

Toxicokinetics of Domoic Acid (DA) in Pregnant and Non-pregnant Mice  
After Repeated Oral Administrations

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A thesis  
submitted in partial fulfillment of the  
requirements for the degree of

Master of Science

University of Washington

2013

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Program Authorized to Offer Degree:  
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**ABSTRACT**

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Domoic acid (DA), a neurotoxin produced by harmful algal blooms of *Pseudo Nitzschia*, has been a public health concern. Developmental exposures to DA are believed to result in brain alterations and behavioral disturbances that may persist into adulthood. Therefore, consumption of DA contaminated seafood by pregnant women is particularly concerning as it could affect the neurodevelopment of a developing fetus. In this study, pregnant and non-pregnant C57BL/6 mice were exposed to DA for 8 consecutive days (from gestational days 10 to 17) via oral gavage. Toxicokinetic data were obtained by quantifying DA concentrations in maternal and fetal plasma, maternal and fetal brain (limit of quantification (LOQ) of 0.25 ng/g tissue), and amniotic fluid (LOQ of 0.5 ng/mL) with liquid chromatography-mass spectrometry (LC/MS) multiple reaction monitoring (MRM). Non-pregnant mice were treated with 1, 3, 5, or 15 mg/kg DA repeatedly, where 5 and 15 mg/kg caused neurotoxicity and mortality. The highest plasma concentrations found in non-pregnant mice at 1, 3, 5, and 15 mg/kg were 6, 19, 34, and 166 ng/mL, respectively. Pregnant mice were treated with maternal non-symptomatic dose of 1 or 3 mg DA/kg. As dose of DA increased, the DA absorption rate for both pregnant and non-pregnant mice increased, and a higher absorption rate was observed in pregnant mice plasma when comparing pregnant and non-pregnant mice treated with same dose of DA. Using a combined model, we found that pregnant and non-pregnant mice have redistribution rate (95% CI) of 3.8 (3.3, 4.3) per hour, uptake rate (95% CI) of 1.9 (0.9, 3.8) per hour, and  $T_{\text{cmax}}$  of 1.2 hours after the last dose. In fetal units, fetal brain and amniotic fluid had significant accumulations over time after last dose on GD17. Fetal plasma showed increase, but without any significance.

Based on results from our *in vivo* study, a maternal-fetal PBTK model for DA was then developed to evaluate potential links between chronic, low-level DA exposure and relative health impacts during pregnancy. The developed PBTK model illustrated that the concentration of DA in maternal blood increases and peaks around 1 hour after last dose on GD17 while DA is retained in fetal brain and amniotic fluid over time after last dose on GD17. Most of the DA was excreted over time based on our developed PBTK model.

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## ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my advisor Prof. Elaine M. Faustman for her support, patience, guidance, and motivation. Besides my advisor, I would like to thank the rest of my thesis committee: Prof. Thomas M. Burbacher and Dr. William Griffith, for their encouragement and insightful comments.

I share the credit of my work with Motohiro Shiotani and would like to thank him for being organized, supportive, and cooperative. I am indebted to my fellow students and colleagues in Faustman lab group: Sungwoo Hong, Susanna Wegner, Sean Harris, Hee Yeon Kim, and undergraduate student helpers and research assistants, for working together, stimulating invaluable discussions. I would also like to thank the Institute for Risk Analysis and Risk Communication (IRARC) group: Eric Vigoren, Keh Duong, Tomomi Workman, Alison Scherer, Alison Laing, Sara Pacheco, Marissa Smith, for all their help and support.

Lastly but not the least, I cannot find words to express my gratitude to my parents and family for their continuous love and support.

This work has been supported by the Pacific Northwest Center for Human Health and Ocean Studies (NIH/IEHS: P50 ES012762 and NSF: OCE-0434087, OCE-0910624, and 1128883).

## CHAPTER 1: INTRODUCTION AND BACKGROUND

### 1.1 DOMOIC ACID (DA)

#### 1.1.1 DA in the Environment

Recently, the number and frequency of harmful algal blooms have been increasing due to variety of reasons. In some cases, harmful algal blooms occur due to increases in nutrients such as phosphorus, nitrogen, and carbon in water from extreme weather events, discharges from industrial and commercial wastewater treatment, and runoff from farms and residential areas (NOAA, 2013).

As harmful algal blooms occur, domoic acid (DA), a neurotoxicant, is produced by the diatom *Pseudo-nitzschia* and released into ocean and estuary settings, contaminating our seafood such as shellfish. Consuming these DA contaminated seafood is known to cause short-term memory loss, brain damage, and death in humans. Numerous experimental studies in rodents and nonhuman primates have also confirmed these observations. Intoxication of DA in humans has increased concerns in public health especially when toxic blooms of the diatom *Pseudo-nitzschia* occur.

#### 1.1.2 Relevance to Human Populations

Of many DA intoxication incidences, a well-known case is from Prince Edward Island in 1987. The outbreak started when government agencies in the Canadian province of New Brunswick and Quebec received reports between November 22 and 24. The reports included three people who had rapid onset of confusion, disorientation, and memory loss within 24 hours of mussel consumption from Prince Edward Island (Perl et al. 1990). Symptoms primarily consisted of gastrointestinal nature such as nausea, vomiting, abdominal cramps, and diarrhea, but severe headache and loss of short-term memory were observed (Perl et al. 1990). In severe cases, seizures, myoclonus, and coma were present. More than 20 people between 20 and 70 years of age were hospitalized for 4 to 101 days (Perl et al. 1990). Studies later found that dietary doses of 0.2-0.3 mg/kg appeared to have no observable effects while 0.9-2.0 and 1.9-4.2 mg/kg doses caused mild gastrointestinal (GI) problems and confusion, disorientation and seizures respectively during this event (Lefebvre and Robertson 2010; Marien 1996). Furthermore, 12 of the patients had severe anterograde memory deficits and clinical and electromyographic evidence of motor or motor sensory neuropathy after several months later, and four people died within four months of mussel ingestion (Teitelbaum et al. 1990).

Neuropathological studies of these four people revealed that neuronal necrosis or loss and astrocytosis were present mainly in the hippocampus and the amygdala (Teitelbaum et al. 1990). This was a first incidence where the effects of DA were recognized.

Since the outbreak in Prince Edward Island, many more cases of intoxication linked to the presence of DA have been reported including Washington State. In Washington State, DA has been detected in razor clams along the Washington coast since 1991 (Horner et al. 1989; Wekell et al. 1994) and at a shellfish farm in Penn Cove, Puget Sound in 1997 (Trainer et al. 2007). Because people in coastal areas consume high quantities of seafood compared to inland people and Puget Sound is home to diverse community of people including tribal nations, Asian, and Pacific Islanders, people in Puget Sound may be at greater risks from DA contaminated seafood.

Currently, guidelines for human public health have been implemented to limit DA contamination with federal regulatory limit of 20  $\mu\text{g}$  DA/g shellfish tissue (Lefebvre and Robertson 2010; Marien 1996). In the United States, the estimated human tolerable daily intake (TDI) level of DA in clams is 19.4  $\mu\text{g}$  DA/g shellfish tissue (Marien, 1996). Unfortunately, those regulatory values such as acute reference dose (ARfD) and TDI have reflected only adults' conditions. Concerns still remain because fetuses, infants, and/or children may be more susceptible than the adults.

### 1.1.3 Effects of DA on Behaviors

DA has been observed to damage not only humans who consume DA contaminated seafood, but also other animals such as rats, sea lions, and pelicans (Maucher et al. 2011) by producing neurotoxicity and/or inducing behavior changes. Some behavioral symptoms of DA poisoning include scratching, ataxia, and seizure (Tasker et al. 1991; Tryphonas et al. 1990a; Tryphonas et al. 1990b; Maucher et al. 2011). In *in vivo* experiments done by Shiotani et al. (unpublished data), chronic, low-level *in utero* DA exposed offspring demonstrated both significant dose- and sex-specific neurobehavioral effects that persisted into adulthood in several neurobehavioral measures including anxiety in elevated plus maze, walking patterns in CatWalk, home-cage behaviors, and memory in Morris water maze.

Furthermore, changes in behaviors were also observed not only in experimental settings, but also in marine animal populations due to their natural exposures to DA after harmful algal blooms of *Pseudo Nitzschia*. One of the famous incidences of suspected DA poisoning in marine animals are the aggressive behavior of sea birds in 1961 (Trabing, 1961), where they attacked humans. California sea lion populations also have been a recurrent problem as they are as sensitive to DA as humans

(Gulland et al. 2002). Table 1.1 lists some of the examples of shellfish, birds, marine mammals, and fish that contained DA by natural exposures and regions where they occur. As Table 1.1 illustrates, DA is found in various organisms, and USA is not the only region affected by DA released into the environment.

| Table 1.1 Examples of Shellfish, Birds, Marine Mammals, and Fish Containing DA by Natural Exposures |   |   |
|---|---|---|
| Organism  | Region  | Reference Study   |
| <b>Shellfish</b>  |   |   |
| Blue Mussel   | Prince Edward Island, Canada<br>Washington, USA | Bates et al. (1989), Smith et al. (1991)  |
| Razor Clam  | Portugal<br>Washington, USA                     | Trainer et al. (2008)   |
| <b>Birds</b>  |   |   |
| Brown pelican   | California, USA<br>Cabo San Lucas, Mexico       | Work et al. (1993)<br>Sierra-Beltran et al. (1997)                                |
| <b>Marine Mammals</b>   |   |   |
| Humpback whale  | California, USA                                 | Lefebvre et al. (2002)  |
| Bottlenose dolphin  | Sonora, Mexico                                  | Sierra-Beltran et al. (2004)  |
| California sea lion   | California, USA                                 | Lefebvre et al. (1999), Scholin et al. (2000)                                     |
| <b>Fish</b>   |   |   |
| European sardine  | Cabo San Lucas, Mexico<br>Portugal              | Sierra-Beltran et al. (1997)<br>Vale and Sampayo (2001), Costa and Garrido (2004) |
| Pacific Halibut   | California, USA                                 | Vigilant and Silver (2007)  |

Table 1.1 Example list of shellfish, birds, marine mammals, and fish that contained DA due to its natural exposures. This table was modified from Landsberg (2002) and Lefebvre and Robertson (2010).

#### 1.1.4 DA Properties and Mechanism of Toxicity

As described above, DA is known to alter behaviors in experimental animals and marine organisms. Then, how does DA cause neurotoxicity and change behaviors? DA is known to be a structural analog of kainic acid (KA) and excitatory amino acids such as glutamic acid and aspartic acid (Lefebvre and Robertson, 2010).

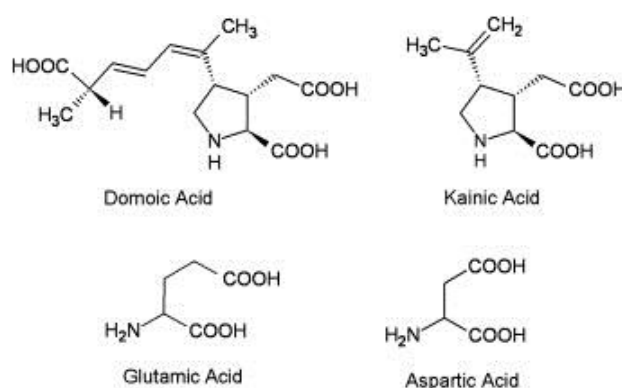


Figure 1.1 Structure of DA and structurally similar compounds such as kainic acid, glutamic acid, and aspartic acid. This figure was adopted from Lefebvre and Robertson (2010).

DA can produce neurotoxicity by activating the ionotropic glutamate receptors on nerve cell terminals by interacting with the glutamate receptor or co-activate the subtypes of  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole (AMPA), kainite, and *N*-methyl-D-aspartate (NMDA) receptors (Hampson et al. 1992; Hampson and Manalo, 1998; Larn et al. 1997; Tasker et al. 1996). It is also known that DA has higher affinity to kainite receptors than kainic acid as shown in previous studies (Zaczek and Coyle, 1982; Costa et al. 2010). Activation of glutamate receptors triggered by DA results an increase in intracellular  $Ca^{2+}$  influx into neurons, causing the release of glutamate, which in turn activates NMDA receptors and promotes neurotoxicity (Berman et al. 2002; Giordano et al. 2006). Excess glutamate, the principal excitatory neurotransmitter in the brain, may cause adverse health effects in humans such as neurodegeneration, seizures, and apoptosis (Zaczek and Coyle, 1982; Doble, 1995; Nijjar and Nijjar, 2000; Colman et al. 2005; Giordano et al. 2007; Giordano et al. 2008).

#### 1.1.5 Susceptible Population to DA

In case of the Prince Edward Island event, four mortalities were over 70 years of age (Perl et al. 1990) and older individuals were more likely to suffer memory loss, suggesting that age is another important factor for DA-induced neurotoxicity. One proposed reason why older populations are more vulnerable is that decreased antioxidant defenses in brain of aged individuals may contribute to increased susceptibility. *In vivo* studies also suggest that aged rat brains have less glutathione and glutamate-cysteine ligase (GCL) activity (Liu et al. 2002; Kaur et al. 2002).

However, not only aging but also developing individuals (*in utero* or in early postnatal period) may be more susceptible to neurotoxicity after DA exposure especially since brains develop dramatically throughout the pregnancy and even after birth. Thus, *in utero* exposures are critical windows of exposure for brain development. It has been also suggested that low serum clearance may be a predisposing factor to the high susceptibility of neonates to DA toxicity (Xi et al. 1997) in addition to the lack of completely developed blood brain barrier, which allows DA to enter freely into the central nervous system (CNS). Antioxidant defense mechanisms are also low in the embryonic and early postnatal period. Therefore, developing fetuses and neonates are susceptible populations that need to be protected from DA exposures.

Another factor that may affect sensitivity to DA effects is renal clearance. For DA, renal clearance, by glomerular filtration (Suzuki and Hierlihy, 1993) is the main route of elimination for serum clearance

(Costa et al., 2010). Marien et al. based their calculations on effective renal clearance but age and disease related changes in clearance could also contribute observed population differences (Scherer et al. 2008).

#### 1.1.6 Summary

After the effects of DA are recognized in Prince Edward Island in 1987, the mechanisms of toxicity for DA have been studied. However, current guidelines for DA still only reflect the adult responses, without considering susceptible populations. To this end, basic information on DA and why it is important to study DA were stated in this thesis.

### 1.2 TOXICOKINETICS

#### 1.2.1 DA Toxicokinetics

Toxicokinetics (TK) is used to describe absorption, distribution, metabolism, and excretion (ADME) of a compound in the body. By studying toxicokinetics, relationships between experimental exposures and actual exposures relative to human can be established. Unfortunately, toxicokinetics of DA exposures relative to humans is not as well characterized as previous studies have focused on exposure routes and patterns with less human relevant exposure pathways. Current understandings on toxicokinetics of DA are:

*Absorption.* After oral administration, which is most relevant route of exposure for humans, DA is not absorbed well (Costa et al. 2011).

*Distribution.* Because most of administered DA is excreted by feces (Suzuki and Hierlihy, 1993), only a small amount of DA is distributed to different organ systems in the body. Since it is a hydrophilic compound (Suzuki and Hierlihy, 1993), it will be poorly permeable to other organ systems.

*Metabolism.* DA has four  $pK_a$  values in the water: 2.10, 3.72, 4.93, 9.82. Because biological environments such as blood and amniotic fluid have a pH of about 7.35-7.45 and 7.0-7.5, respectively, DA will not dissociate as much at a given pH. Moreover, DA is a hydrophilic compound that undergoes limited metabolism according to Suzuki and Hierlihy (1993). Therefore, DA is unlikely to cross barriers including the blood-brain barrier (BBB).

*Excretion.* DA is known to be mainly excreted by feces. Previous studies done by Truelove et al. (1996, 1997) observed only about 2% and 4-7% excreted by urine in rats and non-human primates, respectively upon oral administration. Moreover, about 75% of administered dose are excreted unchanged according to Suzuki and Hierlihy (1993).

### 1.2.2 Maternal-Fetal Toxicokinetics for DA

To date, only a few toxicokinetics studies on DA have been done, and only one study has been published by Maucher Fuquay et al. (2010) regarding toxicokinetics of DA for both maternal and fetal compartments. Maucher Fuquay et al. (2010) investigated the toxicokinetic effects of DA on pregnant Sprague Dawley rats and their fetuses after a single dose of 1 mg DA/kg via intravenous (I.V) injection on gestational day (GD) 20.

DA is found to cross the placental barrier with the maximum concentration ( $C_{max}$ ) of 752 ng/mL and half-life of 577 minutes when intravenous dose of 1 mg DA/kg was given to pregnant rats on gestational day (GD) 20 (Maucher Fuquay et al. 2010). In that study, DA was also detected in amniotic fluid (averages of 27 ng/mL), fetal brain tissue (average of 8.12 ng/g), and fetal plasma with  $C_{max}$  of 86 ng/mL at 60 minutes and a terminal half-life of 553 minutes was identified (Maucher and Ramsdell 2007; Maucher et al. 2012). They also found that amniotic fluid and fetal brain tissue did not show evidence of elimination over 24 hours of DA treatment. Even though an intravenous dose of 1 mg DA/kg can be considered extremely high exposure compared to the level of shellfish consumption in human (Tsuchiya et al. 2008), this study suggests that a fetus may be continually re-exposed during gestation due to longer fetal retention of DA especially in amniotic fluid.

### 1.3 SUMMARY

The diatom *Pseudo Nitzschia* produces DA, a neurotoxicant, and releases into marine environment. As the frequency and geographic area of DA-producing harmful algal blooms are increasing, it is becoming important public health concerns for susceptible populations and sub-populations with high seafood consumption. However, current federal regulatory limit of 20 µg DA/g shellfish tissue has reflected on adults' conditions. Therefore, susceptible populations, specifically pregnant women and developing fetuses need to be considered to better protect them from the adverse health effects of DA.

As mentioned above, previous DA toxicokinetic studies have focused on exposure routes and patterns with less human relevant exposure pathways and with only single exposures. Thus, it is important to define maternal and fetal toxicokinetic parameters after chronic, low-level DA dose to potentially link health impacts with chronic, low-level DA exposures.

#### 1.4 HYPOTHESIS AND SPECIFIC AIMS

The goal of this project was to evaluate potential links between exposure and relative health impacts associated with chronic, low-level DA exposure during pregnancy. Differences in plasma DA concentration of pregnant and non-pregnant mice after giving repeated doses of DA via oral gavage for 8 days were determined as were DA concentrations in maternal and fetal plasma, maternal and fetal brain, and amniotic fluid. Consumption of DA contaminated seafood is one route of potential concern, especially for pregnant women as DA exposure could affect the neurodevelopment of a developing fetus and developmental exposures to DA is believed to result in brain development alterations and behavioral disturbances that may persist into adulthood. The hypothesis of the study was that DA accumulates in pregnant mice and their fetuses after repeated oral dose of DA for 8 days.

Specific aims for this study are:

- To evaluate if there is any difference in plasma and brain DA concentration between pregnant and non-pregnant mice after repeated oral dose of DA for 8 days;
- To quantify DA concentrations in fetal plasma, fetal brain, and amniotic fluid after repeated oral administration of DA to pregnant mice for 8 days; and
- To develop a DA physiologically-based toxicokinetic (PBTK) model for maternal-fetal compartments after repeated DA exposures.

**CHAPTER 2:**  
**TOXICOKINETICS OF DOMOIC ACID IN PREGNANT AND NON-PREGNANT MICE**

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## 2.1 INTRODUCTION

To date, *in vivo* toxicokinetic studies that have been published for DA exposure focused on exposure routes and patterns that are less human relevant and with only single exposures. Also, previous studies focused on the late developmental stages of a fetus, not on chronic, low-dose exposure during early stages of development.

We have designed a study to improve our understanding of oral DA exposure and its relative health effects in pregnant mice and their fetuses. Before we further investigate potential links between adverse health effects and chronic, low-level DA exposures, we first studied the toxicokinetics of DA in pregnant and non-pregnant mice after repeated oral dosing. This allowed us to examine if there is any difference in toxicokinetics between pregnant and non-pregnant mice, and if so, how they are different. Then, toxicokinetic differences among fetal compartments such as fetal plasma, brain, and amniotic fluid were determined, which may help us to link potential health concerns associated with chronic, low-level DA exposure in humans, especially for developing fetus during pregnancy.

## 2.2 METHODS

### 2.2.1 Animals

Timed-pregnant C57BL/6 mice (Charles River, CA) were housed in a centralized, AAALAC-accredited, Specific Pathogen Free facility at the University of Washington, and were maintained in a 12-hour light-dark cycle with unlimited access to food and water. For pregnant mice, the day when a positive vaginal plug was observed was counted as day 0 of gestation; however, because the actual pregnancy was not distinguished at early gestational day (GD), plug-positive animals were delivered (GD8 at arrival) and were randomly divided into different dosing groups (n=8 per group). Animals were then allowed to acclimate for 48 hours prior to experimental manipulation. All experiments were approved by the University of Washington Institutional Animal Care and Use Committee and carried

out in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals, as adopted by the National Institutes of Health.

### 2.2.2 Treatment

Various doses of DA (Sigma-Aldrich, St. Louis, MO; 1, 3, 5, 15 mg/kg body weight) were dissolved in 150  $\mu$ L of sterile distilled water where 1-5 mg/kg BW is environmentally relevant human exposures based on our current understanding (Iverson et al., 1989). We have used 5 and 15 mg/kg DA in our pilot study based on the results from Truelove et al. (1996) and Iverson et al. (1989): Truelove et al. (1996) found no significant findings in clinical and histopathological examinations after dosing rats with 0.01 and 5 mg/kg DA for 64 days by gavage, and Iverson et al. (1989) found no clinical symptoms after giving female CF1 mice a single oral dose of 20 and 28 mg/kg of DA. DA solution was orally dosed once a day for 8 consecutive days in non-pregnant female mice to assess the level of chronic DA exposure toxicities. Although previous studies done by Truelove et al. (1996) and Iverson et al. (1989) did not find any clinical symptoms, we found that at the highest dose of DA tested (15 mg/kg/day), 2 out of 4 females developed severe paralysis of legs at 3 hours after the 5<sup>th</sup> administration, and another female was found dead on the 7<sup>th</sup> day morning accompanied by a significant loss of body weight. At 5 mg/kg, 1 out of 4 females showed abnormal walking after 6<sup>th</sup> administration. Thus, we decided to use non-symptomatic dose levels of DA (1 and 3 mg/kg) in the subsequent pregnant mice study.

A dose of 1 or 3 mg/kg was administered via oral gavage to pregnant mice on gestational day (GD) 10 through 17. DA was dissolved in sterile distilled water such that each animal is administered with a volume of 150  $\mu$ L. The dosing solutions of 1 or 3 mg/kg with a volume of 150  $\mu$ L were prepared daily based on individual body weights from the morning of the experimental day. A maternal blood collection method was optimized so that we withdraw blood from all mice (3 dams per dose level and all fetal units per dam) at specific time points after maternal exposures to DA. For each mouse, a total of 50  $\mu$ L of blood were withdrawn at 4 different time points because only 200  $\mu$ L of blood could be withdrawn per week due to concerns with animal health. Brains were harvested after sacrificed by CO<sub>2</sub> asphyxiation. Pups were removed via caesarian section in order of proximity to the birth canal alternating between right and left uterine horns. Exsanguinations of prenatals were performed transcatheterially. Maternal/fetal blood, maternal/fetal brain, and amniotic fluid samples were harvested at 1, 5, and 24 hours after dosing on GD17. Blood had an extra time point at 1 hour post-dose on GD10.

### 2.2.3 Sample Harvest

Blood collection schedule was optimized to collect at specific time points after maternal exposure. Dams' blood was collected from saphenous vein at 1, 5, or 24 hours post-dose on GD17, immediately before the animals were sacrificed. Fetal blood was pooled from all fetal units for each respective litter at 1, 5, or 24 hours post-dose on GD17. Collected blood was transferred into a BD Microtainer® tube with Lithium Heparin (BD Biosciences, Franklin Lakes, NJ) to prepare samples for liquid chromatography-mass spectrometry (LC/MS).

Each maternal and fetal brain was removed after the animal was sacrificed by CO<sub>2</sub> asphyxiation at 1, 5, or 24 hours after last dosing on GD17. Harvested brains were individually snap frozen in dry ice and maintained at -80 °C. For maternal brain, only the left half of the brain was used for LC/MS samples, and the other half (right) was saved for tissue examination in the future.

Amniotic fluid was collected from individual fetal sac using a 23×1" gauge BD Eclipse™ Hypodermic needle (BD Biosciences, Franklin Lakes, NJ) connected to a 1mL syringe (BD Biosciences, Franklin Lakes, NJ) at 1, 5, or 24 hours post-dose on GD17. Collected amniotic fluid was then transferred to a microcentrifuge tube for storage at -80 °C.

### 2.2.4 LC/MS Sample Preparation

Twice a volume of 100% methanol (2X initial volume) with an internal standard of kainic acid (KA), a related glutamate receptor binding compound, was added to maternal and fetal blood samples. Volume was centrifuged at 16,300×g for 5 minutes (Spectrafuge™ 24D digital microcentrifuge, Labnet International, Inc., Woodbridge, NJ). The supernatants of plasma samples were diluted with equal volume of water and were stored at -80 °C until LC/MS analysis.

DA was extracted from maternal and fetal brains based on the reference paper protocol by Maucher and Ramsdell (2007). Harvested maternal and fetal brains were removed from microcentrifuge tubes and homogenized in four times volume of Dulbecco's phosphate buffered saline (Gibco® Life Technologies, Grand Island, NY) with 10% methanol and 0.05% Tween® 20. Tissues were then extracted by adding 3X sample buffer volume of 50% methanol in water and homogenized with a homogenizer (Brinkmann Instruments, Inc., Westbury, NY). After spiking brain samples with KA, extracts were centrifuged at 3000×g for 15 minutes (Spectrafuge™ 24D digital microcentrifuge, Labnet International, Inc., Woodbridge, NJ). Unlike the extracted maternal brain supernatants that

were removed and diluted with equal volume of water, the fetal brain samples were not diluted with equal volume of water to increase the detection and sensitivity in LC-MS. Samples were stored at  $-80\text{ }^{\circ}\text{C}$  until LC/MS analysis.

Amniotic fluid was added 2X volume of 100% methanol, which included KA as an internal standard, and centrifuged at  $3,000\times g$  for 10 minutes (Spectrafuge™ 24D digital microcentrifuge, Labnet International, Inc., Woodbridge, NJ). The supernatants were collected and diluted with equal volume of water. Amniotic fluid samples were stored at  $-80\text{ }^{\circ}\text{C}$  until LC/MS analysis.

### 2.2.5 LC/MS Analysis

DA was quantified in maternal/fetal plasma, maternal/fetal brain, and amniotic fluid using a Triple Quad LC/MS 6460 QQQ (Aligent Technologies Inc., Palo Alto, CA). LC separations were done with a Thermo Scientific \*Hypersil GOLD\* HPLC Columns (Thermo Scientific),  $1.9\text{ }\mu\text{m}$  particle size, with a C18 SecurityGuard Cartridges (4 x 2.0 mm; Phenomenix) using a gradient of water (A) and methanol (B), with 0.1% formic acid (FA) as an additive. LC gradient, at a flow rate of 0.25 mL/min, was as follows: starting % of 20% B held for 2 minutes, next increased it to 100% over the next 3 minutes, 100% B held for 1 minutes, then back to starting condition in 0.1 minutes, and allowed to re-equilibrate for an additional 2 minutes before the next injection. The injection volume was 5  $\mu\text{L}$ , except for the brain samples, in which the volume was increased to 10  $\mu\text{L}$ . The LC eluent was sent to waste except for the 8 minutes window bracketing the retention time of DA that was sent to the MS. The detection of DA and KA (internal standard) by MS was achieved by multiple reaction monitoring (MRM; DA  $m/z$  312.2  $\rightarrow$  161.1, 133.1; KA  $m/z$  214.1  $\rightarrow$  168.1, 122.1) in positive ion mode. Analyte of interest was acquired on the MS system from 1.5 to 5 minutes. Detected DA and KA in maternal/fetal plasma, maternal/fetal brain, and amniotic fluid were analyzed using Aligent MassHunter Qualitative Analysis Program (Aligent Technologies Inc., Palo Alto, CA; v. B. 04.00) and were quantified with Data Acquisition for Triple Quad (Aligent Technologies Inc., Palo Alto, CA; v. B. 04.01).

## 2.3 RESULTS

### 2.3.1 Pregnant vs. Non-Pregnant Mice DA Concentrations

In plasma from pregnant and non-pregnant mice, no accumulation of DA was observed over time (hours after the last dose) after chronic, low-level exposures to DA (Figure 2.1). When looking at 1 and 3 mg/kg in Figure 2.1, pregnant mice plasma had higher level of DA than non-pregnant mice.

## Plasma DA Concentration in Pregnant and Non-pregnant Mice

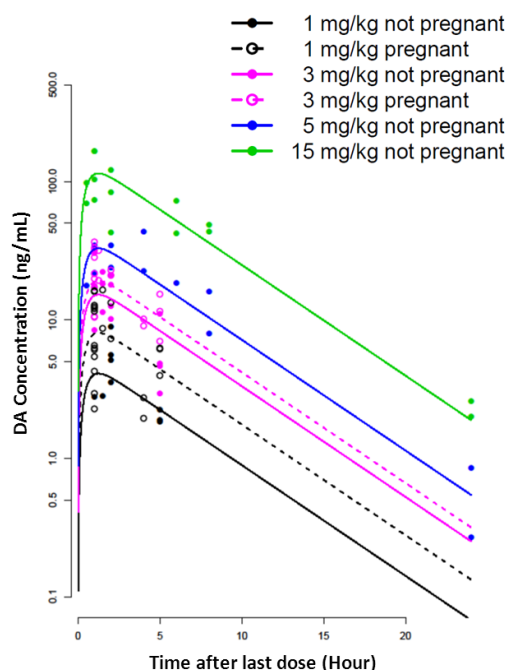


Figure 2.1 Graph of DA concentration (ng/mL) in plasma of pregnant and non-pregnant mice in function of time (hours after the last dosing). In this figure, solid lines (—) or filled circles (●) represent non-pregnant mice while dashed lines (---) or open circles (○) represent pregnant mice. Colors represent different doses: black = 1 mg/kg, pink = 3 mg/kg, blue = 5 mg/kg, and green = 15 mg/kg. Note that the DA concentration is not adjusted by the gravid weight.

To further characterize the absorption of DA, absorption parameters were calculated for each dose group and summarized in Table 2.1. As dose increased, the absorption rate increased for both pregnant and non-pregnant mice. When comparing pregnant and non-pregnant mice that were treated with same doses, pregnant mice showed increased absorption rates, which was consistent with Figure 2.1. However, since DA concentrations in pregnant mice plasma were not adjusted by gravid weight, the differences between pregnant and non-pregnant mice plasma might be due to pregnancy.

We found that pregnant and non-pregnant mice have following rate parameters for DA in plasma using a combined model as it can provide more stable estimates for common redistribution and

uptake rates: Redistribution rate (95% CI) = 3.8 (3.3, 4.3) per hour;

Uptake rate (95% CI) = 1.9 (0.9, 3.8) per hour; and

Time when DA concentrations in plasma is at maximum ( $T_{cmax}$ ) = 1.2 hours after last dose.

Toxicokinetic parameters for pregnant and non-pregnant DA plasma levels are calculated and summarized in Table 2.1.

| <b>Table 2.1 Toxicokinetic Parameters for Pregnant and Non-pregnant Mice Plasma</b> |                        |                                  |                       |
|---|------------------------|----------------------------------|-----------------------|
|   |                        | <b>Pregnant</b>                  | <b>Non-pregnant</b>   |
| <b>Absorption Parameters</b>  | <b>Unit</b>            | <b>Value (95% CI)</b>            | <b>Value (95% CI)</b> |
| <b>1 mg/kg</b>  | ng/mL                  | 11.1 (7.1, 15.0)                 | 5.6 (3.2, 8.0)        |
| <b>3 mg/kg</b>  | ng/mL                  | 26.4 (18.3, 34.5)                | 21.0 (14.3, 27.7)     |
| <b>5 mg/kg</b>  | ng/mL                  | N/A                              | 45.4 (23.9, 66.8)     |
| <b>15 mg/kg</b>   | ng/mL                  | N/A                              | 157.6 (85.9, 229.3)   |
| <b>Combined</b>   |                        | <b>Pregnant and Non-pregnant</b> |                       |
| <b>Rate Parameters</b>  | <b>Unit</b>            | <b>Value (95% CI)</b>            |                       |
| <b>Redistribution</b>   | <b>per hr</b>          | 3.8 (3.3, 4.3)                   |                       |
| <b>Uptake</b>   | <b>per hr</b>          | 1.9 (0.9, 3.8)                   |                       |
| <b>T<sub>cmax</sub></b>   | <b>hours-post dose</b> | Day 7, 1.2 hr                    |                       |

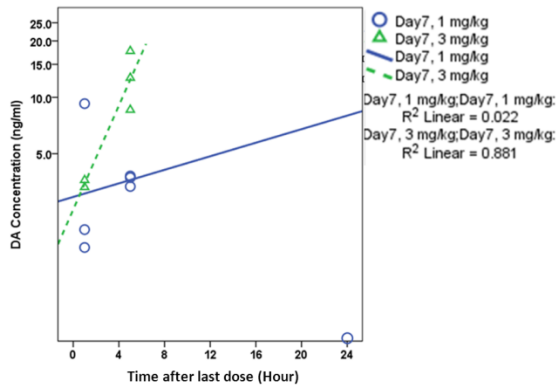
Table 2.1 Toxicokinetic parameters for pregnant and non-pregnant mice plasma. Absorption parameters were separated among different doses for pregnant and non-pregnant mice. For redistribution, uptake rate, and T<sub>cmax</sub>, data were combined to calculate the rate parameters. N/A = Not applicable.

In addition to the plasma of pregnant and non-pregnant mice, we examined their brains at 1, 5, or 24 hours after the last dose. The concentrations of DA detected in maternal brains at 1, 5, or 24 hours after the last dose were above level of detection (LOD) for LC/MS, but below the level of quantification (LOQ). Among non-pregnant mice, one mouse from 3 mg/kg dosing group had DA concentration detected (6.96 ng DA/g tissue) at 1.5 hours after last dose on day 7, but DA was not detected in the 1 mg/kg dosing group. While no data were obtained among non-pregnant mice at 5 and 24 hours after last dose on day 7, the DA concentration in the brains of non-pregnant mice at 24 hours after last dose on day 5 were below level of detection for both 1 mg/kg and 3 mg/kg dosing groups.

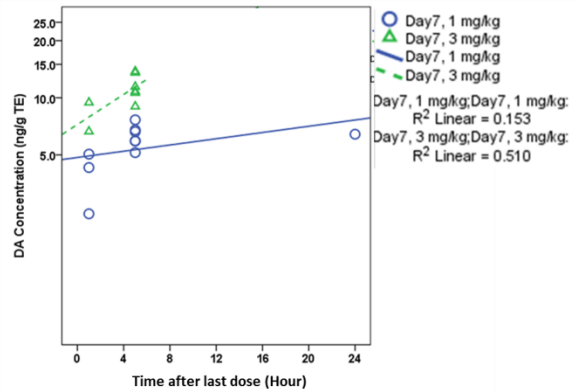
### 2.3.2 Fetal DA Concentrations

After 8 days of repeated oral dosing, pooled fetal plasma, fetal brain, and amniotic fluid showed dose-dependent DA accumulations over time for both 1 and 3 mg/kg dosing groups. Using observed data points, DA concentrations in pooled fetal plasma, fetal brain, and amniotic fluid were plotted in function of time (hours after last dosing), and best fitted lines were drawn to estimate the potential accumulation of DA in these parameters as shown in Figure 2.2.

### A. Pooled Fetal Plasma



### B. Fetal Brain



### C. Amniotic Fluid

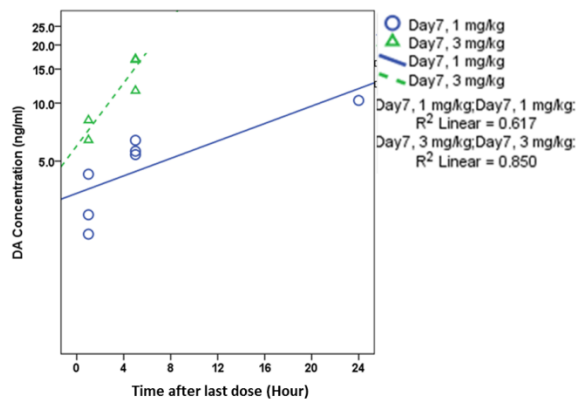


Figure 2.2 Plots of DA concentration (ng/mL or ng/g TE) in pooled fetal plasma, fetal brain, and amniotic fluid in function of time (hours after the last dosing). In this figure, blue circles (○) represent 1 mg/kg dosing group while green triangles (△) represent 3 mg/kg dosing group. Best fitted lines for 1 and 3mg/kg dosing groups were denoted by blue solid (—) and green dashed lines (---), respectively.

After 8 days of repeated maternal oral dosing, pooled fetal plasma, fetal brain, and amniotic fluid showed DA accumulations over time in both 1 and 3 mg/kg dosing groups as shown in Table 2.2. For both 1 and 3 mg/kg maternal dosing groups, fetal brain accumulated slowest with slopes 0.008 +/- 0.007 SE and 0.040 +/- 0.016 SE per hour while fetal plasma and amniotic fluid accumulated at much higher rates (Table 2.2).

In addition to finding slopes and standard error (SE),  $R^2$  values for each fetal compartment were calculated to determine how well data fit the estimated linear lines. While fetal plasma and fetal brain for 1 mg/kg maternal dosing group have lower correlations of 0.022 and 0.153, respectively, some has higher correlations in fetal plasma and amniotic fluid for 3 mg/kg maternal dosing group were observed with  $R^2$  values of 0.881 and 0.850.

| <b>Table 2.2 Fetal Toxicokinetic Parameters</b> |             |                     |                    |              |
|---|-------------|---------------------|--------------------|--------------|
| <b>1 mg/kg</b>                                  |             | <b>Fetal Plasma</b> | <b>Fetal Brain</b> | <b>AF</b>    |
| <b>Parameters</b>                               | <b>Unit</b> | <b>Value</b>        | <b>Value</b>       | <b>Value</b> |
| <b>R<sup>2</sup></b>                            |             | 0.022               | 0.153              | 0.617        |
| <b>Slope</b>                                    | per hr      | 0.018               | 0.008              | 0.023        |
| <b>Standard Error (SE)</b>                      | per hr      | 0.061               | 0.007              | 0.008        |
| <b>3 mg/kg</b>                                  |             | <b>Fetal Plasma</b> | <b>Fetal Brain</b> | <b>AF</b>    |
| <b>Parameters</b>                               | <b>Unit</b> | <b>Value</b>        | <b>Value</b>       | <b>Value</b> |
| <b>R<sup>2</sup></b>                            |             | 0.881               | 0.510              | 0.850        |
| <b>Slope</b>                                    | per hr      | 0.139               | 0.040              | 0.081        |
| <b>Standard Error (SE)</b>                      | per hr      | 0.290               | 0.016              | 0.017        |

Table 2.2 Toxicokinetic parameters for fetal plasma, brain, and amniotic fluid. For both 1 and 3 mg/kg, fetal brain had the lowest slopes while fetal plasma and amniotic fluid have much higher slopes. The R<sup>2</sup> values represent how well the estimated linear regression models fit the data.

Although there was an increase in DA concentration in pooled fetal plasma over time for both dosing groups, no significant effects were found. Unlike pooled fetal plasma, dose and time had significant effects on DA concentration in fetal brain (Two-way ANOVA; p-value < 0.005), and time for amniotic fluid (Two-way ANOVA; p-value < 0.005).

## 2.4 DISCUSSION

DA is a neurotoxicant produced by harmful algal blooms of the diatom *Pseudo-Nitzschia*. Concerns regarding the neurotoxicity of DA still remain, in part because we do not have much understanding on the effects of chronic, low level *in utero* DA exposures. Therefore, this study hypothesized that DA accumulates in pregnant mice and their fetus after repeated oral dose of DA for 8 days. We found that there were no significant differences in plasma and brain DA concentrations between pregnant and non-pregnant mice over time. As dose increased, the DA absorption rate increased for both pregnant and non-pregnant mice. When looking at 1 and 3 mg/kg dosing groups, a higher absorption rate was observed in pregnant mice plasma compared to that in non-pregnant mice. However, since DA concentrations in pregnant mice plasma were not adjusted by gravid weight, the differences between pregnant and non-pregnant mice plasma might be due to pregnancy. To obtain more stable estimates for common uptake and redistribution rates, a combined model was used to fit plasma concentration. From this, we found that pregnant and non-pregnant mice have redistribution rate (95% CI) of 3.8 (3.3, 4.3) per hour, uptake rate (95% CI) of 1.9 (0.9, 3.8) per hour, and T<sub>cm<sub>ax</sub></sub> of 1.2 hours after lase dose.

Brain DA levels were also not significant between pregnant and non-pregnant mice. The levels were either not detected or so low that it was above the LOD, but below the LOQ. This result was consistent with the previous understanding that DA is a hydrophilic compound and would not be permeable to cross BBB (Chapter 1.2.1).

In fetal plasma, brain, and amniotic fluid, DA levels in each compartment increased over time (hours after last dose on GD17) for both maternal dosing groups of 1 and 3 mg/kg, suggesting that DA is able to cross placenta. Developing fetuses may also have increased exposures to DA compared to pregnant moms due to accumulations of DA in fetal plasma, brain, and amniotic fluid. The increased in DA concentrations in fetal plasma was not significant, but fetal brain and amniotic fluid DA concentrations increased significantly in a dose and time dependent manner (Two-way ANOVA;  $p$ -value  $< 0.005$ ). This significant accumulation observed in fetal brain and amniotic fluid was consistent with the study done by Maucher Fuquay et al. (2012) which found that fetal brain and amniotic fluid did not show the evidence of elimination after 24 hours of a single maternal intravenous (IV) dose of 0.5 and 2 mg/kg in rats. Although intravenous doses of 0.5 and 2 mg/kg are considered high relative to human exposures, we both found that there may be significant accumulation in fetal brain and amniotic fluid with no evidence of elimination after 24 hours of the last dose (after single IV dose in case of Maucher Fuquay et al., 2012). However, only limited comparison could be made due to species differences and dissimilar exposure routes and patterns between our study and the study done by Maucher Fuquay et al. (2012).

In our related neurobehavioral study, we found dose- and sex-related neurobehavioral effects in several measurements such as acoustic startle response, walking patterns, home-cage behavior, and Morris water maze after chronic, low-level *in utero* DA exposures. These neurobehavioral effects may be explained by the differences in DA concentrations between maternal and fetal compartments, especially in brains since DA is similar to KA and other excitatory amino acids and can result excitotoxicity by activating sub-types of glutamate receptors (Chapter 1). For example, very low to no DA concentration was observed in pregnant mice brains while fetal brain samples had significant increase in their DA level over time after the last dose on GD17. However, one should consider that the DA concentrations in maternal-fetal compartments in the kinetic study were not stratified by sex. Further studies are necessary to validate results from the kinetic study and to potentially link results from kinetic study to the ones from neurobehavioral study.

## 2.5 FUTURE DIRECTIONS

Although this study will add significant new information to the literature, additional work can also be done. Following are some suggestions:

1. Investigating the potential variability of accumulated DA concentration in fetuses at different position of uterus
2. Link results from our toxicokinetics study to the neurobehavioral studies after chronic, low-dose exposures to pregnant mice, hoping to explain some observed changes in neurobehavioral tests using kinetics data

## 2.6 CONCLUSIONS

In summary, there was no accumulation of DA in plasma of pregnant and non-pregnant female mice after chronic, low-dose exposures (1 and 3 mg/kg BW). The DA absorption rates for both pregnant and non-pregnant mice increased as dose of DA increased. When looking at pregnant and non-pregnant mice treated with same dose of DA, pregnant mice plasma showed a higher absorption rate than non-pregnant mice. However, it is important to note that the differences between pregnant and non-pregnant mice plasma might be due to pregnancy since DA concentrations in pregnant mice plasma were not adjusted by gravid weight. Based on the combined model, pregnant and non-pregnant mice have redistribution rate of 3.8 per hour with 95% confidence intervals (3.3, 4.3) per hour and uptake rate of 1.9 per hour with 95% confidence intervals (0.9, 3.8) per hour.  $T_{cmax}$  occurred at 1.2 hours after the last dose.

In pregnant maternal brains, DA concentrations were too low to quantify, but were detectable with LC/MS. For non-pregnant mice brain, 6.96 ng of DA was detected per a gram of brain tissue after 1.5 hours post last dose on day 7. However, this value is a single point. We have not obtained DA concentration in non-pregnant mice's brains at 5 and 24 hours after last dose on day 7. DA concentration in non-pregnant mice's brains at 24 hours after dose on day 5 was below LOD for both 1 and 3 mg/kg groups. To confirm the results obtained from pregnant and non-pregnant mice's brains, further validation needs to be done.

Developing fetuses may have higher exposures to DA than pregnant moms due to accumulations of DA in fetal plasma, fetal brain, and amniotic fluid after 8 days of repeated, maternal, non-symptomatic oral dosing. DA accumulations in fetal plasma, fetal brain, and amniotic fluid were observed over time in both 1 and 3 mg/kg dosing groups. Dose and time had significant effects on DA concentration in fetal brain and amniotic fluid (Two-way ANOVA; p-value < 0.005).

## CHAPTER 3:

### A MATERNAL-FETAL PHYSIOLOGICALLY-BASED TOXICOKINETIC MODEL FOR DOMOIC ACID

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Thomas M. Burbacher, Lucio G. Costa, Elaine M. Faustman

#### 3.1 INTRODUCTION

Physiologically-based toxicokinetic (PBTK) models are used to mathematically estimate and characterize the absorption, distribution, metabolism, and excretion (ADME) of a compound in the whole body. Even though PBTK models were first developed in the pharmaceutical field, many different fields including pharmaceuticals and risk assessment utilize PBTK models these days. Compartments in PBTK models represent key organ systems in the body, with blood or lymph flows as interconnections.

It is important to develop a PBTK model for DA because of its potential application for risk assessment, especially for populations such as Olympic Peninsula Tribes in Washington State or coastal populations who are likely to have chronic, low-level dietary exposures to DA during pregnancy. Therefore, in this study, a multi-compartment PBTK model was used to estimate the DA concentrations in maternal and fetal units after repeated, non-symptomatic maternal, low-dose exposures to DA.

#### 3.2 PBTK MODEL DESIGN

##### 3.2.1 Maternal-Fetal PBTK Model Framework

Based on the results obtained from Chapter 2, the DA PBTK model framework for maternal-fetal compartments was derived (Figure 3.1), illustrating how the DA may flow from one compartment to another in maternal and fetal unit. The starred ones are the measured parameters from Chapter 2. When we gave oral dosages of 1 or 3 mg of DA /kg to pregnant mice, it went to gastrointestinal (GI) tract, where most of them would be excreted by feces. DA would also be in maternal blood, then distributed to different maternal compartments including maternal kidney (where DA can be excreted by urine), brain, and placenta. Once it crosses the placenta, DA may be distributed and accumulate in fetal units such as fetal blood, brain, and amniotic fluid.

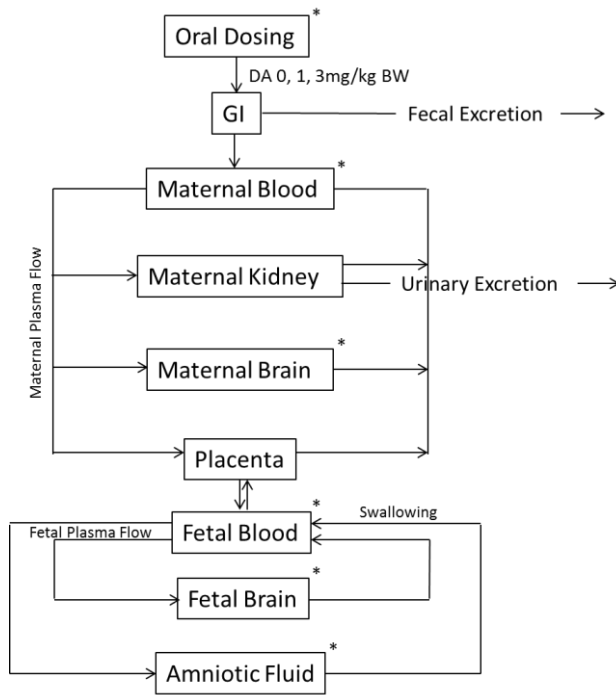


Figure 3.1 DA physiologically-based toxicokinetic (PBTK) model framework for maternal and fetal compartments. Starred ones are the measured parameters (Chapter 2).

### 3.2.2 PBTK Model Equations

To model DA in maternal-fetal units, we can write series of differential equations for DA concentrations on each compartment based on the PBTK model framework for DA (Figure 3.1) as shown below:

$$\begin{aligned} \frac{dA}{dt} (\text{Mblood}) = & -k_{\text{Mblood-Mbrain}} * \text{Mblood} + k_{\text{Mbrain-Mblood}} * \text{Mbrain} \\ & + k_{\text{GI-Mblood}} * \text{GI} \\ & - k_{\text{Mblood-Fblood}} * \text{Mblood} + k_{\text{Fblood-Mblood}} * \text{Fblood} \\ & - k_{\text{urine}} * \text{Mblood} \end{aligned} \quad (\text{Eq. 1})$$

$$\frac{dA}{dt} (\text{Mbrain}) = k_{\text{Mblood-Mbrain}} * \text{Mblood} - k_{\text{Mbrain-Mblood}} * \text{Mbrain} \quad (\text{Eq. 2})$$

$$\frac{dA}{dt} (\text{GI}) = k_{\text{GI-Mblood}} * \text{GI} - k_{\text{fecal}} * \text{GI} \quad (\text{Eq. 3})$$

$$\begin{aligned} \frac{dA}{dt} (\text{Fblood}) = & k_{\text{Mblood-Fblood}} * \text{Mblood} - k_{\text{Fblood-Mblood}} * \text{Fblood} \\ & - k_{\text{Fblood-Fbrain}} * \text{Fblood} + k_{\text{Fbrain-Fblood}} * \text{Fbrain} \\ & - k_{\text{Fblood-AF}} * \text{Fblood} + k_{\text{AF-Fblood}} * \text{AF} \end{aligned} \quad (\text{Eq. 4})$$

$$\frac{dA}{dt} (\text{Fbrain}) = k_{\text{Fblood-Fbrain}} * \text{Fblood} - k_{\text{Fbrain-Fblood}} * \text{Fbrain} \quad (\text{Eq. 5})$$

$$\frac{dA}{dt} (\text{AF}) = k_{\text{Fblood-AF}} * \text{Fblood} - k_{\text{AF-Fblood}} * \text{AF} \quad (\text{Eq. 6})$$

$$\frac{dA}{dt} (\text{Urine}) = k_{\text{urine}} * \text{Mblood} \quad (\text{Eq. 7})$$

$$\frac{dA}{dt} (\text{Feces}) = k_{\text{fecal}} * \text{GI} \quad (\text{Eq. 8})$$

where:

$dA/dt$  is the change in DA concentration in a compartment over time

$M_{\text{blood}}$  = DA concentration in maternal blood

$k_{M_{\text{blood}}-M_{\text{brain}}}$  = transfer coefficient from maternal blood to maternal brain

$k_{M_{\text{brain}}-M_{\text{blood}}}$  = transfer coefficient from maternal brain to maternal blood

$k_{\text{GI}-M_{\text{blood}}}$  = transfer coefficient from GI tract to maternal blood

$k_{M_{\text{blood}}-F_{\text{blood}}}$  = transfer coefficient from maternal blood to fetal blood

$k_{F_{\text{blood}}-M_{\text{blood}}}$  = transfer coefficient from fetal blood to maternal blood

$k_{\text{urine}}$  = transfer coefficient for elimination via urine

$M_{\text{brain}}$  = DA concentration in maternal brain

$\text{GI}$  = DA concentration in GI tract

$k_{\text{fecal}}$  = transfer coefficient for elimination via feces

$F_{\text{blood}}$  = DA concentration in fetal blood

$k_{F_{\text{blood}}-F_{\text{brain}}}$  = transfer coefficient from fetal blood to fetal brain

$k_{F_{\text{brain}}-F_{\text{blood}}}$  = transfer coefficient from fetal brain to fetal blood

$k_{F_{\text{blood}}-AF}$  = transfer coefficient from fetal blood to amniotic fluid

$k_{AF-F_{\text{blood}}}$  = transfer coefficient from amniotic fluid to fetal blood

$F_{\text{brain}}$  = DA concentration in fetal brain

$AF$  = DA concentration in amniotic fluid

$\text{Urine}$  = DA concentration in urine

$\text{Feces}$  = DA concentration in feces

Using Berkeley Madonna Software (Discussed in 3.2.3), we ran the model from the starting time of 0 hour to stopping time of 24 hours, where initial DA concentrations in maternal blood, maternal brain, fetal blood, fetal brain, amniotic fluid, urine, and feces are zero and only GI tract has initial DA concentration of 100. The concentration of DA in GI tract was set to 100, so that it is easier for us to relate the DA concentrations in each compartment to percentages.

The constants for the equations were defined as following:

$$k_{M_{\text{blood}}-M_{\text{brain}}} = 0.01 * (\ln(2)/4) = 1.73 \times 10^{-3}$$

$$k_{M_{\text{brain}}-M_{\text{blood}}} = 0.005 * (\ln(2)/4) = 8.66 \times 10^{-4}$$

$$k_{\text{GI}-M_{\text{blood}}} = 0.01 * (\ln(2)/4) = 1.73 \times 10^{-3}$$

$$k_{M_{\text{blood}}-F_{\text{blood}}} = 0.36 * (\ln(2)/4) = 0.0624$$

$$k_{F_{\text{blood}}-M_{\text{blood}}} = 0.05 * (\ln(2)/4) = 8.66 \times 10^{-3}$$

$$k_{\text{urine}} = 1 * \ln(2)/4 = 0.173$$

$$k_{\text{fecal}} = 15 * (\ln(2)/4) = 2.60$$

$$k_{\text{Fblood-Fbrain}} = 1.8 * (\ln(2)/4) = 0.312$$

$$k_{\text{Fbrain-Fblood}} = 0.05 * (\ln(2)/4) = 8.66 \times 10^{-3}$$

$$k_{\text{Fblood-AF}} = 1.07 * (\ln(2)/4) = 0.185$$

$$k_{\text{AF-Fblood}} = 0.05 * (\ln(2)/4) = 8.66 \times 10^{-3}$$

This DA PBTK model for maternal-fetal compartments makes three assumptions: 1) DA dermal exposures have a negligible contribution to concentration of DA in amniotic fluid, 2) fetus is considered as a closed system, so no elimination occurs from fetal compartment to the exterior, and 3) maternal blood will go to fetal blood, and from fetal blood, it will be distributed to other fetal compartments such as fetal brain and amniotic fluid.

### 3.2.3 Model Software

Berkeley Madonna (version 8.3.18; Berkeley Madonna Inc., University of California, Berkeley, CA, USA) was utilized to model and simulate analyses. With this program that is capable of solving differential equations fast and constructing a mathematical model, we generated a two-compartment model for our pregnant mice and their fetus.

## 3.3 RESULTS

### 3.3.1 PBTK Model Output

Figure 3.2 shows the output plot of PBTK model for maternal-fetal compartments after chronic, low-level exposures of DA. The plot illustrates that concentration of DA in GI tract (Figure 3.2 **Green line**) drops dramatically as the concentration in maternal blood (Figure 3.2 **Red line**) increases, which peaks around 1 hour after last dose on GD17. Moreover, the figure shows that the most of the DA is urinated (Figure 3.2 **Pink line**) over time. Interestingly, fetal blood (Figure 3.2 **Blue line**) DA concentration peaks and drops down, but fetal brain (Figure 3.2 **Orange line**) and amniotic fluid (Figure 3.2 **Sky-blue line**) accumulate over time.

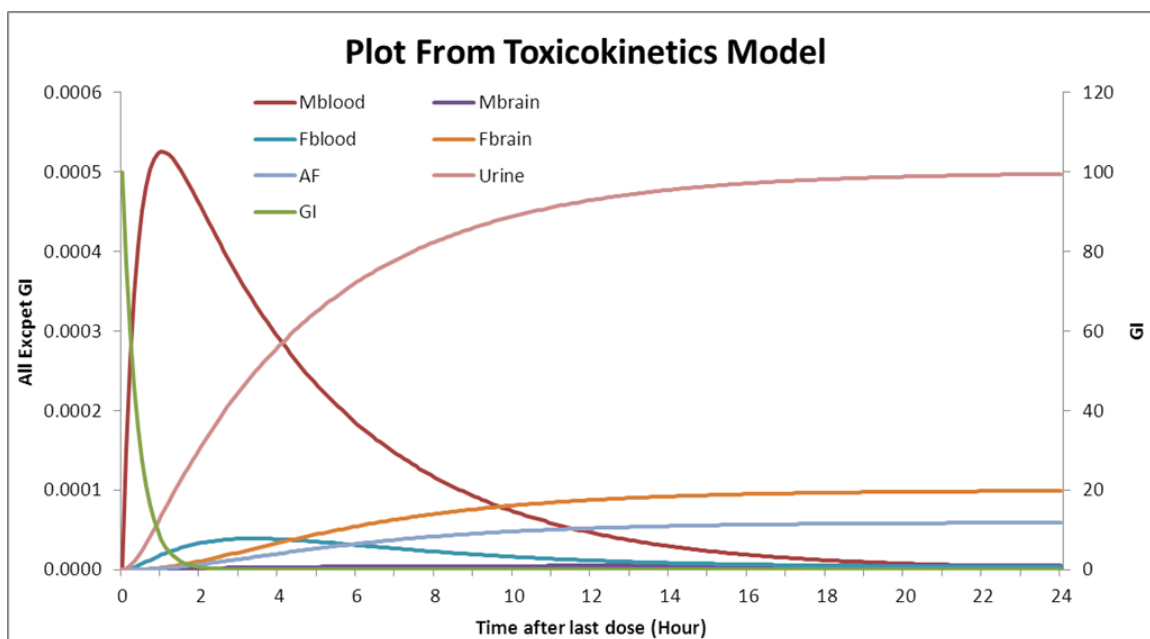


Figure 3.2 Plot of PBTK model output in terms of DA concentration over time. Different colors represent different maternal and fetal compartments.

Toxicokinetic parameters such as slopes, uptake rate, point of plateau, area under the curve (AUC), and time when the maximum DA concentration is reached ( $T_{Cmax}$ ) were defined for some maternal and fetal compartments and summarized in Table 3.1.

| Table 3.1 Toxicokinetic Parameters from DA PBTK Model |              |             |             |            |            |
|---|--------------|-------------|-------------|------------|------------|
|   |              | MBlood      | FBlood      | FBrain     | AF         |
| Parameters  | Unit         | Value       | Value       | Value      | Value      |
| Slope   | per hr       | 0.0088      | 0.00016     | N/A        | N/A        |
| Uptake rate   | per hr       | 0.052       | 0.0019      | 0.00024    | 0.00014    |
| Point of plateau                                      | hr-post dose | N/A         | N/A         | 10         | 10         |
| AUC   | hr ng        | 0.281       | 0.039       | 0.186      | 0.111      |
| $T_{Cmax}$  | hr-post dose | GD17, 1.0hr | GD17, 3.3hr | GD17, 24hr | GD17, 24hr |

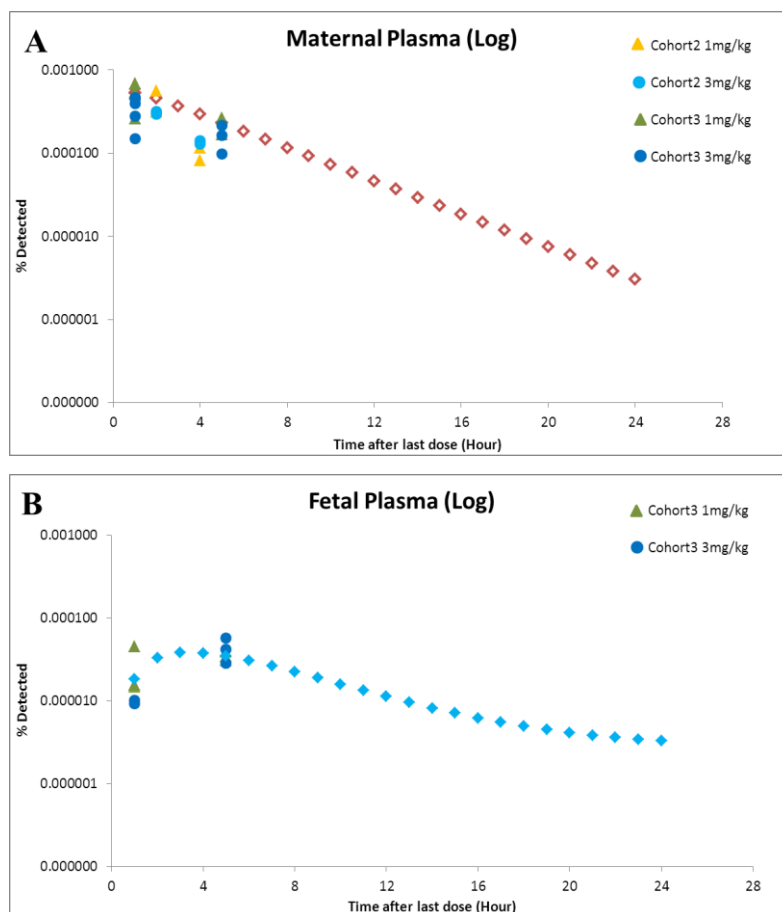
Table 3.1 Plot of PBTK model output in terms of DA concentration over time. Maternal blood has faster uptake rate than fetal compartments. The percent of DA identified only in fetal brain and amniotic fluid plateau while those in maternal and fetal blood peak at 1 and 3.3 hours after last dose on GD17, respectively, and decrease. N/A = Not applicable.

According to our maternal-fetal PBTK model for DA, maternal blood accumulates the fastest with uptake rate of 0.052 per hour compared to fetal blood, fetal brain, and amniotic fluid. Interestingly, the percent of DA identified in fetal brain and amniotic fluid plateaued at 10 hours after the last dose on GD17 while that in maternal and fetal blood did not have the point of plateau. Rather, the percent of DA detected in maternal and fetal blood peaked at 1 and 3.3 hours after last dose on GD17 and

decreased. This suggested that DA do not accumulate in maternal and fetal blood over time. On the other hand, fetal brain and amniotic fluid plateaued, and the maximum percent DA identified was at 24 hours after last dose on GD17, suggesting that DA is accumulated in fetal brain and amniotic fluid.

### 3.3.2 DA PBTK Model Performance

The PBTK model was developed to estimate the concentration of DA and its related effects in the previous sections, but it is important to check how the model performed compared to the actual in vivo data obtained in Chapter 2. Thus, data points for various compartments including maternal and fetal plasma, fetal plasma, fetal brain, and amniotic fluid were plotted on top of the estimated DA percent detected from the PBTK model for each appropriate compartment. These plots were shown below (Figure 3.3):



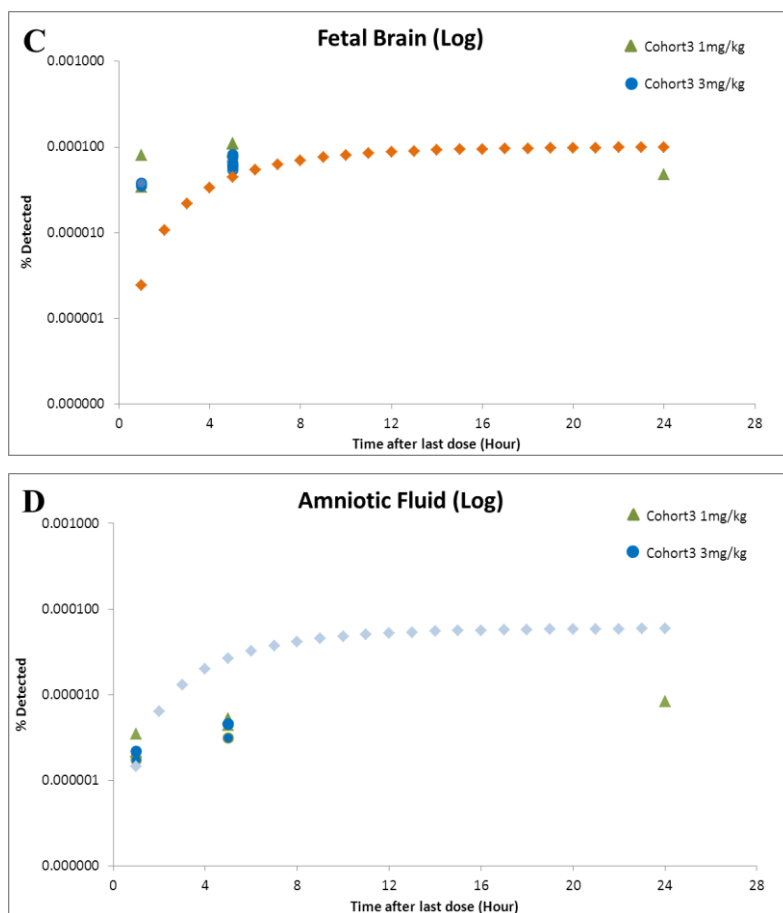


Figure 3.3 Comparison between observed (Chapter 2) and predicted DA % detected in (A) maternal and (B) fetal plasma, (C) fetal brain, and (D) amniotic fluid. Actual data points obtained from Chapter 2 were plotted on top of the DA PBTK model output. The plots show percent DA detected in various compartments over time.

### 3.4 DISCUSSION

To date, only a few studies have published regarding toxicokinetics of DA. Of those, only one study investigated toxicokinetics of DA in pregnant rats and their fetuses (Maucher Fuquay et al., 2012). Although toxicokinetic parameters were first defined in this study after exposing pregnant Sprague Dawley rats with a single dose of DA via IV injection on GD20, they have not done PBTK modeling for DA. Our study developed a maternal-fetal PBTK model for the first time after treating pregnant C57BL/6 mice with repeated, low-dose of DA by oral gavage. Compared to other previous studies, this study was done with the most human relevant route and pattern of exposures as the main route and patterns of exposure for humans are chronically consuming low concentrations of DA contaminated seafood.

The results from this study illustrated that the output plot of our developed maternal-fetal PBTK model showed that the concentration of DA in maternal blood increased and peaked at 1 hour after

last dose on GD17. However, very small amount of DA is detected in maternal blood compared to what is administered. Moreover, most of the DA was excreted via urine and feces over time based on our developed PBTK model. These results were consistent with the previous findings that DA is absorbed poorly in the body and most of DA is excreted (Chapter 2). The model also demonstrated that the percent of DA identified in fetal brain and in amniotic fluid accumulates over time, plateauing at 10 hours after last dose on GD17. Our findings that DA was accumulating in fetal brain and amniotic fluid were consistent with the findings of Maucher Fuquay et al. (2012) that limited elimination is occurring in fetal brain and amniotic fluid. Therefore, fetuses may be continually re-exposed to DA during gestation due to longer fetal retention of DA. Unlike fetal brain and amniotic fluid, the percent of DA detected in maternal and fetal blood peaked at 1 and 3.3 hours after last dose on GD17 and decreased, suggesting that DA do not accumulate in maternal and fetal blood over time. Moreover, the maximum percent of DA in fetal blood peaked at later compared to maternal blood, suggesting delayed exposures.

The purpose of developing a maternal-fetal PBTK model for DA was to better understand the links between *in utero* DA exposures and adverse health impacts. As mentioned before, *in utero* exposure to DA could affect the neurodevelopment of a developing fetus and result in brain alterations and behavioral disturbances that may persist into adulthood. With our model, we may be able to better estimate the DA distributed to various maternal and fetal compartments and protect susceptible populations including developing fetuses.

### 3.5 FUTURE DIRECTIONS

The model described above was developed with a hope that the model can be applied in risk assessment. However, additional work may be needed to validate the model and to study more on the toxicokinetics of DA after repeated oral administrations. Future studies may include:

1. Performing further analysis on sensitivity of our developed maternal-fetal PBTK model for DA;
2. Applying current PBTK model to other species to explore if there are any significant toxicokinetic differences after repeated *in utero* exposures to DA; and
3. Modeling early, repeated post-natal exposures to DA in mice and compare with *in utero* exposure models.

### 3.6 CONCLUSIONS

In conclusion, the PBTK model was developed and compared with our *in vivo* kinetics study from Chapter 2, which also helped us to refine the model. The model was able to fit the data as shown in Figure 3.3. The output plot of our developed maternal-fetal PBTK model demonstrated that the concentration of DA in the GI tract dropped dramatically after oral administration while that in maternal blood increased and peaked at 1 hour after last dose on GD17. In fetal blood, the peak where the percent DA identified was delayed compared to maternal blood as shown in Table 3.1. Most of the DA was eliminated through urine and feces over time based on our developed PBTK model. The model also demonstrated that the percent DA detected in fetal brain and in amniotic fluid accumulated over time, suggesting DA is retained in fetal brain and amniotic fluid. After further validation of the model, it may be applied in risk assessment to estimate the DA concentration distributed in various maternal and fetal compartments after chronic, low exposures to DA.

## CHAPTER 4: CONCLUSIONS

The objective of this study was to evaluate potential links between exposure and relative health impacts associated with chronic, low-level DA exposure during pregnancy. We hypothesized that DA accumulates in pregnant mice and their fetuses after repeated oral dose of DA for 8 days. To test the hypothesis, we examined differences in DA concentration in pregnant and non-pregnant mice and in various fetal compartments including fetal plasma, fetal brain, and amniotic fluid. Lastly, we have developed maternal-fetal PBTK model for DA. In summary, conclusions from the study include following:

- No accumulation of DA in plasma of pregnant and non-pregnant female mice was observed after chronic, low-level exposures (1 and 3mg/kg BW);
- The DA absorption rates increased for both pregnant and non-pregnant mice as dose increased, and pregnant mice plasma showed a higher absorption rate than non-pregnant mice when comparing animals that were treated with same dose of DA. For these, however, it is important to note that the differences between pregnant and non-pregnant mice plasma might be due to pregnancy since DA concentrations in pregnant mice plasma were not adjusted by gravid weight;
- Using the combined model, we found that pregnant and non-pregnant mice plasma have redistribution rate of 3.8 with 95% confidence interval (3.3, 4.3) per hour and uptake rate of 1.9 with 95% confidence interval (0.9, 3.8) per hour, and  $T_{\text{cmax}}$  occurred at 1.2 hours after the last dose;
- Developing fetuses may have increased exposure to DA compared to pregnant moms due to potential accumulations of DA in fetal plasma, fetal brain, and amniotic fluid over time. Dose and time had significant effects on DA concentration in fetal brain and amniotic fluid (Two-way ANOVA; p-value < 0.005);
- When compared with our *in vivo* kinetics study (Chapter 2), the developed PBTK model was able to fit the data;
- According to the developed maternal-fetal PBTK model for DA, the percent of DA identified in maternal and fetal blood peaked at 1 and 3.3 hours after last dose on GD17 and decreased, suggesting that DA did not accumulate in maternal and fetal blood over time. The maximum percent of DA in fetal blood peaked later compared to maternal blood, suggesting fetuses have delayed exposures;

- Unlike maternal and fetal blood in the PBTK model, the percent of DA detected in fetal brain and amniotic fluid plateaued at 10 hours after the last dose on GD17 and were retained;

Finally, results from the study on the toxicokinetic effects of repeated, low-dose exposures to DA will add significant new information to the literature to understand the links between *in utero* DA exposures and adverse health impacts such as brain alterations and behavioral disturbances that may persist into adulthood.

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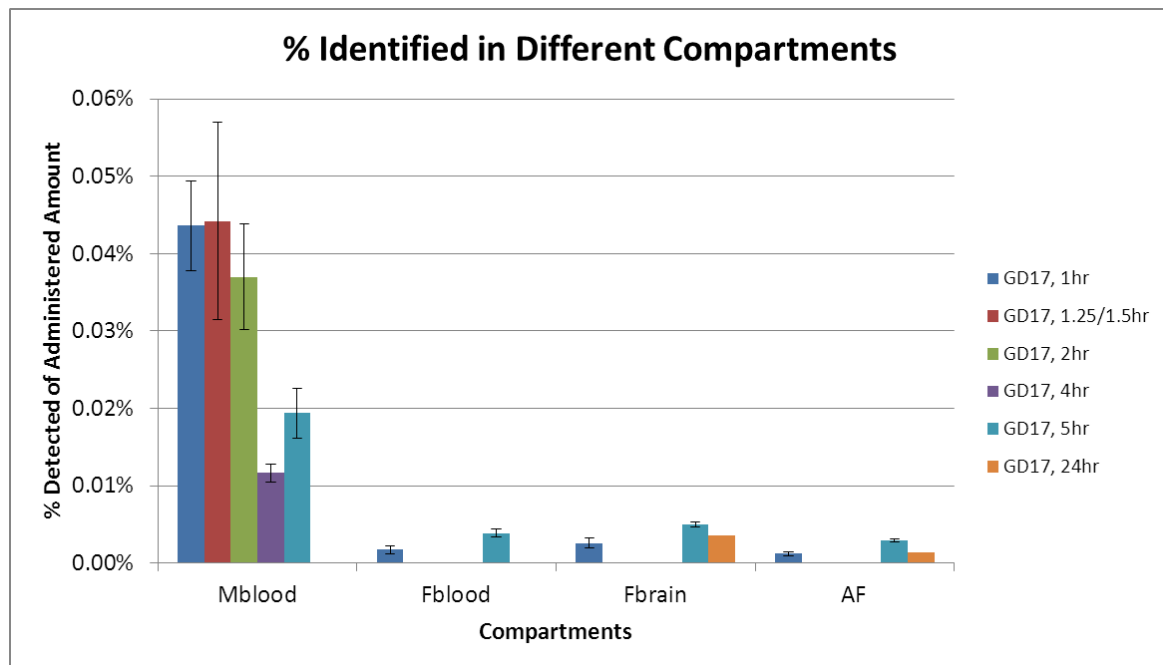
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## APPENDIX

### Appendix 1.

Based on the concentrations of DA in maternal and fetal plasma, fetal brain, and amniotic fluid detected by the LC/MS, the percent of DA detected were calculated in measured maternal and fetal compartments. To find this, the AUCs obtained from LC/MS were back-calculated (e.g. adjusting for dilution factors) first to determine detected concentrations of DA. Then, the numbers were divided by the administered DA concentrations to the animals. The plot illustrates the average percent of DA identified in different maternal and fetal compartments at various time points represented by colors.

The highest percentages were found in maternal blood, but decreased over time. There were no data available for fetal blood, fetal brain, and amniotic fluid at 1.25/1.5, 2, and 4 hours after last dose, but from the percent of DA identified at 1 hour post last dose is lower than 5 hours for all three fetal compartments. Values for 24 hr were plotted, but these are based on a single data point (n = 1).



Appendix 1 Plot of percent DA identified in different maternal-fetal compartments after administering pregnant mice with DA for 8 days (GD10 - GD17). Colors represent different time points after last dosing on GD17: Blue = 1hr, red = 1.25/1.5 hr, green = 2hr, purple = 4hr, blue-green = 5hr, and orange = 24hr after last dose on GD17. Highest percentages of DA were found in maternal blood, but DA was detected at later time points after the last dosing.

No 1.25/1.5, 2, and 4hr data available for Fblood, Fbrain, and AF. Mblood, Fblood, Fbrain and AF have N > 3 for all time points except N = 1 for 24 hr.