ABSTRACT

The teratogenicity of all-trans retinoic acid (RA) has been known for decades, however to date no extensive investigation of the dose-response and kinetics across a wide range of doses has been undertaken. The objectives of this study were to 1) increase the robustness of the dose-response curve for forelimb and cleft palate malformation resulting from RA exposure in gestation day (GD) 11 mice, 2) compare two techniques (computerized image analysis and visual morphological evaluation) for the assessment of fetal forelimb malformations, and 3) develop a physiologically based pharmacokinetic (PBPK) model to describe the maternal and fetal disposition of RA. Pregnant CD-1 mice were administered a single oral dose of all-trans RA (0, 2.5, 10, 30, 60 or 100 mg/kg) on GD 11. GD 18 fetuses were examined for malformations using visual morphological scoring and computerized image analysis. In separate experiments maternal plasma and fetal tissue time-course data for RA and a major metabolite (13-cis retinoic acid, 13cisRA) were collected on GD 11 and analyzed by HPLC. A PBPK model was then developed to describe the maternal and fetal disposition of RA and 13cisRA. Overall, computerized image analysis was not more sensitive in detecting changes in the humerus, radius
and ulna than gross visual examination. Dose-response statistical analysis of developmental endpoints yielded comparable benchmark dose levels (5%) for long bones and cleft palate that ranged from 0.24 to 7.6 mg/kg *all-trans* RA. In general, the PBPK model predicted maternal plasma and embryonic concentrations of RA and 13cisRA compared favorably with experimental observations across the range of doses tested. Model predicted embryo concentrations of RA at the lowest two doses (2.5 and 10 mg/kg) were lower than the measured amounts and the model slightly over-predicted the clearance. This suggests that the kinetics of low doses of RA are somewhat different from the kinetics of the high doses administered in the study.

**INDEX WORDS:** all-trans retinoic acid, fetal limb malformation, cleft palate, benchmark dose, physiologically based pharmacokinetic modeling
PHYSIOLOGICALLY-BASED PHARMACOKINETIC MODELING AND DOSE-RESPONSE FOR FETAL SKELETAL MALFORMATIONS INDUCED BY INGESTION OF

*ALL-TRANS* RETINOIC ACID IN CD-1 MICE

by

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DOCTOR OF PHILOSOPHY

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PHYSIOLOGICALLY-BASED PHARMACOKINETIC MODELING AND DOSE-RESPONSE FOR FETAL SKELETAL MALFORMATIONS INDUCED BY INGESTION OF ALL-TRANS RETINOIC ACID IN CD-1 MICE

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CHAPTER 1

INTRODUCTION

Over the last decade, the therapeutic use of RA has grown exponentially. However, with this increased use, there has also been an increase in risk that women on retinoid treatment become pregnant resulting in severe developmental deficits in their offspring. There were two main goals of this research. The first was to conduct teratology studies for cleft palate and forelimb malformations in fetuses of gestation day (GD) 11 CD-1 mice to expand upon the dose-response characterization for RA. Two techniques (computerized image analysis and visual morphological scoring) were compared for assessing fetal forelimb malformation. The second objective was to expand on a previously published physiologically based pharmacokinetic (PBPK) model for RA (Clewell et al., 1997) to account for changes in the disposition and metabolism of RA during GD 11 in the pregnant CD-1 mouse over a range of concentrations (i.e., from the no observable adverse effect level (NOAEL) to maximally teratogenic doses). This dissertation includes a literature review of the current knowledge of RA, followed by two chapters that discuss the background, methods, and results of experiments including the dose response analysis and the PBPK model developed to describe the disposition of RA in maternal tissues and embryos. The first manuscript has been published in Birth Defects Research Part B (Campbell et al., 2004) and the second will be submitted to Toxicological Sciences. The final chapter will include the overall conclusions of this research and future work needed to improve upon the knowledge base concerning RA teratogenicity and kinetics.
References


CHAPTER 2
LITERATURE REVIEW

The purpose of this research is an extensive examination of the dose response and kinetics of RA on the developing limb in the mouse embryo. This review will cover the pertinent information related to the role of retinoids in skeletal development and malformation as well as a review of the knowledge base regarding the absorption and distribution of RA in maternal and embryonic tissues used to develop the physiologically based pharmacokinetic model of RA in the gestation day (GD) 11 mouse.

Teratogenicity of RA

Abnormally high concentrations of RA in both experimental animals (Kochhar et al., 1996) and humans (Rosa, 1983; Lammer et al., 1985) result in fetal malformations. The teratogenic effects of retinoids on humans were demonstrated in the 1980’s when Accutane® (13-cis retinoic acid, 13-cis RA), a metabolite of RA, was marketed for use as a treatment for acne. When pregnant women took Accutane®, their fetuses were at increased risk for adverse effects including malformations of the limbs, ears, heart, brain and thymus, and reduction in IQ (Rosa, 1983; Lammer et al., 1985; Rizzo et al., 1991). While the use of 13cisRA as a drug is now restricted in pregnancy, research related to the potential therapeutic use of retinoids continues in the treatment of acute promyelocytic leukemia (Grimwade and Lo Coco, 2002), the treatment of acne (Shalita, 2001), and AIDS-related Kaposi's sarcoma (Cattelan et al., 2002).

Excessive amounts of RA can affect almost any organ system, depending upon the stage of development in which exposure occurs (Kochhar, 1967; Shenefelt, 1972; Kamm et al., 1984).
In mouse embryos, RA produces severe limb defects and craniofacial malformations (primarily cleft palate) when treatment occurs between GD 11 and 14.5 (Kwasigroch and Kochhar, 1980; Kochhar et al., 1996; Hansen et al., 2001). A single 100 mg/kg dose of RA to GD 11 mice has been shown to result in 92% (Kraft et al., 1989) and 100% (Soprano et al., 1994; Kochhar et al., 1996) malformed fetuses. At 10 mg/kg, exposure resulted in 23-39% of the fetuses being malformed (Soprano et al., 1994; Kochhar et al., 1996). No fetal limb malformations were seen when a dose of 1 mg/kg RA or less was administered to pregnant mice on GD 11 (Kochhar et al., 1996). Hansen et al. (2001) reported a more rigorous dose-response for RA in GD 11 and GD 13 CD-1 mice. At an RA dose of 10 mg/kg, Hansen’s group reported only 2.4% of the fetuses exhibiting cleft palate, while none of the fetuses had detectable forelimb malformations. At 1.0 and 100 mg/kg RA, Hansen et al. (2001) reports similar results to previous studies with no malformations at 1.0 mg/kg and nearly 90% of fetuses having either cleft palate or a forelimb defect at 100 mg/kg.

Role of Retinoic Acid in Abnormal Limb Development

RA is the bioactive form of Vitamin A and is known to act on cells in the developing embryo resulting in altered gene activity (Maden, 2000; Underhill and Weston, 1998). The skeleton, especially the developing limb, is a structure that is particularly susceptible to excessive or deficient amounts of vitamin A. Kochhar (1967) was the first to show RA to be more potent at mediating this teratogenic effect than vitamin A. In the developing rodent limb between GD 10.5 and 14.5, the mesenchymal cells condense and differentiate into chondrocytes. This sensitive period of limb development can be disrupted by RA treatment resulting in limb malformations and suggesting that RA disrupts the chondrogenic process (Maden, 2000; Underhill and Weston, 1998; Napoli, 1999).
RA’s mode of action on the developing limb has been shown to be mediated by a family of nuclear hormone receptors, of which there are two classes: RA receptors (RAR) and retinoid X receptors (RXR) (Underhill and Weston, 1998; Napoli, 1999; Collins and Mao, 1999). The current research into RA teratogenicity has focused on the primary cellular targets for RA (i.e., the genes that contain retinoic acid response elements). RARs function as heterodimers with the RXRs, while the RXRs can act as heterodimers or homodimers (Chambon, 1996). The modular nature of these ligand-inducible transcription factors are shown in their structure where they contain domains for ligand binding, protein-protein interactions, and DNA binding (Underhill and Weston, 1998). Retinoid receptors are also expressed with distinct, sometimes overlapping, spatiotemporal patterns during embryogenesis (Underhill and Weston, 1998; Napoli, 1999; Collins and Mao, 1999). In addition to the characterization of the expression patterns of the receptors within several tissue types, both loss- and gain-of function studies have provided insight into the functions of the various receptor types. Specifically, regulation of the RARs has been found to be important in chondrogenesis of the developing limb. Recent studies using microarray technology have shown several targets for RA in the developing limb including PBX, MEIS, and IGF-I (Qin et al., 2002). Over expression of PBX and MEIS affect the proximodistal patterning of the developing chick limbs (Capdevila et al., 1999; Mercader et al., 1999). PBX and MEIS are themselves transcription factors. However, the genes targeted by PBX and MEIS are still unknown.

**Kinetics of RA**

Due to the need to maintain precise levels of RA within developing embryonic tissue, exogenous doses of RA result in severe alteration in normal pattern formation by overwhelming the balance of RA concentration in tissue. Two published datasets examine the distribution of an
oral dose of RA in GD 11 pregnant mice (Kraft et al., 1987; Satre and Kochhar 1989) and one study reports a time course for RA in GD 14 mice (Tzimas et al., 1995).

RA is a highly lipophilic weak acid and the kinetics of a single oral dose includes a rapid uptake and distribution phase for maternal and embryonal tissues followed by a period of rapid elimination. Measurable quantities are only detectable up to 8 h after oral dosing (Satre and Kochhar 1989; Tzimas et al., 1995). Peak concentrations in maternal and embryonal tissues are reached within 2 h and the half-life of RA after oral exposure is 0.57 to 1.02 h (Le Doze et al., 2000).

Kraft et al. (1987) measured maternal plasma and embryo concentrations of RA 1, 2, and 4 h after a single oral dose (100 mg/kg) in GD 11 NMRI mice. In maternal plasma, RA concentrations were at a maximum (3 µg/ml) at 1 h and decreased to 1.9 µg/ml by 4 h post treatment. In the embryo, concentrations of RA on GD 11 reached a maximum (1.5 µg/ml) at 2 h declining to 0.77 µg/ml 4 h post dosing. Kraft et al. (1987) also measured concentrations of 13cisRA on GD 11. Maternal plasma had a peak concentration (0.14 µg/ml) at 1 h and declined rapidly to 0.8 µg/ml by 2 h with the 4 h time point having the same concentration. In the embryo the concentration of 13cisRA followed the concentration of RA with the peak concentration (0.06 µg/ml) occurring at 2 h falling to 0.03 µg/ml by 4 h.

A subsequent study (Satre and Kochhar, 1989) measured the time course of RA in whole embryos and limb buds (GD 11) with doses of 10 and 100 mg/kg in soybean oil in ICR mice. The concentration of RA for the 100 mg/kg dose in both the whole embryo and limb bud rapidly rose to a peak concentration of 0.11 µg/mg protein at 3 h and then fell sharply to 0.001 µg/mg protein. For the 10 mg/kg dose, RA concentrations in both the embryo and limb bud peaked at 0.011 µg/mg protein and then declined to approximately 0.001 µg/mg protein by 4 h. Because
there was good agreement between the whole embryo and the limb bud, it is apparent that measurements of RA in the whole embryo can be substituted for the concentration in the target tissue (i.e, the developing limb).

The kinetic profile of RA is somewhat different with increasing dose and gestation day. That is, the clearance of RA from plasma appears to have a much steeper slope at 100 mg/kg versus the 10 mg/kg dose. Tzimas et al. (1995) conducted studies with RA in NMRI mice on GD 14 with somewhat similar results to the studies conducted in GD 11 mice at 10 mg/kg. However peak concentrations in maternal plasma and embryos for animals treated with 100 mg/kg RA were not reached until 4 h post dosing. After 10 mg/kg were given orally, the concentration of RA in maternal plasma reached a peak of 0.86 µg/ml with the embryo concentrations at 0.6 ng/g. The time course of the 100 mg/kg dose appeared to plateau at 3.8 µg/ml from 1 to 4 h in maternal plasma followed by a sharp decline to 0.1 µg/ml. This was similar in the embryo which had a concentration only 10% lower than the plasma.

**RA Metabolism**

The metabolism of RA is rapid with metabolites appearing in plasma as early as 0.5 h after oral dosing (Tzimas et al., 1995). Under normal conditions, RA is primarily metabolized in the tissue where it is formed from retinol by dehydrogenases. Metabolism of RA (Figure 2.1) occurs through three primary routes including: isomerization to 13cisRA, glucuronidation to RAG (retinoyl-β-glucuronide), and hydroxylation (phase I metabolism via P450 enzymes, CYPs 26, 3A7, 2C8, 4A11, 1A1, 3A4/5) to 18-OH RA and 4-OH RA (Maden, 2000). In the mouse metabolism is primarily by P450, while in humans glucuronidation serves as the primary route (Zile et al., 1982; Swanson et al., 1981; Kraft et al., 1991).
The metabolism of RA to 13cisRA occurs primarily in the liver, although some isomerization has been shown in the intestinal mucosa and other tissues of the adult mammal (Zile et al., 1982). While early reports suggested that 13cisRA was itself bioactive, it was subsequently found that the isomerase enzyme has the ability to produce RA from 13cisRA (Kraft et al., 1991). However, it should be noted that the rate of 13cisRA to RA back conversion during excessive RA conditions has not been investigated in whole animal studies. Thus, the isomerization pathway of RA metabolism is most likely a detoxification mechanism under excessive RA conditions as 13cisRA is also rapidly metabolized and eliminated.

Glucuronidation of RA in animals is thought to primarily occur in tissues that do not express cellular retinoic acid binding protein (i.e., liver and intestinal mucosa) (Maden, 2000). Along with RA, many of the phase I metabolites are also eliminated through this process.

Recent studies of RA metabolism via P450 pathways have shown that RA induces P450 (primarily CYP26) responsible for its metabolism. Yamamoto et al. (2000) originally showed that the vitamin A status of rats augmented the production of CYP26 mRNA. That is, animals that were given a vitamin A supplement had higher levels of CYP26 mRNA when compared to control animals, and vitamin A deficient rats displayed reduced levels of CYP26 mRNA when RA was administered. In a subsequent publication Wang et al. (2002) elucidated that the induction of CYP26 was under the control of RA, an active metabolite of vitamin A. CYP26 mRNA was elevated within the first 3 h after dosing with RA and remained elevated up to 24 h post dosing. The auto-induction of RA metabolism was confirmed by the analysis of data from the intravenous administered RA in rats (el Mansouri et al., 1995). This work also showed that the clearance of RA increased at 180 min post dosing (i.e., an increase in the slope of the clearance curve). Other isoforms of P450 may also be induced by RA through interaction of RA
receptors and the aryl hydrocarbon receptor in liver (Napoli, 1999). Thus, the overall ability to upregulate RA metabolism needs further investigation.

**PBPK Models for Pregnancy**

To improve the understanding of risk posed by exposure to developmental toxicants, PBPK models have previously been used to provide a better measure of the embryonic dose for several chemicals including trichloroethylene (Fisher et al., 1989), methylmercury (Clewell, 1999) and methanol (Ward et al., 1997) (see Corley et al., 2003 for a complete review pregnancy models). A PBPK model utilizes known species-specific physiological parameters, such as blood flow and tissue volumes, and chemical specific parameters (i.e., partition coefficients, metabolic rate constants, etc.) (Corley et al., 2003). By incorporating these species specific parameters, PBPK models reduce the uncertainty of extrapolating from high dose used in experimental animal studies to the low doses found in environmental exposures to humans. PBPK models can also provide clues to the mode(s) of action and assists in determining the direction of future research (Corley et al., 2003).

Clewell et al. (1997) have previously published a model describing the disposition of RA in pregnant mice, rats, nonhuman primates, and humans. This model was used to describe single oral and dermal exposures to RA and 13cisRA at only 1 dose level per species. The model included metabolism in the liver for P450, isomerization, and glucuronidation of RA to its more polar metabolites. Fetal transfer of RA through the placenta was described as diffusion limited. However, the documentation surrounding the estimation of parameters including metabolic rate constants, partition coefficients, and diffusion coefficients was very limited. While the model fit the data presented for the rat, no information other than AUC and Cmax were presented for the mouse. The model was able to describe the uptake and elimination of RA from maternal tissues.
No information was reported on the model’s ability to predict metabolite concentrations.

However, it was stated that the model-predicted volume of distribution and clearance of RA was similar to that from experimental studies in nonhuman primates and humans.

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Figure 2.1. Schematic representation of RA metabolism (adapted from Petkovich, 2001). RAG = retinoyl-β-glucuronide
CHAPTER 3

DOSE-RESPONSE FOR RETINOIC ACID-INDUCED FORELIMB MALFORMATIONS AND CLEFT PALATE: A COMPARISON OF COMPUTERIZED IMAGE ANALYSIS AND VISUAL INSPECTION

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Abstract

BACKGROUND: The objectives of this study were to 1) compare two techniques (computerized image analysis and visual morphological evaluation) for the assessment of fetal forelimb malformations and 2) increase the robustness of the dose-response curve for forelimb and cleft palate malformation resulting from all-trans retinoic acid (RA) exposure in GD 11 mice. METHODS: Pregnant CD-1 mice were administered a single oral dose of all-trans RA (0, 2.5, 10, 30, 60 or 100 mg/kg) on GD 11. GD 18 fetuses were examined for malformations using visual morphological scoring and computerized image analysis. RESULTS: Dose-dependent changes occurred in size and shape of the humerus, radius and ulna based on both assessment methodologies. The most sensitive indicators for the lowest effect level (10 mg/kg) on forelimbs were roundness, a shape measurement determined by image analysis, and visual morphological scoring. For all other bone measurements (proximal and distal width, area, length, and perimeter), the lowest effect level was 30 mg/kg. The maximum effect for limb defects and total malformed fetuses was seen at 60 mg/kg and higher. Incidence of cleft palate increased over the entire range of administered doses reaching a maximum of 74% (100 mg/kg). CONCLUSIONS: Overall, results indicate that computerized image analysis was no more sensitive in detecting changes in the humerus, radius and ulna than gross visual examination. Dose-response modeling of developmental endpoints yielded comparable benchmark dose levels for long bones and cleft palate that ranged from 0.24 to 7.6 mg/kg all-trans RA.

KEYWORDS: Retinoic acid, limb malformation, cleft palate, mouse, computerized image analysis, benchmark dose
Introduction

*all-trans* Retinoic acid (RA), an endogenous metabolite of vitamin A, is required for normal pattern formation during embryogenesis. However, abnormally high concentrations in both experimental animals (Kochhar *et al.*, 1996) and humans (Rosa, 1983; Lammer *et al.*, 1985) result in fetal malformations. The teratogenic effects of retinoids on humans were demonstrated in the 1980’s when Accutane® *(13-cis retinoic acid, 13-cis RA)*, a metabolite of *all-trans* RA, was marketed for use as a treatment for acne. When pregnant women took Accutane®, their fetuses were at increased risk for adverse effects including malformations of the limbs, ears, heart, brain and thymus, and reduction in IQ (Rosa, 1983; Lammer *et al.*, 1985; Rizzo *et al.*, 1991). While the use of 13-*cis* RA as a drug is now restricted in pregnancy, research related to the potential therapeutic use of retinoids continues in the treatment of acute promyelocytic leukemia (Grimwade and Lo Coco, 2002), the treatment of acne (Shalita, 2001), and AIDS-related Kaposi's sarcoma (Cattelan, 2002).

Excessive amounts of *all-trans* RA can affect almost any organ system, depending upon the stage of development in which exposure occurs (Kochhar, 1967; Shenefelt, 1972; Kamm *et al.*, 1984). In mouse embryos, *all-trans* RA has been shown to produce severe limb defects and craniofacial malformations when treatment occurs between gestation days (GD) 11 and 14.5 (Kwagisgroch and Kochhar, 1980; Kochhar *et al.*, 1996; Hansen *et al.*, 2001). A single 100 mg/kg dose of *all-trans* RA to GD 11 mice has been shown to result in 92% (Creech Kraft *et al.*, 1989) and 100% (Soprano *et al.*, 1994; Kochhar *et al.*, 1996) malformed fetuses. At 10 mg/kg, *all-trans* RA exposure resulted in 23-39% of the fetuses being malformed (Soprano *et al.*, 1994; Kochhar *et al.*, 1996). No fetal limb malformations are seen when a dose of 1 mg/kg *all-trans* RA or less is administered to pregnant mice on GD 11 (Kochhar *et al.*, 1996). Hansen *et al.*
(2001) reported a more rigorous dose-response for *all-trans* RA in GD 11 and GD 13 CD-1 mice. At an *all-trans* RA dose of 10 mg/kg, Hansen’s group reported only 2.4% of the fetuses exhibiting cleft palate, while none of the fetuses were designated as having forelimb malformations. At 1.0 and 100 mg/kg *all-trans* RA, Hansen *et al.* (2001) reports similar results to previous studies with no malformations at 1.0 mg/kg and nearly 90% of fetuses having either cleft palate or a forelimb defect at 100 mg/kg.

The overall objective of this research was to conduct teratology studies for cleft palate and forelimb malformations in fetuses of GD 11 CD-1 mice to expand upon the dose-response characterization for *all-trans* RA. Two techniques (computerized image analysis and visual morphological scoring) were compared for assessing fetal forelimb malformation. Computerized image analysis was conducted in an effort to detect subtle changes in development that may escape traditional visual scoring of malformations. Computerized image analysis has been used previously to measure the effects of *all-trans* RA on mouse embryo limb buds *in vitro* (Desbiens *et al.*, 1990) and *in vitro* after *in vivo* exposure (Kwasigroch *et al.*, 1984).

**Methods and Materials**

**Animals and Treatments**

Timed-pregnant CD-1 mice were obtained on GD 8 from Charles River Laboratories (Grand Rapids, MI). The presence of a copulatory plug was designated as GD 0. Animals were housed individually in polypropylene cages with wood shavings bedding and provided laboratory chow (Purina, New Brunswick, NJ) and water *ad libitum*. Rooms were kept at a constant temperature with a 12-hour light/dark cycle. The animals used in this study were handled in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (National Academy Press, 1996) and the Animal Welfare Act of 1966, as amended.
The experiment was carried out using a computer generated random block design containing 5 blocks. Mice were treated by oral gavage on the morning of GD 11 with all-trans RA (Sigma Chemical, MO) suspended in soybean oil. Volumes of dosing solutions were adjusted by maternal body weight to provide 0 (vehicle), 2.5, 10, 30, 60, or 100 mg/kg all-trans RA. All work with retinoids was conducted under dim yellow light to prevent breakdown. Mice remained in home cages until GD 18 when they were sacrificed by CO\textsubscript{2} asphyxiation. There were no maternal deaths in any of the treatment groups. There was no difference in the number of dams dosed within a treatment group and the number of litters examined within a treatment group (Table 3.1). As fetuses were removed from the uterus, they were immediately given an intra-cardiac injection of pentobarbital. After weights and crown-rump lengths were recorded, fetal heads were removed, preserved in 10% formalin and sent to the National Center for Toxicological Research (NCTR; Jefferson, AR) for determination of cleft palate incidence and brain weight. Fetuses were then eviscerated and fixed in 95% ethanol for subsequent staining.

**Staining Procedure for Fetuses**

The double staining technique, similar to that described by Whitaker and Dix (1979) was used. Briefly, eviscerated fetuses were placed in a 70°C water bath for 7 seconds, the skin was subsequently removed, and fetuses were fixed in 95% ethanol for approximately 24 hours. After draining the ethanol, fetuses were placed in an alcian blue (Sigma Chemical) solution (150 mg alcian blue in 800 ml of 95% ethanol and 200 ml glacial acetic acid) for 20 hours to stain cartilage. The alcian blue solution was drained and replaced with 95% ethanol for an additional 8 hours. Fetuses were then placed in a 0.35% KOH (Sigma Chemical) solution overnight to clear the tissue. Once the KOH solution was drained, fetuses were submerged in a solution containing 25 g of alizarin red S (Sigma Chemical) in 1000 ml of 0.2% KOH for approximately
4 hours to stain ossified areas. The alizarin red S solution was then drained and fetuses were placed in a 1:1 solution of 70% ethanol and glycerin. Forelimbs were excised from fetuses prior to visual inspection and computerized image analysis.

**Morphological Analysis Using Visual Inspection (VI) and Computerized Image Analysis (CIA) of the Fetal Forelimb**

For VI and CIA morphological evaluations, the fixed limb was placed in a Petri dish containing deionized water and covered with a glass slide. Images were captured using an Olympus dissecting microscope outfitted with a Sony CD-4 digital video camera. Care was taken to maintain identical settings on the microscope and to orient the right and left limbs in the same manner.

For VI analysis, changes in overall size and shape of the ossified regions were recorded as normal or abnormal. Forelimb data were expressed as percent of fetuses examined with at least one malformed forelimb bone, per dose group. The total number of malformed fetuses per dose group was calculated as the percent of fetuses that had at least one defect in a limb bone or cleft palate.

For CIA, a Leica Quantimet 570c Image Analysis System was used to save and measure images. Each day, the system was calibrated before limbs were imaged and prior to measuring images. Measurements were taken for the scapula (data not shown) and the three long bones (i.e., humerus, radius and ulna). The maximal widths at both the proximal and distal ends of each bone were determined. Lengths were determined between the midpoints of the proximal and distal ends of each of the four bones. The image analysis program calculated area, perimeter and roundness. While area was determined as the total number of pixels within the bone, perimeter and roundness were determined using the following equations:
Equation 1. \[ \text{Perimeter} = (2 \times \text{Horizontal projection} + 2 \times \text{Vertical projection}) \]
\[ - (0.65 \times \text{Corner count}) \]

Equation 2. \[ \text{Roundness} = (\text{perimeter} \times 1000) / (4 \times \pi \times \text{area}) \]

**Statistical Analyses**

Quantal VI percentages and continuous CIA measurements were averaged by dam prior to analysis. Analysis of variance (ANOVA) (2-sided, \( \alpha = 0.05 \)) was used to determine overall significant differences between treatment groups. When overall significant differences were found, pairwise comparisons were performed by Dunnett’s test (2-sided, \( \alpha = 0.05 \)) to determine if significant differences existed between each treated group and the control group. All statistical analyses were performed using SAS Version 8.2 (SAS Institute, Cary, NC).

Benchmark dose analyses were performed to compare differences in dose response characteristics of forelimb malformations quantified by VI and CIA. Benchmark dose analysis was implemented using the BenchMark Dose (ver. 1.3.1) software package obtained from the U.S. Environmental Protection Agency (www.epa.gov/ncea/bmds.htm). The BMDL was defined as the 95% lower confidence limit of the benchmark dose central estimate (BMD) corresponding to the benchmark response (BMR) level. Benchmark dose analysis was performed only on dose responses that showed a significant treatment effect above control response levels.

Quantal data (i.e., morphological endpoints) were modeled based on counts of affected fetuses per dam and counts of dams with at least one affected fetus. The BMR was defined as 5% extra risk, which has previously been shown to be comparable to the NOAEL for developmental toxicity studies (Allen *et al.*, 1994). The dichotomous quantal linear model was used for dose response analyses of the percent dams with \( \geq 1 \) affected fetus (Table 3.1). The nested NLogistic model was used for the VI measurements of the incidence of fetal forelimb...
malformations and cleft palate. Model adequacy was determined visually and by the chi-square goodness-of-fit test ($\alpha=0.10$) as described in U.S. EPA (2000).

Continuous endpoints (i.e., length, area and roundness of each bone) were averaged by dam prior to analysis. The Hill model was used to model continuous measurements. This model was chosen because it dichotomizes the data allowing the BMR to be defined as 5% extra risk. For the continuous datasets, model adequacy was not achieved using all the doses tested. In order to achieve model adequacy, the continuous datasets were fitted without the highest dose (100 mg/kg) as recommended by the U.S. EPA (2000). This is reasonable given that the highest dose studied is the least relevant for extrapolation to the low-dose region.

**Results**

**Visual Inspection**

Teratogenic outcomes for cleft palate and limb defects were quantified by VI in pregnant CD-1 mice administered a single oral dose of *all-trans* RA on GD-11 (Table 3.1 and Figure 3.1). At least 10 litters were examined for each dose group (2.5, 10, 30, 60 and 100 mg/kg), including controls (Table 3.1). There was no noticeable change in maternal food consumption or body weight, and there were no maternal deaths in any of the treatment groups. The average number of fetuses per dam and their average body weight did not differ across treatment groups. The incidence of cleft palate was significantly greater ($p<0.05$) than that of the control group for dams receiving 30 mg/kg or greater *all-trans* RA. The percentage of forelimb defects and number of fetuses with at least one forelimb or cleft palate malformation were significantly greater ($p<0.05$) than that of the control group at all doses, except 2.5 mg/kg. At approximately 30 mg/kg *all-trans* RA, the percent fetuses affected reached a plateau for limb defects, total malformed fetuses and percent dams affected (Figure 3.1). In contrast, cleft palate incidence
increased in a linear fashion over the dose range tested for \textit{all-trans} RA with a maximum incidence of about 70\% for animals receiving 100 mg/kg. The incidence of forelimb malformations was much steeper and quickly approached a maximum response of approximately 94\% with \textit{all-trans} RA doses greater than 30 mg/kg (Figure 3.1).

\textbf{Computerized Image Analysis}

For length and width CIA bone measurements, the radius of the forelimb was the most sensitive indicator of \textit{all-trans} RA exposure (Table 3.2). The radius was the only long bone that indicated a statistically significant difference at 30 mg/kg across all three measures (length, proximal width and distal width). The radius was the only long bone that showed significant decreases (p<0.05) in length with concomitant increases in both proximal and distal widths at the three highest doses tested (30, 60, and 100 mg/kg). The CIA measurement of bone length was the most consistent measurement to be affected significantly across all three long bones when compared to either the proximal or distal widths. The scapula was the least sensitive of all bones examined showing a significant change (p<0.05) only at the 100 mg/kg dose group (Table 3.2).

CIA measurement of shape (roundness) was consistently the most sensitive indicator of \textit{all-trans} RA exposure, with the ulna, humerus and radius significantly different (p<0.05) from controls at 10 mg/kg \textit{all-trans} RA and above (Table 3.2 and Figure 3.2). For the ulna area measurement, there was a biphasic response, in that the area was significantly increased (p<0.05) at 10 mg/kg followed by a significant decrease (p<0.05) at 100 mg/kg when compared to control area. For perimeter CIA measurements of the radius and humerus, the 30 mg/kg of \textit{all-trans} RA was the lowest dose that resulted in significantly different (p<0.05) values compared to control.
Benchmark Dose Evaluation of Fetal Malformations

The nested NLogistic model fit the dose response data sets for cleft palate, limb defects and total malformed fetuses (Table 3.3), and the BMDLs were all within a factor of 3. Incidences of limb defects and total malformed fetuses had equal BMDs (3.4 mg/kg) and BMDLs (2.2 mg/kg). The BMDL for the incidence of dams with at least one affected fetus (0.25 mg/kg) was an order of magnitude lower compared to cleft palate (5.0 mg/kg), limb defects (2.2 mg/kg) and total malformed fetuses (2.2 mg/kg). For the continuous measurements (CIA measurements), the Hill model estimated BMDLs from a low of 0.24 mg/kg (humerus roundness) to 7.6 mg/kg (humerus area) and were comparable to those determined by visual inspection.

Discussion

In this study, a single oral dose of all-trans RA (100 mg/kg) to pregnant mice on GD 11 resulted in 94% of the fetuses having at least one malformation. Our data does agree with previously published reports that administration of 100 mg/kg all-trans RA to pregnant dams’ results in a 90-100% malformation rate in mouse fetuses (Creech Kraft et al., 1989; Soprano et al., 1994). Similarly, at the lower dose of 10 mg/kg all-trans RA, Soprano et al. (1994) reported 39% malformed fetuses compared to our finding of 31%. The data reported in this study for fetal forelimb malformations do, however, differ from those of Hansen et al. (2001) who reported little or no forelimb malformations at doses up to 25 mg/kg all-trans RA followed by a sharp increase in incidence between 25 (0.9%) and 50 (96%) mg/kg. It is unclear why this difference was observed. The only major difference between the two studies appears to be the choice of vehicle with the Hansen group using corn oil compared to our use of soybean oil. However, the
present study and that of Hansen et al. (2001) are similar with respect to the incidence of cleft palate in GD 11 CD-1 mice.

Fetal limb defects resulting from *all-trans* RA showed a very steep dose-response relationship. The response plateaued near 100% between 30 and 60 mg/kg *all-trans* RA. A rapid dose-dependent increase in forelimb malformation incidence was observed for the 2.5, 10 and 30 mg/kg dose groups. For cleft palate, the incidence reached about 70% at the 100 mg/kg dose group, while there was no detectable effect when 2.5 mg/kg of *all-trans* RA was administered to pregnant mice on GD 11. The distinct differences in dose response curve shapes suggest that the mechanisms of action and/or sensitivity of response to *all-trans* RA are different for limb defects and cleft palate. However, this observation is most likely the result of differential timing in the formation of the palate as compared to the long bones of the fetal forelimb.

CIA measurements compared favorably with the VI results for fetal forelimbs. When dose-response relationships were evaluated using BMD methodology, the calculated BMDLs from the VI group ranged from 0.25 to 5 mg/kg and the CIA group ranged from 0.24 to 7.6 mg/kg. The most sensitive endpoint for the CIA was ‘roundness’, which was significantly different for the humerus, radius, and ulna at RA doses of 10 mg/kg and above. Likewise for VI, 10 mg/kg was the lowest *all-trans* RA dose that was significantly different from controls for both fetuses with forelimb malformations and with forelimb and/or cleft palate malformations. The most sensitive endpoint for a BMDL calculation from the VI data was number of dams with at least one malformed fetus.

One purpose of this study was to determine dose-response relationships between maternally administered doses of *all-trans* RA on GD 11 and fetal forelimb malformations and
fetal cleft palate as measured on GD 18. The dose-response curves for all-trans RA-induced fetal forelimb malformations were very steep and can be described as a linear process for all-trans RA doses of 2.5 mg/kg to doses of about 30 mg/kg (control to \(\approx 10\) mg/kg for dams with at least one malformed fetus), at which point adverse responses were recorded in nearly all fetuses. The second purpose of this study was to compare VI to CIA. The calculated BMDL values were similar for data analyzed by VI or CIA techniques, thus the CIA technique was not a more sensitive method for detecting limb malformations. In addition, CIA is more time-consuming and logistically difficult to complete. However, CIA may be useful for teratological investigations where quantitative data are useful.

Acknowledgments

The authors would like to thank Dr. J.H. Grabau for his efforts in developing the procedures for image analysis, Sgt. W.R. Helton for his help in acquiring image analysis data and Dr. Deborah Keys for her assistance with statistical analysis. The authors are grateful to Dr. Bob Holson and his associates at the National Center for Toxicological Research for their assessment of cleft palate.

References


Table 3.1. Forelimb malformation and cleft palate data collected by visual inspection (VI) in CD-1 fetuses exposed orally to all-trans retinoic acid on GD 11.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>No. Litters&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. Live Fetuses</th>
<th>Avg. No. Fetuses per Dam&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Avg. Fetal Weight&lt;sup&gt;b&lt;/sup&gt; (g)</th>
<th>No. Dams with ≥ 1 Affected Fetus&lt;sup&gt;c&lt;/sup&gt; (%)</th>
<th>No. Fetuses with Cleft Palate&lt;sup&gt;d&lt;/sup&gt; (%)</th>
<th>No. Fetuses with Forelimb Malformations&lt;sup&gt;d&lt;/sup&gt; (%)</th>
<th>No. Fetuses Malformed w/ Cleft Palate and/or Limb Malformation&lt;sup&gt;d&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10</td>
<td>98</td>
<td>9.8 ± 1.9</td>
<td>1.16 ± 0.10</td>
<td>2 (20.0%)</td>
<td>0 (0.0 ± 0.0%)</td>
<td>2 (2.3 ± 4.8%)</td>
<td>2 (2.3 ± 4.8%)</td>
</tr>
<tr>
<td>2.5</td>
<td>23</td>
<td>200</td>
<td>8.7 ± 4.0</td>
<td>1.21 ± 0.16</td>
<td>8 (34.8%)</td>
<td>1 (0.5 ± 2.6%)</td>
<td>11 (4.9 ± 8.1%)</td>
<td>11 (4.9 ± 8.1%)</td>
</tr>
<tr>
<td>10</td>
<td>18</td>
<td>153</td>
<td>8.5 ± 3.2</td>
<td>1.24 ± 0.08</td>
<td>14 (77.8%)</td>
<td>12 (7.6 ± 11.8%)</td>
<td>28 (26.3 ± 29.4%)</td>
<td>37 (31.0 ± 30.7%)</td>
</tr>
<tr>
<td>30</td>
<td>25</td>
<td>216</td>
<td>8.6 ± 3.4</td>
<td>1.18 ± 0.11</td>
<td>25 (100.0%)</td>
<td>58 (26.3 ± 19.8%)</td>
<td>183 (83.0 ± 18.0%)</td>
<td>187 (85.4 ± 19.4%)</td>
</tr>
<tr>
<td>60</td>
<td>16</td>
<td>154</td>
<td>9.6 ± 3.4</td>
<td>1.21 ± 0.12</td>
<td>16 (100.0%)</td>
<td>72 (42.4 ± 28.3%)</td>
<td>143 (93.3 ± 7.0%)</td>
<td>146 (94.7 ± 7.1%)</td>
</tr>
<tr>
<td>100</td>
<td>18</td>
<td>192</td>
<td>10.7 ± 2.7</td>
<td>1.13 ± 0.13</td>
<td>18 (100.0%)</td>
<td>141 (73.7 ± 31.7%)</td>
<td>179 (93.2 ± 17.0%)</td>
<td>181 (94.5 ± 17.0%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>The number of litters corresponds to the number of dams treated. There were no maternal deaths in any group.

<sup>b</sup>The average number of fetuses and average fetal weight calculated per litter.

<sup>c</sup>Each dam had at least one fetus with either a limb defect or cleft palate.

<sup>d</sup>Number of fetuses affected; ( ) percent fetuses affected calculated on a per dam basis.

* Different significantly from control (p<0.05).
Table 3.2. Computer image analysis (CIA) of CD-1 fetal forelimbs after maternal oral exposure to \textit{all-trans} retinoic acid on GD-11$^a$

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Length (µm±SD)</th>
<th>Proximal Width (µm±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Humerus</td>
<td>Radius</td>
</tr>
<tr>
<td>0</td>
<td>2443 ± 161</td>
<td>2264 ± 164</td>
</tr>
<tr>
<td>2.5</td>
<td>2412 ± 167</td>
<td>2291 ± 163</td>
</tr>
<tr>
<td>10</td>
<td>2325 ± 121</td>
<td>2260 ± 128</td>
</tr>
<tr>
<td>30</td>
<td>1953 ± 165*</td>
<td>1987 ± 157*</td>
</tr>
<tr>
<td>60</td>
<td>1790 ± 233*</td>
<td>1869 ± 195*</td>
</tr>
<tr>
<td>100</td>
<td>1314 ± 277*</td>
<td>1397 ± 365*</td>
</tr>
</tbody>
</table>

*Significantly different from control (p<0.05).

$^a$Averages calculated on a per dam basis.

Table 3.2 cont’d.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Distal Width (µm±SD)</th>
<th>Area (µm$^2$±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Humerus</td>
<td>Radius</td>
</tr>
<tr>
<td>0</td>
<td>585 ± 40</td>
<td>336 ± 32</td>
</tr>
<tr>
<td>2.5</td>
<td>577 ± 50</td>
<td>347 ± 30</td>
</tr>
<tr>
<td>10</td>
<td>588 ± 47</td>
<td>360 ± 37</td>
</tr>
<tr>
<td>30</td>
<td>566 ± 36</td>
<td>405 ± 54*</td>
</tr>
<tr>
<td>60</td>
<td>571 ± 30</td>
<td>450 ± 55*</td>
</tr>
<tr>
<td>100</td>
<td>580 ± 45</td>
<td>480 ± 101*</td>
</tr>
</tbody>
</table>

*Significantly different from control (p<0.05).

$^a$Averages calculated on a per dam basis.
<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Humerus (µm±SD)</th>
<th>Radius (µm±SD)</th>
<th>Ulna (µm±SD)</th>
<th>Scapula (µm±SD)</th>
<th>Roundness (±SD)</th>
<th>Humerus (±SD)</th>
<th>Radius (±SD)</th>
<th>Ulna (±SD)</th>
<th>Scapula (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7020±511</td>
<td>6607±423</td>
<td>5492±375</td>
<td>6947 ± 442</td>
<td>2.20±0.14</td>
<td>2.84±0.13</td>
<td>2.85±0.14</td>
<td>1.53 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>7004±450</td>
<td>6693±393</td>
<td>5573±377</td>
<td>7102 ± 443</td>
<td>2.11±0.12</td>
<td>2.74±0.11</td>
<td>2.78± 0.15</td>
<td>1.53 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>6744±319</td>
<td>6553±342</td>
<td>5541±282</td>
<td>7054 ± 281</td>
<td>1.99±0.11*</td>
<td>2.58±0.13*</td>
<td>2.54±0.13*</td>
<td>1.52 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>6031±369*</td>
<td>5907±473*</td>
<td>5083±287</td>
<td>6839 ± 270</td>
<td>1.85±0.13*</td>
<td>2.16±0.23*</td>
<td>2.15±0.21*</td>
<td>1.50 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>5800±465*</td>
<td>5524±581*</td>
<td>4846±360*</td>
<td>6623 ± 194</td>
<td>1.91±0.22*</td>
<td>2.01±0.23*</td>
<td>1.95±0.19*</td>
<td>1.48 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>4867±748*</td>
<td>4325±584*</td>
<td>3575±865*</td>
<td>6390 ± 165*</td>
<td>1.69±0.28*</td>
<td>1.67±0.27*</td>
<td>1.54±0.40*</td>
<td>1.48 ± 0.03</td>
<td></td>
</tr>
</tbody>
</table>

*Significantly different from control (p<0.05).

*a Averages calculated on a per dam basis.
Table 3.3. Evaluation of maternally administered dose of *all-trans* RA and fetal malformations in GD-11 fetuses using benchmark dose analysis.

<table>
<thead>
<tr>
<th>Goodness of Fit p-Value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Doses Corresponding to BME&lt;sup&gt;b&lt;/sup&gt;</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BMD (mg/kg)</td>
<td>BMDL (mg/kg)</td>
<td></td>
</tr>
<tr>
<td>Visual Inspection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cleft Palate</td>
<td>0.1745</td>
<td>8.3</td>
<td>5.0</td>
</tr>
<tr>
<td>Limb defects</td>
<td>0.3131</td>
<td>3.4</td>
<td>2.2</td>
</tr>
<tr>
<td>Total Malformed Fetuses&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.2609</td>
<td>3.4</td>
<td>2.2</td>
</tr>
<tr>
<td>Litters with at least one affected fetus</td>
<td>0.9394</td>
<td>0.37</td>
<td>0.25</td>
</tr>
<tr>
<td>Computerized Image Analysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ulna</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length</td>
<td>0.9320</td>
<td>7.2</td>
<td>2.6</td>
</tr>
<tr>
<td>Distal Width</td>
<td>0.4867</td>
<td>5.1</td>
<td>1.5</td>
</tr>
<tr>
<td>Roundness</td>
<td>0.9716</td>
<td>2.3</td>
<td>1.1</td>
</tr>
<tr>
<td>Radius</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length</td>
<td>0.6512</td>
<td>9.9</td>
<td>4.0</td>
</tr>
<tr>
<td>Proximal Width</td>
<td>0.1341</td>
<td>4.1</td>
<td>1.3</td>
</tr>
<tr>
<td>Distal Width</td>
<td>0.6817</td>
<td>6.2</td>
<td>1.7</td>
</tr>
<tr>
<td>Perimeter</td>
<td>0.586</td>
<td>7.9</td>
<td>2.9</td>
</tr>
<tr>
<td>Roundness</td>
<td>0.2247</td>
<td>3.4</td>
<td>1.2</td>
</tr>
<tr>
<td>Humerus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length</td>
<td>0.6797</td>
<td>5.9</td>
<td>2.8</td>
</tr>
<tr>
<td>Perimeter</td>
<td>0.9964</td>
<td>4.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Area</td>
<td>0.169</td>
<td>24.6</td>
<td>7.6</td>
</tr>
<tr>
<td>Roundness</td>
<td>0.1986</td>
<td>0.74</td>
<td>0.24</td>
</tr>
</tbody>
</table>

<sup>a</sup>Resulting p values used to assess the adequacy of the models for describing the data as described in U.S. EPA (2000).

<sup>b</sup>The benchmark effect (BME) level is the dependent variable value (5%) for which the benchmark doses (BMD) and their 95% lower confidence limits (defined as the benchmark dose lower, BMDL) are calculated (U.S. EPA, 2000).

<sup>c</sup>Total Malformed Fetuses = cleft palate and/or limb malformations
Figure 3.1. Dose-response for pregnant CD-1 mice exposed on gestation day 11 to a single gavage dose of *all-trans* retinoic acid. The responses for cleft palate, forelimb defects and total fetuses malformed (forelimb defects and/or cleft palate) are the average of the percent fetuses affected per dam ± SD.
Figure 3.2. Dose-response for pregnant CD-1 mice exposed on gestation day 11 to a single gavage dose of \textit{all-trans} retinoic acid. The response for roundness is calculated per dam ± SD.
CHAPTER 4

PHYSIOLOGICALLY BASED PHARMACOKINETIC MODEL FOR *ALL-TRANS RETINOIC ACID* IN PREGNANT CD-1 MICE

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Abstract

all-trans Retinoic acid (RA) is a well documented teratogen in laboratory animals as well as humans. When administered to mice on gestation day 11 (GD-11), RA causes severe alterations in fetal forelimbs and cleft palate. A nine compartment physiologically-based pharmacokinetic model (PBPK) for RA and a four compartment PBPK sub-model for its metabolite, the 13-cis retinoic acid (13cisRA) isomer was developed to describe the uptake, fetal transfer, and systemic clearance of RA in pregnant GD 11 CD-1 mice. This model was adapted from a previously published RA model (Clewell et al., 1997) to include endogenous production of RA and induction of RA metabolism in the maternal liver. Maternal blood and fetal tissue time-course kinetic data was collected from pregnant CD-1 mice on GD 11 after a single oral bolus dose (2.5, 10, 30, 60, and 100 mg/kg) of RA suspended in soybean oil. Analyses of fetal tissues and maternal plasma for RA and 13cisRA were by HPLC (after solid phase extraction). The systemic clearance of RA by metabolism was described by two metabolic pathways; isomerization to 13cisRA and a composite pathway representing both P450 and glucuronidation metabolic pathways. Placental transfer of RA and 13cisRA to the fetal compartment was described as diffusion limited. Metabolism of 13cisRA was described as a composite pathway including P450 and glucuronidation. To obtain adequate fits to the kinetic data the composite metabolic pathway for RA was assumed to be induced in a dose dependent manner across the range of doses examined. Diffusion limited transfer of RA and 13cisRA to the embryo were also well described by the model for all doses tested. Both model-predicted AUC and maximum concentration were determined to be good measures of fetal exposure and could be used in place of maternally administered dose resulting in reduced uncertainty when extrapolating risks across species. Key Words: all-trans retinoic acid, PBPK model, pregnancy, mouse
Introduction

*All-trans* retinoic acid (RA), an endogenous metabolite of vitamin A, is required for pattern formation during embryogenesis. Excessive concentrations in both experimental animals (Kochhar, 1973) and humans (Lammer et al., 1985; Rosa, 1983) result in fetal malformation. In humans, the teratogenic effects of retinoids were clearly demonstrated in the 1980’s when Accutane, an endogenous metabolite of RA (13-*cis* retinoic acid, 13cisRA), was marketed for use as a treatment for acne. Adverse effects included malformations of the ears, heart, brain and thymus, and reduction in IQ (Lammer et al., 1985; Rosa, 1983). However, in rodents 13cisRA toxicity only occurs at doses near 100 mg/kg with only 2% of fetuses expressing malformed limbs while there is no increase in incidence of cleft palate (Kraft et al., 1987). Rodents have a much lower capacity for placental transfer of 13cisRA and isomerization of 13cisRA to RA when compared to primates, thus a lower exposure to the most active form of vitamin A (Nau, 2001; Nau, 1995; Zile et al., 1982). RA has been shown to produce severe limb defects and craniofacial malformations in mouse embryos when treatment occurs between gestation days (GD) 11 and 14.5 (Campbell et al., 2004; Kwasigroch and Kochhar, 1980; Kochhar et al., 1996; Hansen et al., 2001), the sensitive period for development of these tissues.

Because RA is a highly lipophilic weak acid, the kinetics of a single oral dose includes a rapid uptake and distribution phase within maternal tissues along with transfer to the embryonic compartment followed by a period of rapid elimination with measurable quantities only detectable up to 8 h after dosing (Satre and Kochhar 1989; Tzimas et al., 1995). Peak concentrations in maternal and embryonal tissues are reached within 2 h and the half-life of RA after oral exposure is 0.57 to 1.02 h (Le Doze et al., 2000). The metabolism of RA *in vivo* and is rapid with metabolites appearing in plasma as early as 0.5 h after oral dosing (Tzimas et al.,
1995). Under normal conditions, RA is produced by metabolism of retinol in the tissue where RA is needed. Metabolism of RA occurs through three primary routes including: isomerization to 13cisRA, glucuronidation to RAG (retinoyl-β-glucuronide), and hydroxylation (phase I metabolism via P450 enzymes, CYPs 26, 3A7, 2C8, 4A11, 1A1, 3A4/5) to 18-OH RA and 4-OH RA. In the mouse, metabolism is primarily by P450 while in humans glucuronidation serves as the primary route (Zile et al., 1982; Swanson et al., 1981; Kraft et al., 1991).

Current approaches for assessment of human teratogenic risk depend largely on dose-response models fit to pregnancy outcome data from high-dose animal studies. The lack of quantitative information about the biological sequelae of exposure and the shape of the consequent dose-response curve result in a large degree of uncertainty in extrapolations across doses and species. PBPK pregnancy modeling has been used previously to describe the dose of exogenous chemicals to the fetus to reduce this uncertainty (for a review see Corley et al., 2003). Clewell et al. (1997) published a RA model which described the disposition of low oral doses and skin absorption of RA in the rat, primate, and human. However, several limitations were uncovered when the model was employed to describe our dose dependent changes in fetal RA kinetics over a wide range of teratogenic doses in the mouse.

Elucidating the relationship between maternal plasma and embryonic levels of RA and the increase in malformations is one step toward a better understanding of fetal response to this teratogen. Fetal exposure occurs because of the transfer of RA in maternal plasma across the placenta to the fetal tissue. The PBPK model developed in this study describes the relationship between maternal and fetal kinetics of RA and 13cisRA across a wide range of administered doses in GD 11 CD-1 mice.
Methods

Animals and Dosing

Timed-pregnant CD-1 mice were obtained on GD 8 from Charles River Laboratories and housed in an AALAC accredited facility using standard animal care guidelines. Mice were dosed by oral gavage on the morning of GD 11 with all-trans RA suspended in soybean oil. Doses were adjusted by maternal body weight to provide 0 (vehicle), 2.5, 10, 30, 60, or 100 mg/kg. Dams were sacrificed by CO$_2$ asphyxiation at 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, and 8.0 hours for time course analysis with an n of 3 to 5 animals for each dose/time point. Maternal plasma and embryos were harvested and stored at -80°C until further analysis. For determinations of RA and 13cisRA in embryos, the embryos within a litter were combined and treated as one sample. Dosing, tissue collection, and analysis were completed under dim yellow light to prevent the breakdown of RA as a result of UV radiation.

RA Analysis

Maternal plasma and embryo samples were extracted by solid phase extraction and analyzed for RA and 13cisRA using a modified reverse-phase HPLC method developed by Eckoff (1996). An aliquot of plasma (0.1 ml) was mixed with an internal standard (Ro 10-1670, Hoffmann-La Roche Inc., Nutley, NJ) and deproteinated with 2-propanol (0.25 ml, Fisher Scientific, Pittsburg, PA) followed by centrifugation (1250 x g for 5 min). Embryos, pooled for each dam, were homogenized by repeatedly sucking the sample up into a 1.0 ml pipet with a portion of the tip removed. A weighed portion (20-140 mg of tissue) was then mixed with internal standard and 0.45 ml of 2-propanol for deproteination. Embryo samples were pulse sonicated to liquefy the sample and after centrifugation (1250 x g for 5 min), samples were dried to approximately 0.25 ml before solid phase extraction. The supernatant was transferred onto a
C8 Bond Elut® (Varian, Inc., Walnut Creek, CA) solid phase extraction cartridge and eluted using a mixture of triethylamine, methanol, and ethanol (1:24:75, Fisher Scientific) on a Vac-Elut (Varian, Inc.) apparatus with a flow rate of 1 ml/min. Once eluted, samples were dried under nitrogen and reconstituted in 1% acetic acid:acetonitrile (60:40, Fisher Scientific). HPLC analysis was carried out with a Hewlett Packard Series 1050 HPLC with a diode array detector set to 345 nm. Samples were separated on a Spherisorb ODS 2 (Hewlett Packard, 5 µm 250 x 4 mm) column at 40°C with a 1% acetic acid:acetonitrile gradient mobile phase. The mobile phase gradient was 57% acetonitrile at 0 min, 65% acetonitrile at 12 min, and 90% acetonitrile at 20 min. The method limit of quantification was 6.7 ng/ml for both RA and 13cisRA.

Model Development

PBPK modeling was performed in acslXtreme (ver. 1.4, Aegis Technologies, Huntsville, AL). The PBPK model for RA and 13cisRA (Figure. 4.1) is modified from an RA model previously published by Clewell et al. (1997). Parameter values used in this model are listed in Tables 4.1 and 4.2. The model for RA was described with nine compartments and the sub-model for 13cisRA was described with four compartments. Oral uptake of RA was described with two non-physiological compartments as in Staats et al. (1991). A 1st order rate loss for metabolism of RA by intestinal mucosa was placed in the second compartment for oral uptake (Ahmad et al., 2000). Rate constant values for oral uptake and intestinal metabolism were visually fit to maternal plasma concentrations to describe the uptake for all RA doses. All compartments are assumed to be flow-limited for RA and 13cisRA except for placental transfer which was described as diffusion-limited (Clewell et al., 1997; Fisher et al., 1989; Terry et al., 1995). Basal endogenous production of RA was included in the PBPK model. The rate of production for RA was described as zero order with the rate constant value determined by fitting to the endogenous
maternal plasma level (Satre and Kochhar, 1989). After preliminary simulations with the endogenous production of RA, the initial concentration of RA in all compartments was set to steady state concentrations. Fetal weight was estimated for the GD 11 mouse using the equation described by O’Flaherty et al. (1992). The equation describes the weight of an individual fetus which was then multiplied by a factor of 10 (as an average number of fetuses per dam) to get a total weight of fetuses in utero.

After several iterations whereby both the uptake and clearance of RA were simulated with a single set of rate constants, the model failed to describe the experimental data across all doses tested (i.e., over prediction of plasma and embryo RA concentrations). It is well known that RA is metabolized by three main processes including isomerization, P450 hydroxylation, and glucuronidation (Maden, 2000). In the rodent, the primary routes include isomerization and hydroxylation while in the nonhuman primate and human, isomerization and glucuronidation serve as the primary routes (Zile et al., 1982; Swanson et al., 1981; Kraft et al., 1991). Recent studies of RA metabolism via P450 pathways have shown that RA induces P450 isoforms (primarily CYP26) responsible for metabolism. Yamamoto et al. (2000) originally showed that rats given a vitamin A supplement had higher levels of CYP26 mRNA when compared to control animals, and vitamin A deficient rats displayed reduced levels of CYP26 mRNA when RA was administered. In a subsequent publication Wang et al. (2002) elucidated that the induction of CYP26 was under the control of RA, an active metabolite of vitamin A. These researchers reported that CYP26 mRNA was elevated within the first 3 hrs after dosing with RA and remained elevated up to 24 hrs post dosing. The auto-induction of RA metabolism was confirmed by the analysis of data from intravenously dosed rats (el Mansouri et al., 1995). This research demonstrated that the clearance of RA increased at 180 min post dosing (i.e., an
increase in the slope of the clearance curve). As the initial modeling efforts failed to describe the clearance of RA across all doses, the final model included nonlinear or saturable Michaelis-Menten metabolism of RA to 13cisRA in the maternal liver with P450 and glucuronidation metabolic pathways being combined as an inducible non-linear metabolic pathway. The production of polar metabolites (composite pathway) is modeled with a fold induction of $V_{mgb}$ (the basal maximum velocity of RA metabolism via P450) occurring between 1 and 2 h after dosing for the 30, 60, and 100 mg/kg dose groups and then returning to the basal rate between 2 and 8 h. No induction was used to model doses of 2.5 or 10 mg/kg RA. The auto-induction (fold increase) was fit visually to the clearance of RA from maternal plasma for the three doses that included induction, as were the maximum velocity and $K_m$ for the production of 13cisRA.

RA and 13cisRA distribution ratios (e.g., partition coefficients) for maternal compartments were estimated from published data (Wang et al., 1980). For the maternal compartments, the concentration in the tissue was divided by the concentration in the plasma at 240 min post intravenous injection of RA. Partitioning into the embryo was estimated from the terminal phase of the experimental data collected in this study (i.e., the concentration in embryo and plasma at 6 h post dosing for the 100 mg/kg dose). All partition coefficients were calculated as the tissue concentration divided by the plasma concentration.

Model Validation

Model validation was accomplished with published data from Kraft et al. (1987) who gavaged GD 11 pregnant mice with a single dose of 100 mg/kg RA. Data were digitized from graphs using GrabIt! XP (Datatrend Software, Raleigh, NC).
Sensitivity Analysis

An analysis was performed of the sensitivity of model-predicted plasma (CP) and embryo (CE) RA and 13cisRA concentrations to the model parameters employed in this PBPK model (acslXtreme, ver. 1.4). The central differences method was selected for calculation with delta set to 0.01 or 1%. Sensitivity coefficients were log-normalized and then multiplied by their respective parameter value. The resulting sensitivity coefficient can be interpreted as the ratio of the standardized change in model response (output) to the standardized change in parameter values (input) (Haefner, 1996 and Perleberg et al., 2004). Parameter values greater or less than 0.5 were deemed to be sensitive. The RA PBPK model was exercised with a concentration of 100 mg/kg, and sensitivity coefficients were calculated for 1-, 2-, and 6-h time points for maternal plasma and embryo.

Results

Model Development

Early modeling efforts were not able to describe the uptake and clearance of RA from plasma across the dose range administered in this study, especially after 2 h. The metabolism of RA was initially described as a Michaelis-Menton saturable process (Figure 4.2a) with a single set of rate constants applied to all dose groups. However, because recent experimental evidence (el Mansouri et al., 1995; Wang et al., 2002; Yamamoto et al., 2000) indicated that RA induces its own metabolism, we added a table function to the model to reflect the increased clearance seen at higher doses of RA and this resulted in a fold increase in Vmax above the basal rate to fit the clearance of RA from plasma (Figure 4.2b). The change in Vmax for global metabolism was increased with doses greater than 10 mg/kg and included a 2-fold (30 mg/kg), 5-fold (60 mg/kg), and 7-fold (100 mg/kg) increase at 2 h post dosing.
Model Simulations

Overall the model output predicted the experimental data across all five doses administered. The appearance of RA in maternal plasma was adequately described (Figure 4.3) except for the 10 mg/kg dose (Figure 4.3b), where the experimental data was approximately 2 times the model predicted value. At the two highest doses, 60 (Figure 4.3d) and 100 (Figure 4.3e) mg/kg, the model output was roughly 3 times that of the experimental data for the oral uptake (1st hour post dosing). However, the model was able to predict the clearance of RA from maternal plasma at these doses. For the 2.5 mg/kg (Figure 4.3a) and 30 mg/kg doses (Figure 4.3c), the model described the uptake and clearance of RA from maternal plasma. The appearance and clearance of 13cisRA from maternal plasma was also adequately predicted (Figure 4.4), especially for 10 and 30 mg/kg (Figure 4.4a,b) RA doses. However, at 60 and 100 mg/kg RA (Figures 4.4c,d) the model under predicted the peak concentrations of 13cisRA. 13cisRA was detectable in samples from the 2.5 mg/kg dose group, however they were below the limit of quantification for both maternal plasma and embryos.

Model predicted concentrations of RA in the embryo were adequately fit to the experimental data (Figure 4.5) and followed a similar pattern to the maternal plasma predictions. Overall, the pattern was similar to that of the maternal plasma with the model slightly under predicting the concentrations at the 2.5 and 10 mg/kg (Figure 4.5a,b). For the 10, 60 and 100 mg/kg RA doses (Figure 4.3c,d,e), the model fit the experimental data across the entire time course and only slightly over-predicted the peak concentration at the highest dose. Embryo 13cisRA model predictions (Figure 4.6) followed that of the maternal plasma. Measured concentrations of 13cisRA in the embryo were adequately predicted at 10 and 30 mg/kg RA.
doses (Figure 4.6a,b) with the 60 and 100 mg/kg RA doses being slightly under predicted at the peak tissue concentration.

The model predicted AUC (0 to 12 h post dosing) and Cmax in the embryo are shown in Figure 4.7. AUC or Cmax values for the embryo show the nonlinear kinetics of RA across the range of doses tested and, based on the similarity when plotted against administered dose; either measure would be acceptable for target tissue dose.

**Model Validation**

The time course data from GD11 mice orally dosed with 100 mg/kg RA reported by Kraft et al. (1987) was simulated in order to validate the current model (Figure 4.7). Good agreement between the model predicted and the experimental data were achieved for RA in maternal and embryonic compartments (Figure 4.8a,c). The results were similar for that of the currently reported time course data where the model slightly over predicted the peak concentration in both maternal plasma and embryos. Similar results were also seen with the model predicted concentrations of 13cisRA (Figure 4.7b,d). Model predicted 13cisRA was in agreement with the measured concentrations in both the embryo and maternal plasma.

**Sensitivity analysis**

Several model parameters were found to be sensitive for predicting maternal plasma and embryo RA and 13cis concentrations. The volume of the liver was sensitive for predicting maternal plasma concentration, the volume of the embryo compartment and the RA embryo/plasma partition coefficient were sensitive for predicting the concentration of RA in the embryo, and the 13cisRA embryo/plasma partition coefficient was sensitive for 13cisRa concentration in embryo (Table 4.3). Several model parameters including K2, K3, basal global metabolism maximum velocity for RA, and maximum velocity for metabolism of RA to
13cisRA were sensitive for all target parameters (Tables 4.4 and 4.5). The affinity constant for 13cisRA production from RA, the intestinal metabolic rate for RA, and the clearance rate for 13cisRA were all sensitive for predicting the concentration of 13cisRA in maternal plasma and embryo compartments.

**Discussion**

This PBPK model describes the uptake, elimination, and fetal transfer of RA in the pregnant mouse after a single oral dose during GD 11, a sensitive window for embryonic development. RA is the most active of the Vitamin A derivatives and its metabolism is thought to be a detoxification step under conditions of excess RA. The final model was able to describe the time course of RA in the GD 11 pregnant mouse across a wide range of doses. Auto induction of RA metabolism was incorporated into the model to successfully predict maternal plasma and embryo RA concentrations. Under normal conditions, conversion of RA from retinol occurs in the target tissue along with metabolism of RA. Model prediction was in general agreement with experimental observations. The predicted concentrations of 13cisRA were under-predicted at the peak maternal plasma and embryo concentrations for the two highest administered doses (30 and 60 mg/kg) while the lower administered doses (10 and 30 mg/kg) were well described by the model.

The oral uptake kinetics of RA administered in oil was difficult to fit across the administered dose range of 2.5 to 100 mg/kg using a single set of oral uptake rate constants (e.g., K2 and K3). After several iterations these two rate constants were set to allow for the best fit across the entire range of doses administered. However, there is some limited evidence that the oral uptake of RA from the GI tract is dose dependent. Saadeddin et al. (2004) determined the bioavailability of RA was roughly 40% and attributed this to a limited oral uptake of RA,
suggesting that active transport mechanisms play a role in oral RA absorption. A comparison of orally to subcutaneously administered RA showed distinct differences in the plasma time curves of RA (Tzimas et al., 1997). The clearance of RA was much slower after subcutaneous injection (AUC was 2.5 times oral dose AUC) and the concentration of RA metabolites were significantly lower. Therefore, we assumed that differences in kinetics are due to rapid uptake and metabolism of RA in the liver (i.e, first pass effect) than reduced oral absorption. Tzimas et al. (1997) reported that the maternal plasma AUC of RA was a better indicator of fetal dose than the maternally administered dose or both the Cmax for maternal plasma or the Cmax for embryo.

The data collected for this model show that the relationship between administered dose and the kinetics of RA were nonlinear (AUC\(_{(0-12h)}\) vs. dose administered). Because of this phenomenon, the use of maternal dose may not fully describe fetal exposure to RA. This became evident when trying to fit the higher doses administered in this study with the same rate constants for RA metabolism used for the lowest doses administered. Recent experimental evidence showed that RA induces its own metabolism (el Mansouri et al., 1995; Wang et al., 2002; Yamamoto et al., 2000). Because these studies were generally conducted with a single administered dose, it was necessary to determine the overall increase in RA metabolism in whole animals across a wide range of exposure levels in order to better understand the overall maximum up-regulation of metabolism caused by RA. RA metabolism was described simply by combining both P450 and glucuronidation to gain initial insights into the nonlinear behavior of RA kinetics as a function of dose. Further research is needed to determine the specific isoforms of P450 that are induced by RA, and the overall impact of this increase on systemic clearance of RA.
To explore the effect of metabolism on the kinetics of RA, the metabolic rate of RA was increased with maternal dose. This included adding a metabolic term in the intestine and combining the P450 and glucuronidation metabolic pathways. A dose dependent increase in the metabolic capacity of the lumped metabolic pathway was employed to fit the clearance of RA from maternal plasma with a single set of oral uptake rate constants. The metabolism of RA to 13cisRA, one of the primary metabolites of RA, was incorporated as a saturable Michaelis-Menton process in the liver compartment. The Vmax for metabolism to 13cisRA and the clearance of RA were determined by visually fitting the model to the experimental data.

The RA model simulated previously published kinetic data (Kraft et al., 1987). As with data collected for model development, the peak concentration of RA in both the maternal plasma and embryo were slightly over predicted. The model was also able to predict the maternal plasma and embryonic concentrations of 13cisRA.

The PBPK model developed for RA described the oral uptake, maternal metabolism, and fetal disposition across doses ranging from 2.5 to 100 mg/kg. Overall the experimental data were adequately predicted by the PBPK model across the range of doses tested. The PBPK model did slightly over-predict the maternal plasma concentrations in the first 1 to 2 h post dosing for the 60 and 100 mg/kg doses, while at the same time slightly under-predicting the maternal plasma concentrations for the 10 mg/kg dose. Also, the peak fetal concentrations of RA (2 h post dosing) were under-predicted by the PBPK model for both the 2.5 and 10 mg/kg dose. The model-predicted dosimetrics (AUC$_{(0-12h)}$ and Cmax) represented the maternally administered dose. With the inclusion of auto-induction, the model predicted AUC$_{(0-12h)}$ or Cmax (data not shown) were able to describe the non-linearity of the dose-response curve, showing a rapid rise in percent affected between maternal doses of 2.5 and 30 mg/kg followed by
a plateau in the response (Figure 4.9). As the PBPK model was able to describe the disposition of RA to the fetal compartment across a wide range of doses, this model may be useful for estimating fetal exposure with the addition of alternate routes of maternal exposure to RA (i.e., dermal) and extrapolation humans.

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Table 4.1. Physiologic parameters for the pregnant mouse (GD 11).

<table>
<thead>
<tr>
<th>Compartment volumes (V)</th>
<th>Pregnant Mouse</th>
<th>Value</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight BW (kg)</td>
<td></td>
<td>0.035</td>
<td></td>
</tr>
<tr>
<td>Slowly perfused VSC (%BW)</td>
<td></td>
<td>0.656</td>
<td>Brown et al., 1997</td>
</tr>
<tr>
<td>Richly perfused VRC (%BW)</td>
<td></td>
<td>0.081</td>
<td>Brown et al., 1997</td>
</tr>
<tr>
<td>Liver VLC (%BW)</td>
<td></td>
<td>0.055</td>
<td>Brown et al., 1997</td>
</tr>
<tr>
<td>Fat VFC (%BW)</td>
<td></td>
<td>0.089</td>
<td>O’Flaherty et al., 1992</td>
</tr>
<tr>
<td>Placenta VPLC (%BW)</td>
<td></td>
<td>0.00016</td>
<td>O’Flaherty et al., 1992</td>
</tr>
<tr>
<td>Embryo VEC (mg)</td>
<td></td>
<td>48.0</td>
<td>O’Flaherty et al., 1992</td>
</tr>
<tr>
<td>Plasma VPC (%BW)</td>
<td></td>
<td>0.049</td>
<td>Brown et al., 1997</td>
</tr>
<tr>
<td>Intestine (%BW)</td>
<td></td>
<td>0.0262</td>
<td>Brown et al., 1997</td>
</tr>
<tr>
<td>Body water (%BW)</td>
<td></td>
<td>0.65</td>
<td>Clewell et al., 1997</td>
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Blood Flows (Q)

<table>
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<tr>
<th>Blood Flows (Q)</th>
<th>Pregnant Mouse</th>
<th>Value</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiac output QCC (L/hr/kg)</td>
<td></td>
<td>15</td>
<td>Clewell et al., 1997</td>
</tr>
<tr>
<td>Slowly perfused QSC (%QC)</td>
<td></td>
<td>0.21</td>
<td>O’Flaherty et al., 1992</td>
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<td>0.47</td>
<td>O’Flaherty et al., 1992</td>
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<tr>
<td>Liver QLC (%QC)</td>
<td></td>
<td>0.25</td>
<td>Andersen et al., 1987</td>
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<tr>
<td>Fat QFC (%QC)</td>
<td></td>
<td>0.03</td>
<td>O’Flaherty et al., 1992</td>
</tr>
<tr>
<td>Placenta QPLC (%QC)</td>
<td></td>
<td>0.02</td>
<td>O’Flaherty et al., 1992</td>
</tr>
</tbody>
</table>

*a* Volume of embryo compartment estimated using the equation from O’Flaherty et al. (1992). Estimated as the sum of 10 embryos weighing 4.8 mg each and a tissue density of 1 g/ml.

*b* Volume of intestine used to calculate loss of RA from second compartment of GI tract.
Table 4.2. Chemical specific parameters for the pregnant mouse (GD 11).

<table>
<thead>
<tr>
<th>Pregnant Mouse</th>
<th>Value</th>
<th>Source</th>
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<tbody>
<tr>
<td><strong>Partition Coefficients (P)</strong></td>
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<tr>
<td>Slowly perfused PS</td>
<td>3.75</td>
<td>Set to richly perfused</td>
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<tr>
<td>Richly perfused PR</td>
<td>3.75</td>
<td>Wang et al., 1980</td>
</tr>
<tr>
<td>Liver PL</td>
<td>3.69</td>
<td>Wang et al., 1980</td>
</tr>
<tr>
<td>Fat PF</td>
<td>4.42</td>
<td>Wang et al., 1980</td>
</tr>
<tr>
<td>Placenta PPL</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>Embryo PE (RA)</td>
<td>0.56</td>
<td>Experimental data</td>
</tr>
<tr>
<td>Embryo PEC (13cisRA)</td>
<td>0.4</td>
<td>Experimental data</td>
</tr>
<tr>
<td><strong>Oral Uptake</strong></td>
<td></td>
<td></td>
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<tr>
<td>Stomach to GI tissue K1 (1/hr)</td>
<td>0.00001</td>
<td>Fit to experimental data</td>
</tr>
<tr>
<td>Stomach to Duodenum K2 (1/hr)</td>
<td>0.85</td>
<td>Fit to experimental data</td>
</tr>
<tr>
<td>Duodenum to GI tissue K3 (1/hr)</td>
<td>1.7</td>
<td>Fit to experimental data</td>
</tr>
<tr>
<td>Kirac (loss of RA from intestine to metabolism) (L/h-kg)</td>
<td>0.002</td>
<td>Fit to experimental data</td>
</tr>
<tr>
<td><strong>Diffusion Limited Transfer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placenta to embryo: RA Kei (L/hr)</td>
<td>0.0074</td>
<td>Fit to experimental data</td>
</tr>
<tr>
<td>13cisRA Keci (L/hr)</td>
<td>0.00144</td>
<td>Fit to experimental data</td>
</tr>
<tr>
<td>Endogenous Production RA: Kendi (mg/hr)</td>
<td>0.004083</td>
<td>Fit to endogenous plasma level (Satre and Kochhar, 1989)</td>
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<td><strong>Max Velocity, Vmax (mg/hr·kg)</strong></td>
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<td></td>
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<tr>
<td>Liver RA, Vmgb (Basal rate, includes P450 and glucuronidation)</td>
<td>1.1</td>
<td>Fit to experimental data</td>
</tr>
<tr>
<td>Liver RA, Vmcis (isomerization of RA to 13cisRA)</td>
<td>1.77</td>
<td>Fit to experimental data</td>
</tr>
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<td><strong>Affinity Constant, Km (mg/L)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver RA, Kmg (includes P450 and glucuronidation)</td>
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<td>Ahmad et al., 2000</td>
</tr>
<tr>
<td>Liver RA, Kmcis (isomerization of RA to 13cisRA)</td>
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<td>Set to Km of RA</td>
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<tr>
<td><strong>Rate Constants</strong></td>
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<td></td>
</tr>
<tr>
<td>Klcis (clearance rate for 13cisRA from liver metabolism) (L/h)</td>
<td>1.9</td>
<td>Fit to experimental data</td>
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</table>
Table 4.3. Sensitivity analysis of selected tissue volumes, blood flows, and partition coefficients for RA and 13cisRA at 100 mg/kg evaluated 1, 6, and 8 h post dosing.

<table>
<thead>
<tr>
<th>Model Parameter</th>
<th>Time (h)</th>
<th>Target Model Parameter Value&lt;sup&gt;a&lt;/sup&gt;</th>
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<sup>a</sup>Target model parameters are CP=maternal plasma RA, CBC=maternal plasma/body water 13cisRA, CE=embryo RA, CEC=embryo 13cisRA. See Tables 4.1 and 4.2 for definition of model parameters.

<sup>b</sup>Values in bold typeface are sensitive parameters (greater than ±0.5) to the target model parameter.
Table 4.4. Sensitivity analysis of oral uptake and diffusion coefficients for RA and 13cisRA after a 100 mg/kg oral bolus and evaluated 1, 6, and 8 h post dosing.

<table>
<thead>
<tr>
<th>Model Parameter</th>
<th>Time (h)</th>
<th>CP</th>
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<th>CE</th>
<th>CEC</th>
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a. Target model parameters are CP=maternal plasma RA, CBC=maternal plasma/body water 13cisRA, CE=embryo RA, CEC=embryo 13cisRA. See Tables 4.1 and 4.2 for definition of model parameters.

b. Values in bold typeface are sensitive parameters (greater than ±0.5) to the target model parameter.
Table 4.5. Sensitivity analysis of metabolic parameters for RA and 13cisRA after a 100 mg/kg oral bolus and evaluated 1, 6, and 8 h post dosing.

<table>
<thead>
<tr>
<th>Model Parameter</th>
<th>Time (h)</th>
<th>CP</th>
<th>CBC</th>
<th>CE</th>
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</table>

*aTarget model parameters are CP=maternal plasma RA, CBC=maternal plasma/body water 13cisRA, CE=embryo RA, CEC=embryo 13cisRA. See Tables 4.1 and 4.2 for definition of model parameters.

bValues in bold typeface are sensitive parameters (greater than ±0.5) to the target model parameter.
Figure 4.1. Schematic of PBPK model for RA and 13cisRA.
Figure 4.2. Maternal plasma concentration of *all-trans* retinoic acid without (a) and with (b) auto-induction of metabolism after a single oral dose of 100 mg/kg on GD 11 in the CD-1 mouse (n = 3 to 5 dams per time point). The model predicted is represented by a solid line and the experimental data is represented by triangles.
Figure 4.3. Concentration of all-trans retinoic acid in maternal plasma of GD 11 CD-1 mice given an oral bolus of 2.5 (a), 10 (b), 30 (c), 60 (d), or 100 (e) mg/kg all-trans retinoic acid (n = 3 to 5 per time point). The model predicted is represented by a solid line and the experimental data is represented by triangles.
Figure 4.4. Concentration of 13-cis retinoic acid in maternal plasma of GD 11 CD-1 mice given an oral bolus of 10 (a), 30 (b), 60 (c), or 100 (d) mg/kg all-trans retinoic acid (n = 3 to 5 per time point). The model predicted is represented by a solid line and the experimental data is represented by triangles.)
Figure 4.5. Concentration of *all-trans* retinoic acid in GD 11 mouse embryo (pooled by dam) from CD-1 mice given an oral bolus of 2.5 (a), 10 (b), 30 (c), 60 (d), or 100 (e) mg/kg *all-trans* retinoic acid (n = 3 to 5 dams per time point). The model predicted is represented by a solid line and the experimental data is represented by triangles).
Figure 4.6. Concentration of 13-cis retinoic acid in GD 11 mouse embryo (pooled by dam) from CD-1 mice given an oral bolus of 10 (a), 30 (b), 60 (c), or 100 (d) mg/kg all-trans retinoic acid (n = 3 to 5 dams per time point). The model predicted is represented by a solid line and the experimental data is represented by triangles.)
Figure 4.7. Plot of model predicted embryo AUC (a) and Cmax (b) versus maternally administered dose. AUC and Cmax were determined for the control based on the endogenous production of *all-trans* retinoic acid.
Figure 4.8. Model validation with data for all-trans retinoic acid in maternal plasma (a) and embryo (c) and for 13-cis retinoic acid in maternal plasma (b) and embryo (d) from Kraft et al. (1987). Data were collected in GD 11 mice orally exposed to 100 mg/kg all-trans retinoic acid. The model predicted is represented by a solid line and the experimental data is represented by triangles.)
Figure 4.9. AUC for embryo (a and b) and maternally administered dose (c and d) plotted against the teratogenic response for GD 11 CD-1 mice orally exposed to \textit{all-trans} retinoic acid (RA). Fetal response was determined on GD 18 after a single oral dose of RA on GD 11, previously published by Campbell et al. (2004). Plots a and c show the entire dose range and plots b and d show an enlargement of the low dose region of plot a and c. AUC for the embryo was based on the endogenous production of RA. Total defected includes embryos with either a limb defect or cleft palate.
CHAPTER 4
CONCLUSIONS

Dose Response Analysis

One purpose of this study was to determine dose-response relationships between maternally administered doses of RA on GD 11 and fetal forelimb malformations and fetal cleft palate as measured on GD 18. The dose-response curves for RA-induced fetal forelimb malformations were very steep and can be described as a linear process for RA doses of 2.5 mg/kg to doses of about 30 mg/kg (control to ≈ 10 mg/kg for dams with at least one malformed fetus), after which the dose-response reached a plateau and defects were recorded in nearly all fetuses for the 60 and 100 mg/kg dose groups. The second purpose of this study was to compare VI to CIA. The calculated BMDL values were similar for data analyzed by VI or CIA techniques, thus the CIA technique was not a more sensitive method for detecting limb malformations. In addition, CIA is more time-consuming and logistically difficult to complete. However, CIA may be useful for teratological investigations where quantitative data are needed.

PBPK Model

The PBPK model developed for RA described the oral uptake, maternal metabolism, and fetal disposition across doses ranging from 2.5 to 100 mg/kg. Overall the experimental data were adequately predicted by the PBPK model across the range of doses tested. The PBPK model did slightly over-predict the maternal plasma concentrations in the first 1 to 2 h post dosing for the 60 and 100 mg/kg doses, while at the same time slightly under-predicting the maternal plasma concentrations for the 10 mg/kg dose. Also, the peak fetal concentrations of
RA (2 h post dosing) were under-predicted by the PBPK model for both the 2.5 and 10 mg/kg dose. The model predicted dosimetrics (AUC\(_{(0-12h)}\) and Cmax) represented the maternally administered dose. With the inclusion of auto-induction, the model predicted AUC\(_{(0-12h)}\) or Cmax (data not shown) were able to describe the non-linearity of the dose-response curve, showing a rapid rise in percent affected between maternal doses of 2.5 and 30 mg/kg followed by a plateau in the response (Figure 4.9). As the PBPK model was able to describe the disposition of RA to the fetal compartment across a wide range of doses, this model may be useful for estimating fetal exposure with the addition of alternate routes of maternal exposure to RA (i.e., dermal) and extrapolation humans.

**Future Work**

Several areas that need further investigation were uncovered during the course of this research. One is the need for specific information regarding the induction of metabolism in the rodent. While we have fit the level of Vmax induction to describe the clearance of RA from maternal and embryonic tissues, studies need to be undertaken to describe the maximal induction of Vmax and at what concentration maximal induction occurs. Secondly, a better understanding of oral uptake of RA in from the intestinal tract is needed. While recent evidence suggests there may be other factors involved (i.e., active uptake), no direct evidence of a transport system has been demonstrated. In order to take this model to the next step, assessing the molecular changes that occur in the embryo under conditions of excess RA, specific data as to the quantitative changes in RA induced gene expression need to be undertaken.
APPENDICES
APPENDIX A

ALL-TRANS RETINOIC ACID AND 13-CIS RETINOIC ACID MODEL CODE

Program: JCRAmodel1.csl

!Modified by Jerry Campbell
!Date Modified: 9/02/04
!Last Changed: 11/02/04

! A physiologically based pharmacokinetic model for
! retinoic acid and its metabolites.

INITIAL !Pre-execution block

!Blood Flows
constant QCC = 15.      !Total (L/hr-1kg) -- scaled allometrically
constant QRC = 0.47     !Richly perfused tissues (fraction of QC) (Brown et al. 1997)
constant QLC = 0.25     !Liver Andersen etal (1987)
constant QSC = 0.21     !Slowly perfused tissues (Brown et al., 1997)
constant QFC = 0.03     !Fat (Brown et al., 1997)
constant QPLC = 0.02    !Placenta (estimated from O’Flaherty et al., 1992)

!Tissue Volumes
constant BW = 0.035     !Total (kg)
constant VBC = 0.65     !Volume of Body Water (Harvey’s #)
constant VPC = 0.049    !Plasma (Brown etal 1997)
constant VRC = 0.081    !Richly perfused tissues !Liver (Brown et al 1997)
constant VLC = 0.055    !Liver (Brown et al 1997)
constant VSC = 0.656    !Slowly perfused (Brown et al 1997)
constant VFC = 0.089    !Fat (est like O’Flaherty etal 1992 Mouse)
constant VPLC = 0.00016 !Placenta (est. as day 12 rat O’Flaherty etal 1992)
constant VEC = 48       !Embryo (mg) (O’Flaherty et al 1992)
constant VIC = 0.0262   !Intestine (Small and Large) Brown etal 1997)

!Allometric Scaling of Physiological Parameters
VB = VBC*BW
VP = VPC*BW
VR = VRC*BW
VL = VLC*BW
VF = VFC*BW  
VS = VSC*BW  
VPL = VPLC*BW  
VE = VEC/1.e6  
VI = VIC*BW  

QCN = QCC*BW**.75  
QR = QRC*QCN  
QL = QLC*QCN  
QF = QFC*QCN  
QS = QSC*QCN  
QPL = QPLC*QCN  
QC = QCN + QPL  

!Partition Coefficients for TRANS (tissue/plasma)  
constant PR = 3.75  !Richly perfused tissues (est from Wang etal 1980 Avg of  
!  ,Lu,Li,Br)  
constant PL = 3.69  !Liver (est from Wang etal 1980)  
constant PS = 3.75  !Slow (Set to PR)  
constant PF = 4.42  !Fat (Used brain partition est. from Wang etal 1980)  
constant PPL = 0.56  !Placenta (Paper in Rat GD20)  

!Embryo/Maternal Plasma Partition Coefficients  
constant PE = 0.56  !Embryo/plasma ratio for TRANS est. from my data  
constant PEC = 0.4  !Embryo/plasma ratio for CIS est. from my data  

!Kinetic Parameters  
constant k1 = 0.00001  !Stomach to GI tissue (liver) rate, (1/hr)(Fit Visual)  
constant k2 = 0.85  !Stomach to Duodenum rate, (1/hr)(Fit Visual)  
constant k3 = 1.7  !Duodemun to GI tissue (liver) rate, (1/hr)(Fit Visual)  
constant kei = 0.000704  !'diffusion limited transfer between placenta'  
                    !'and embryo for TRANS (L/hr)'  
constant keci =0.00144  !' -- for CIS (L/hr)'  
constant kendi = 0.004083  !'endogenous production of TRANS (mg/hr)  
                    !(Fit to endogenous level of Satre and Kochhar 1989)  
constant Vmgi = 1.1  !Fit 'capacity for global metabolism of TRANS (mg/hr-kg)'  
                    !includes: p450 and glucuronidation  
constant Kmgi = 0.1532  !'affinity for global metabolism of TRANS (mg/L)'  
constant Vmcsi = 1.77  !capacity for isomerization of trans to cis (mg/hr-kg)  
constant Kmcisi = 0.33  !affinity for isomerization of trans to cis (mg/L)  
constant Kirac = 0.002  !1st order rate loss of RA from intestine (L/hr-kg)
constant $K_{lcis} = 1.9$ \hspace{1cm} !clearance of 13-cis by liver

!Dosing Parameters
constant $Doi = 100.$ \hspace{1cm} !oral dose of TRANS (mg/kg)

!Allometric Scaling of Kinetic Constants
\begin{align*}
ke &= kei \times BW^{0.75} \\
kec &= keci \times BW^{0.75} \\
kendo &= kendi \\
Kira &= Kirac \times BW^{0.75} \\
Vmgb &= Vmgi \times BW^{0.75} \\
Kmg &= Kmgi \\
Vmcis &= Vmcisi \times BW^{0.75} \\
Kmcis &= Kmcisi \\
Doo &= Doi \times BW
\end{align*}

!Table vmgiind in pregnant mouse
\begin{itemize}
\item 100 mg/kg dose
\begin{larger}\begin{tabular}[t]{c|cccccccc}
foldindc & 1 & 6 & 0. & 1 & 2 & 5 & 8 & 12 & 1 \\
\hline & 1 & 1 & 7. & 2 & 1 & 1 & & & \\
\end{tabular}\end{larger}
\end{itemize}

! 60 mg/kg dose
\begin{larger}\begin{tabular}[t]{c|cccccccc}
foldindc & 1 & 6 & 0. & 1 & 2 & 5 & 8 & 12 & 1 \\
\hline & 1 & 1 & 5. & 2 & 1 & 1 & & & \\
\end{tabular}\end{larger}

! 30 mg/kg dose
\begin{larger}\begin{tabular}[t]{c|cccccccc}
foldindc & 1 & 6 & 0. & 1 & 2 & 5 & 7. & 12 & 1 \\
\hline & 1 & 1 & . & 2. & 1 & 1 & & & \\
\end{tabular}\end{larger}

! 2.5 and 10 mg/kg dose
\begin{larger}\begin{tabular}[t]{c|cccccccc}
foldindc & 1 & 2 & 0. & 1 & 2 & & & & \\
\hline & 1 & 1 & & & & & & & \\
\end{tabular}\end{larger}

!Simulation Control Parameters
constant $T_{stop} = 12.$ \hspace{1cm} !Time to terminate simulation (hrs)
constant $C_{int} = .01$ \hspace{1cm} !Communication interval (hrs)

END !End of Initial Block

DYNAMIC !Execution Block
ALGORITHM IALG = 2  !Gear algorithm for stiff systems

DERIVATIVE !Definition of model derivative equations

procedural
    hour = t
    foldind = foldindc(hour)
    vmgind = foldind * vmgb
    vmg = vmgind
end

!oral dosing, single bolus dose

!ast and aug = Amount remaining in two compartments (mg)
!aao= amount absorbed from two compartments,mg
    rst = -(k1*ast) - (k2*ast)  !rate of change in stomach-comp 1, mg/hr
    ast = integ(rst, doo)
    rug = (k2*ast) - (k3*aug) - Kira*aug/Vi  !rate of change in intestine-comp 2, mg/hr
    aug = integ(rug, 0.)
    rao = k1*ast + k3*aug  !rate of oral abs. into liver (mg/hr)
    aao = integ(rao, 0.)
    ano = kira*aug/Vi
    rano = INTEG(ano, 0)

!All-trans-retinoic acid (TRANS):

!TRANS Concentration in Plasma (Cp):
    dCp = (Qr*Cr/Pr + Qs*Cs/Ps + Qf*Cf/Pf + Qpl*Cpl/Ppl + Ql*Cl/Pl)/Vp - Qc*Cp/Vp
    Cp = INTEG(dCp, 0.0034)
    Ap = Cp*Vp
    ACpt = INTEG(Cp, 0.)  !'AUC of TRANS in Plasma'

!TRANS Concentration in Richly perfused tissues (Cr):
    dCr = Qr*(Cp - Cr/Pr)/Vr
    Cr = INTEG(dCr, 0.01275)
    Ar = Cr*Vr

!TRANS Concentration in Slowly perfused tissues (Cs):
    dCs = Qs*(Cp - Cs/Ps)/Vs
    Cs = INTEG(dCs, 0.01275)
    As = Cs*Vs

!TRANS Concentration in Fat (Cf):
    dCf = Qf*(Cp - Cf/Pf)/Vf
    Cf = INTEG(dCf, 0.01503)
Afat = Cp*Vf

!TRANS Concentration in Placenta (Cpl):
\[ dCpl = \frac{Qpl*(Cp - Cpl/Ppl)}{Vpl} - \frac{ke*(Cpl/Ppl - Ce/Pe)}{Vpl} \]
\[ Cpl = \text{INTEG}(dCpl,0.001904) \]
\[ Apl = Cpl*Vpl \]
\[ CVpl = \frac{Apl}{Vpl*Ppl} \]

!TRANS Concentration in Embryo (Ce):
\[ dCe = \frac{ke*(CVpl - Ce/Pe)}{Ve} \]
\[ Ce = \text{INTEG}(dCe,0.001904) \]
\[ Ae = Ce*Ve \]
\[ ACet = \text{INTEG}(Ce,0.) \quad \text{!'AUC of TRANS in Embryo'} \]

!TRANS Concentration in Liver (Cl):
\[ dCl = \frac{Ql*(Cp - Cl/Pl)}{Vl} - \frac{Vmg*Cl/Pl}{(Kmg + Cl/Pl)/Vl} - \frac{Vmcis*Cl/Pl}{(Kmcis + Cl/Pl)/Vl} + \frac{rao}{Vl} + \frac{kendo}{Vl} \]
\[ Cl = \text{INTEG}(dCl,0.01455) \]
\[ Al = Cl*Vl \]
\[ dAmg = \frac{Vmg*Cl/Pl}{(Kmg + Cl/Pl)} \]
\[ Amg = \text{INTEG}(dAmg,0.) \]
\[ dAmcis = \frac{vmcis*Cl/Pl}{(Kmcis + Cl/Pl)} \]
\[ Amcis = \text{INTEG}(dAmcis,0.) \]

!13-cis-retinoic acid (CIS):

!CIS Concentration in Body Water (Cbc):
\[ dCbc = \frac{Qpl*Cplc/Vb}{Vb} + \frac{Ql*Clc/Vb}{Vb} - \frac{(Qpl + Ql)*Cbc/Vb}{Vb} \]
\[ Cbc = \text{INTEG}(dCbc,0.000896606) \]
\[ Abc = Cbc*Vb \]
\[ ACpc = \text{INTEG}(Cbc,0.) \quad \text{!'AUC of CIS in Plasma'} \]

!CIS Concentration in Placenta (Cplc):
\[ dCplc = \frac{Qpl*(Cbc - Cplc/Vpl)}{Vpl} - \frac{kee*(Cplc - Cec/Pec)}{Vpl} \]
\[ Cplc = \text{INTEG}(dCplc,0.000896608) \]
\[ Aplc = Cplc*Vpl \]

!CIS Concentration in Embryo (Cec):
\[ dCec = \frac{kee*(Cplc - Cec/Pec)}{Vpl} \]
\[ Cec = \text{INTEG}(dCec,0.000358693) \]
\[ Aec = Cec*Ve \]
\[ ACec = \text{INTEG}(Cec,0.) \quad \text{!'AUC of CIS in Embryo'} \]

!CIS Concentration in Liver (Clc):
\[ dClc = \frac{Ql*(Cbc - Clc)}{Vl} - \frac{Klcis*Clc}{Vl} + \frac{Vmcis*Cl/Pl}{(Kmcis + Cl/Pl)/Vl} \]
\[ Clc = \text{INTEG}(dClc,0.000896127) \]
\[ Alc = Clc \times Vl \]
\[ dAmgc = Klcis \times Clc \div Vl \]
\[ Amgc = \text{INTEG}(dAmgc,0.) \]

!Total AUC of RA and 13-cis in Plasma (AUCP) and Embryo (AUCE):
\[ AUCP = ACpt + ACpc \]
\[ AUCE = ACet + ACec \]

!Total concentration of RA and 13-cis in Plasma and Embryo:
\[ \text{Plasma} = Cp + Cbc \]
\[ \text{Embryo} = Ce + Cec \]

!Mass balance (Mbal):
\[ \text{Dose} = Doo \]
\[ \text{endprod} = kendi \times t \]
\[ \text{endog} = \text{INTEG}(\text{endprod},0) \]
\[ \text{doend} = \text{dose} + \text{endog} \]
\[ \text{TRANS} = Ap + AS + Ar + Afat + Apl + Ae + Al \]
\[ \text{CIS} = Abc + Aplc + Aec + Alc \]
\[ \text{Body} = \text{TRANS} + \text{CIS} \]
\[ \text{Tmass} = 0.00046274 - \text{Body} \]

\[ \text{Mbal} = \text{doend} - \text{Tmass} \]

\[ \text{TERMT}(T.GT.Tstop) \]

END !End of Derivative Block

END !End of Dynamic Block

END !End of Program
APPENDIX B

MODEL CMD AND M FILES

File: RAprep.cmd

!Mouse Retinoic Acid model (CMD file RAprep.cmd)

!Created 06/26/2004 by: Jerry Campbell

!Last modified: 11/17/2004

prepare t, cp, cpl, cr, cs, cf, ce, cl, cec, cbc, cplc, clc, vmgmx

File: 2_5plot.m

%RA dataset plot 2.5 mg/kg data

%Created 10/29/2004 by Jerry Campbell

%Last Modified; 10/29/2004

%Simulation commands

RA_2_5data

%Set report parameters

!!S TSTOP=12

!!s doi=2.5

!!start/nc

%Plotting Commands

plot(_t, cp, plasma_ra2_5(:,1),plasma_ra2_5(:,2), 'PRAplot.aps');

plot(_t, ce, embryo_ra2_5(:,1),embryo_ra2_5(:,2), 'ERAplot.aps');
File: 10plot.m

%RA dataset plot 100 mg/kg data

%Created 6/29/2004 by Jerry Campbell

%Last Modified; 6/29/2004

%Simulation commands

RA_10data

%Set report parameters

!!S TSTOP=12

!!s doi=10

!!start/nc

%Plotting Commands

plot(_t_cp,plasma_ra10(:,1),plasma_ra10(:,2), 'PRAplot.aps');
plot(_t_cbc,plasma_13cis10(:,1),plasma_13cis10(:,2), 'PCisplot.aps');
plot(_t_ce,embryo_ra10(:,1),embryo_ra10(:,2), 'ERAplot.aps');
plot(_t_cec,embryo_13cis10(:,1),embryo_13cis10(:,2), 'ECsplot.aps');

File: 30plot.m

%RA dataset plot 100 mg/kg data

%Created 6/29/2004 by Jerry Campbell

%Last Modified; 8/24/2004

%Simulation command

RA_30data
%Set report parameters
!!S TSTOP=12
!!s doi=30
!!start/nc

%Plotting Commands
plot(_t_cp,plasma_ra30(:,1),plasma_ra30(:,2), 'PRAplot.aps');
plot(_t_cbc,plasma_13cis30(:,1),plasma_13cis30(:,2), 'PCisplot.aps');
plot(_t_ce,embryo_ra30(:,1),embryo_ra30(:,2), 'ERAplot.aps');
plot(_t_cec,embryo_13cis30(:,1),embryo_13cis30(:,2), 'ECsplot.aps');

File: 60plot.m

%RA dataset plot 100 mg/kg data

%Created 6/29/2004 by Jerry Campbell

%Last Modified; 6/29/2004

%Simulation command

RA_60data

%Set report parameters

!!S TSTOP=12
!!s doi=60
!!start/nc

%Plotting Commands
plot(_t_cp,plasma_ra60(:,1),plasma_ra60(:,2), 'PRAplot.aps');
plot(_t_cbc,plasma_13cis60(:,1),plasma_13cis60(:,2), 'PCisplot.aps');
plot(_t_ce,embryo_ra60(:,1),embryo_ra60(:,2), 'ERAplot.aps');
plot(_t_cec ,embryo_13cis60(:,1),embryo_13cis60(:,2), 'ECsplot.aps');

**File: 100plot.m**

%RA dataset plot 100 mg/kg data

%Created 6/29/2004 by Jerry Campbell

%Last Modified: 6/29/2004

%Simulation command

RA_100data

%Set report parameters

!!S TSTOP=12

!!s doi=100

!!start/nc

%Plotting Commands

plot(_t_cp ,plasma_ra100(:,1),plasma_ra100(:,2), 'PRAplot.aps');
plot(_t_cbc ,plasma_13cis100(:,1),plasma_13cis100(:,2), 'PCisplot.aps');
plot(_t_ce,embryo_ra100(:,1),embryo_ra100(:,2), 'ERAplot.aps');
plot(_t_cec ,embryo_13cis100(:,1),embryo_13cis100(:,2), 'ECsplot.aps');