detected at a different wavelength (270 nm for CA, 210 nm for VA, and 254 nm for DES).

Measuring concentrations of test compounds in zebrafish embryos/larvae

The exposed embryos (3-20 embryos per replication, three or four replicates/level/time point) were analyzed every 24 hpf up to a maximum of 144 hpf. The chorions of embryos were removed with forceps under a microscope prior to sampling for the C_e measurements. The dechorionated embryos or the larvae were weighed as wet body weight and were individually transferred through a series of two glass beakers containing 100 mL of fresh dilution water to remove chemical residues on the body surface. The water on their body surfaces was removed with a filter paper. After that step, the embryos/larvae were homogenized with a silicon pestle in a 1.5-mL sampling tube containing a mixed solvent of the same composition as each liquid chromatography eluent to extract the test compound. The mixture was centrifuged at 10,000 *g* for 5 min. The supernatant was collected, brought up to a volume of 1 mL, and filtered through a Millex® filter with a 0.2-μm pore size (Merck KGaA, Darmstadt, Germany). The filtrate was analyzed by HPLC or liquid chromatography-mass spectrometry (LC-MS). The equipment and conditions for measuring the C_es of CA and DES were the same as those used to measure their C_ws. The C_e of VA was analyzed by LC-MS with an LCMS-8050 (Shimadzu) and a Nexera X2 (Shimadzu) equipped with an ACQUITY UPLC BEH C18 column (length, 50 mm; inner diameter, 2.1 mm; particle size, 1.7 μm; Nihon Waters, Tokyo, Japan). Each 20-μL sample was eluted in a mobile phase of acetonitrile (with 0.1% formic acid): water (with 0.1% formic acid) (4:6 v/v). VA was monitored by electrospray negative ionization mode (142.9 m/z).

Kinetic analysis

We carried out a kinetic analysis using the C_ws of CA and VA and the corresponding C_es measured every 24 hpf. We applied the methods described in OECD Test Guideline 305 annex 5 to estimate values of the uptake rate constant (k_1; L kg^{-1} h^{-1}) and the depuration rate constant (k_2; h^{-1}), the time required for the C_e to reach 95% of the steady-state (SS) value (T_{SS95}; h), and the kinetic BCF (BCF_K; L kg^{-1}) (OECD, 2012). We estimated the k_1 and k_2 values based on a time series of concentration data and the following equation

$$C_e = C_w k_1[1 - \exp(-k_2 t)]/k_2 \quad (1)$$

where C_e is the geometric mean of the C_es at each time point (mg kg^{-1}), C_w is the geometric mean of the C_ws (mg L^{-1}), and t is time (h). We also estimated T_{SS95}, BCF, and BCF_K with the following equations (OECD, 2012)

$$T_{SS95} = -\ln(0.05)/k_2 = 3.0/k_2,\ BCF = C_e/C_w,\ BCF_K = k_1/k_2$$

RESULTS AND DISCUSSION

Table 1 shows the C_ws of test compounds used to determine C_es. The geometric means of the measured C_ws expressed as percentages of the nominal concentrations were 97.2-98.0% for CA, 88.5-95.0% for VA, and 89.6-101% for DES. The measured C_w of each test compound was therefore close to the nominal value throughout the study. In the following description, the C_ws are reported as the nominal concentrations.

Figure 1 shows the C_es of each compound measured every 24 hpf up to a maximum of 144 hpf.

The C_e of CA at an exposure level of 155 mg/L increased steadily until 144 hpf, but the rate of increase gradually declined. The maximum concentration during the test was 174 mg/kg at 144 hpf, which was very similar to the C_w at 144 hpf (151 mg/L, BCF: 1.15 L kg^{-1}). The C_e of CA at an exposure level of 621 mg/L was measured

Table 1. Measured concentrations of test compounds in test solutions used to determine the concentrations in zebrafish embryo/larva (C_es).

Caffeine			Sodium valproate			Diethylstilbestrol		
Test level (mg/L)	Geometric mean of measured concentration (mg/L) (% of nominal concentration)		Test level (mg/L)	Geometric mean of measured concentration (mg/L) (% of nominal concentration)		Test level (mg/L)	Geometric mean of measured concentration (mg/L) (% of nominal concentration)	
155	151	(97.2)	41.5	36.7	(88.5)	0.268	0.240	(89.6)
621	609	(98.0)	166	158	(95.0)	0.537	0.530	(98.6)
						1.07	1.08	(101)

only until 96 hpf because there was 100% mortality at 120 hpf. The C_e of CA at an exposure level of 621 mg/L increased steadily until 96 hpf, and the C_e at 96 hpf was 503 mg/kg (BCF: 0.826 L kg^{-1}).

The C_e of VA at an exposure level of 41.5 mg/L increased until 48 hpf; at 72 hpf it was in the range 107-116 mg/kg (BCF: 2.92-3.16 L kg^{-1}). The C_e of VA at an exposure level of 166 mg/L was measured until 72 hpf because there was 100% mortality at 96 hpf; the C_e increased steadily until reaching 400 mg/kg at 72 hpf (BCF: 2.53 L kg^{-1}).

Kinetic analyses were performed on the plots of the C_e of CA at an exposure level of 155 mg/L and on the plots of the VA concentration in embryos/larvae at an exposure level of 41.5 mg/L at all time points. Table 2 shows the calculated kinetic parameters. Figure 1 (a) and (c) show the fitted curves with k_1 and k_2 values calculated from equation (1). Similar curve fitting was not performed for CA and VA because the number of plots was insufficient for the analysis. Curve fitting was also not performed for DES because the uptake and depuration of DES did not follow first-order kinetics based on a one-compartment model. The k_1 for VA (0.087 L kg^{-1} h^{-1}) was 2.8 times that of CA (0.031 L kg^{-1} h^{-1}), but the k_2s for CA and VA were similar (0.027 and 0.030 L kg^{-1}, respectively). Therefore, the BCF_K of VA was 2.4 times that of CA. This difference is consistent with the observation that the higher the K_{ow} of a compound, the higher the BCF tends to be (Arnot and Gobas, 2006). The T_{SS95}s for CA and VA were almost the same (110 and 99 h, respectively). Collection of more information about C_e combined with kinetic analyses might enable us to predict C_e without analytical measurements.

In contrast, the C_e of DES showed temporal behavior different from that of CA and VA. The C_es of DES at exposure levels of 0.268, 0.537, and 1.07 mg/L reached maximum values at 48 or 72 hpf and then gradually decreased to less than half the corresponding maximum concentrations. The C_e at an exposure level of 0.268 mg/L reached a maximum of 62.1 mg/kg (BCF: 259 L kg^{-1}) at 48 hpf and decreased to 9.24 mg/kg (BCF: 38.5 L kg^{-1}) at 144 hpf. The C_e at an exposure level of 0.537 mg/L reached a maximum of 122 mg/kg (BCF: 230 L kg^{-1}) at 72 hpf and decreased to 61.1 mg/kg (BCF: 115 L kg^{-1}) at 144 hpf. The C_e of DES at an exposure level of 1.07 mg/L was measured only until 96 hpf because there was 100% mortality at 120 hpf. The C_e at an exposure level of 1.07 mg/L reached a maximum of 322 mg/kg (BCF: 299 L kg^{-1}) at 72 hpf and decreased to 155 mg/kg (BCF: 144 L kg^{-1}) at 96 hpf.

Table 2. Kinetic parameters calculated via kinetic analysis.

Parameter	Caffeine	Valproate sodium salt
	155 mg/L	41.5 mg/L
k_1 (L kg^{-1} h^{-1})	0.031	0.087
k_2 (h^{-1})	0.027	0.030
BCF_K (L kg^{-1})	1.2	2.9
T_{SS95} (h)	110	99

k_1: uptake rate constant, k_2: depuration rate constant, BCF_K: kinetic bioconcentration factor, T_{SS95}: time required for the concentration of the compound to reach 95% that at steady state in zebrafish embryo/larva.

DES is highly soluble in fat, and we presume that the gradual decrease of C_e after 48 or 72 hpf was due to a decline of total lipid concentration in whole embryos/larvae because of the energetic costs of development and growth. To address this hypothesis, we estimated the total lipid concentrations of whole embryos/larvae exposed to DES concentrations of 0.268 and 0.537 mg/L in the present study using previously reported data (Fraher *et al*., 2016) (Fig. 2, details of the calculation method are shown in Supplemental data). The total lipid concentration for both exposure levels decreased gradually until 72 hpf and more rapidly from 72 hpf to 120 hpf. The concentrations at 120 hpf in the 0.268 and 0.537 mg/L treatments decreased to 63% and 48% of the corresponding concentrations at 24 hpf. We therefore hypothesized that the C_e of DES gradually increased up to 48 or 72 hpf by absorption through the gills and the skin and by allocation to fat in whole embryos/larvae. Then the C_e gradually decreased in association with the decline of the total lipid concentration in whole embryos/larvae. To test this hypothesis, more information would need to be collected about the concentrations of compounds that are highly soluble in fat. In contrast, the liver is unlikely to have had much of a metabolic function at 48 or 72 hpf because the zebrafish liver does not become visible until approximately 96 hpf (Chu and Sadler, 2009). Therefore, metabolic activity in the liver would not have been mainly responsible for the decline of the DES C_e. Nevertheless, drug-metabolizing enzymes in the liver and/or in sites other than the liver might have partially contributed to the rapid decrease of the DES C_e at 72 hpf and later. Although no peaks other than DES were observed on the chromatogram obtained under the analytical conditions used to measure DES in the present study (data not shown), small amounts of metabolites might have been detected via additional detailed analyses targeting DES metabolites. More studies would be required to clarify the factors that caused the decrease of the DES C_e.

In conclusion, our study provided unique information about the temporal pattern of C_es for compounds with dif-

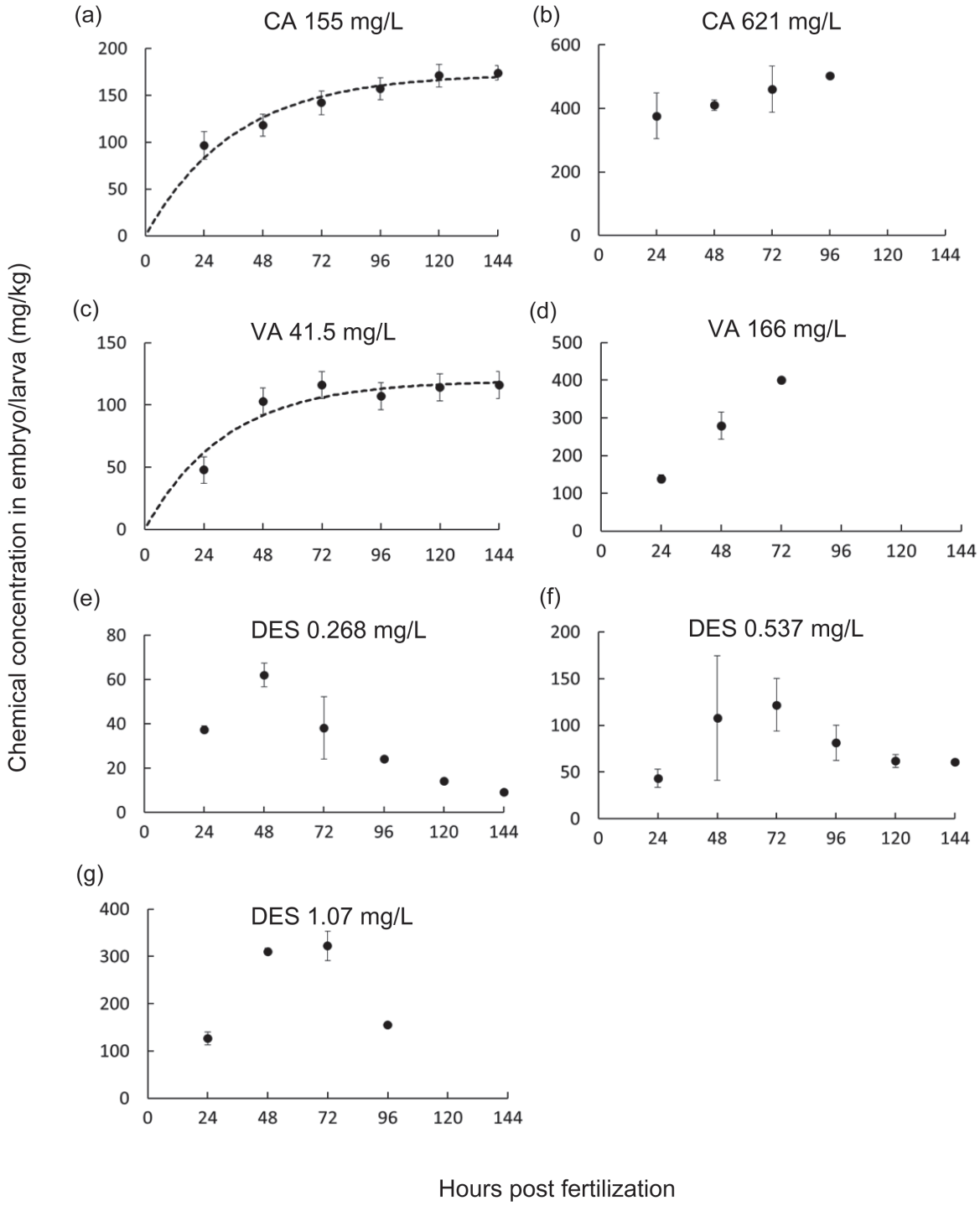

Fig. 1. Chemical concentrations of each compound every 24 hpf in zebrafish embryos/larvae exposed to (a) a caffeine (CA) solution of 155 mg/L, (b) a CA solution of 621 mg/L, (c) a valproate sodium salt (VA) solution of 41.5 mg/L, (d) a VA solution of 166 mg/L, (e) a diethylstilbestrol (DES) solution of 0.268 mg/L, (f) a DES solution of 0.537 mg/L, and (g) a DES solution of 1.07 mg/L. Each plot shows the mean value of several replicates (CA: four replicates, VA and DES: three replicates). Each error bar shows the standard deviation of the replicates. Theoretical curves show the first-order kinetic model for (a) a CA solution of 155 mg/L and (c) a VA solution of 41.5 mg/L.

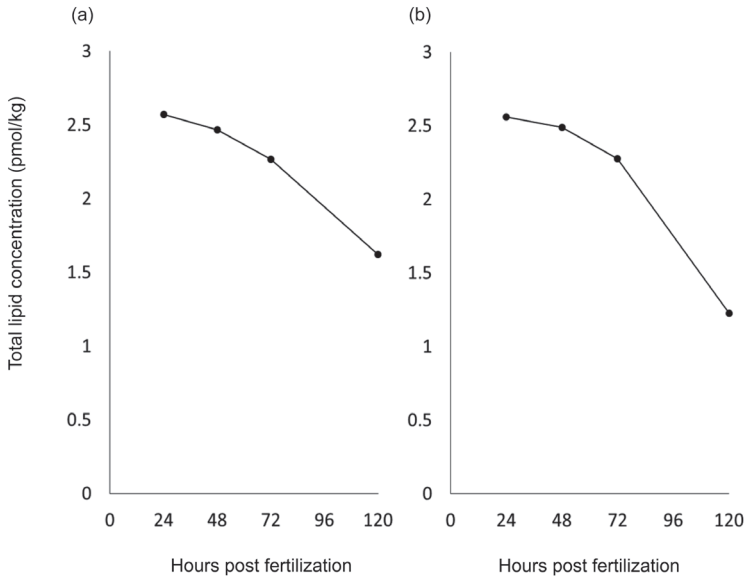

Fig. 2. Time series of calculated total lipid concentrations in whole embryos/larvae exposed to a DES solution of (a) 0.268 mg/L and (b) 0.537 mg/L.

ferent hydrophobicities that caused developmental toxicity. In particular, for compounds like DES that are highly soluble in fat, the temporal pattern of C_e can vary widely in spite of a stable C_w. To clarify such behavior, more data for compounds highly soluble in fat are needed. In addition, more data about the temporal pattern of C_e for various kinds of compounds might lead to generalizations about the temporal pattern of every compound group. Particular attention should be paid to gaps between C_w and C_e if developmental toxicity is evaluated via C_w without measuring C_e. The gradually decreased C_e of DES may be related to the decline of the total lipid concentration in whole embryos/larvae.

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

REFERENCES

Arnot, J.A. and Gobas, F. (2006): A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in aquatic organisms. Environ. Rev., **14**, 257-297.

Ball, J.S., Stedman, D.B., Hillegass, J.M., Zhang, C.X., Panzica-Kelly, J., Coburn, A., Enright, B.P., Tornesi, B., Amouzadeh, H.R., Hetheridge, M., Gustafson, A.L. and Augustine-Rauch, K.A. (2014): Fishing for teratogens: a consortium effort for a harmonized zebrafish developmental toxicology assay. Toxicol. Sci., **139**, 210-219.

Brannen, K.C., Panzica-Kelly, J.M., Danberry, T.L. and Augustine-Rauch, K.A. (2010): Development of a zebrafish embryo teratogenicity assay and quantitative prediction model. Birth Defects Res. B Dev. Reprod. Toxicol., **89**, 66-77.

Chu, J. and Sadler, K.C. (2009): New school in liver development: lessons from zebrafish. Hepatology, **50**, 1656-1663.

Diekmann, H. and Hill, A. (2013): ADMETox in zebrafish. Drug Discov. Today Dis. Models, **10**, e31-e35.

Fraher, D., Sanigorski, A., Mellett, N.A., Meikle, P.J., Sinclair, A.J. and Gibert, Y. (2016): Zebrafish embryonic lipidomic analysis reveals that the yolk cell is metabolically active in processing lipid. Cell Rep., **14**, 1317-1329.

Gibert, Y., Trengove, M.C. and Ward, A.C. (2013): Zebrafish as a genetic model in pre-clinical drug testing and screening. Curr. Med. Chem., **20**, 2458-2466.

Hansch, C., Leo, A. and Hoekman, D. (1995): Exploring QSAR—hydrophobic, electronic, and steric constants. ACS Professional Reference Book. American Chemical Society, Washington, DC.

Hood, R.D. (2016): Principles of developmental toxicology revisited, Developmental and reproductive toxicology, a practical approach, Third edition. (Hood, R.D., ed.), pp. 1-9, CRC Press, London.

Huang, H., Huang, C., Wang, L., Ye, X., Bai, C., Simonich, M.T., Tanguay, R.L. and Dong, Q. (2010): Toxicity, uptake kinetics and behavior assessment in zebrafish embryos following exposure to perfluorooctanesulphonicacid (PFOS). Aquat. Toxicol.,

98, 139-147.
International Conference on Harmonization (1994): Note for guidance on the detection of toxicity to reproduction for medicinal products and toxicity to male fertility (CPMP/ICH/386/95).
Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B. and Schilling, T.F. (1995): Stages of embryonic development of the zebrafish. Dev. Dyn., **203**, 253-310.
Kyowa Hakko Kirin Co., Ltd. (2017): Drug interview form of Depaken®
Lombardo, F., Shalaeva, M.Y., Tupper, K.A. and Gao, F. (2001): ElogD(oct): a tool for lipophilicity determination in drug discovery. 2. Basic and neutral compounds. J. Med. Chem., **44**, 2490-2497.
OECD. (1992): OECD guidelines for testing of chemicals. No. 203, Fish, Acute Toxicity Test.
OECD. (2012): OECD guidelines for testing of chemicals. No. 305, Bioconcentration in Fish: Aqueous and Dietary Exposure.
Piersma, A.H. (2004): Validation of alternative methods for developmental toxicity testing. Toxicol. Lett., **149**, 147-153.
Sanz-Landaluze, J., Pena-Abaurrea, M., Muñoz-Olivas, R., Cámara, C. and Ramos, L. (2015): Zebrafish (*Danio rerio*) eleutheroembryo-based procedure for assessing bioaccumulation. Environ. Sci. Technol., **49**, 1860-1869.
Selderslaghs, I.W., Van Rompay, A.R., De Coen, W. and Witters, H.E. (2009): Development of a screening assay to identify teratogenic and embryotoxic chemicals using the zebrafish embryo. Reprod. Toxicol., **28**, 308-320.
Selderslaghs, I.W., Blust, R. and Witters, H.E. (2012): Feasibility study of the zebrafish assay as an alternative method to screen for developmental toxicity and embryotoxicity using a training set of 27 compounds. Reprod. Toxicol., **33**, 142-154.
Yamashita, A., Inada, H., Chihara, K., Yamada, T., Deguchi, J. and Funabashi, H. (2014): Improvement of the evaluation method for teratogenicity using zebrafish embryos. J. Toxicol. Sci., **39**, 453-464.