Use of novel inhalation kinetic studies to refine physiologically-based pharmacokinetic models for ethanol in non-pregnant and pregnant rats

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Abstract
Ethanol (EtOH) exposure induces a variety of concentration-dependent neurological and developmental effects in the rat. Physiologically-based pharmacokinetic (PBPK) models have been used to predict the inhalation exposure concentrations necessary to produce blood EtOH concentrations (BEC) in the range associated with these effects. Previous laboratory reports often lacked sufficient detail to adequately simulate reported exposure scenarios associated with BECs in this range, or lacked data on the time-course of EtOH in target tissues (e.g. brain, liver, eye, fetus). To address these data gaps, inhalation studies were performed at 5000, 10 000, and 21 000 ppm (6 h/d) in non-pregnant female Long-Evans (LE) rats and at 21 000 ppm (6.33 h/d) for 12 d of gestation in pregnant LE rats to evaluate our previously published PBPK models at toxicologically-relevant blood and tissue concentrations. Additionally, nose-only and whole-body plethysmography studies were conducted to refine model descriptions of respiration and uptake within the respiratory tract. The resulting time-course and plethysmography data from these in vivo studies were compared to simulations from our previously published models, after which the models were recalibrated to improve descriptions of tissue dosimetry by accounting for dose-dependencies in pharmacokinetic behavior. Simulations using the recalibrated models reproduced these data from non-pregnant, pregnant, and fetal rats to within a factor of 2 or better across datasets, resulting in a suite of model structures suitable for simulation of a broad range of EtOH exposure scenarios.

Keywords
Ethanol, physiologically-based pharmacokinetic (PBPK) models, pregnancy, rats

Introduction
Physiologically-based pharmacokinetic (PBPK) models have been used to predict concentrations of ethanol (EtOH) in both blood and exhaled breath in non-pregnant adult animals and humans following exposures via multiple routes (Martin et al., 2012; Pastino et al., 1996, 1997; Pastino & Conolly, 2000; Plawecki et al., 2004, 2007; Sultatos et al., 2004; Umulis et al., 2005). We recently reported the first suite of ‘life-stage’ PBPK models for EtOH in non-pregnant adult, pregnant female and neonatal rats. These models accurately simulated a range of inhalation, ingestion, and intravenous exposure datasets from the pharmacokinetic and toxicity literature (Martin et al., 2012). The models were part of a larger effort aimed at creating a series of multi-route PBPK models that could be used to inform and interpret new studies of the pharmacokinetics of EtOH across a wide range of inhalation exposure scenarios. The current report describes the results of these new pharmacokinetic studies and efforts to improve the predictive capabilities of the adult and pregnant rat PBPK models.

Peak blood EtOH concentrations (BEC) are often used as a potential dose metric, since the severity of the EtOH-induced toxicity is related to the achieved BEC. In earlier work, Martin et al. (2012) shed light on the in vivo kinetics of ingested EtOH in brain and liver tissues, with successful simulation of these tissue data highlighting both the range
of applicability of the models and informing the in-depth pharmacokinetic (PK) studies in the current report. As data on brain (BrEC) and liver (LEC) EtOH concentrations produced by inhalation are sparse in the literature, especially at concentrations associated with toxicity, we designed inhalation experiments to enable rapid collection of these tissue data, across multiple exposure scenarios, and to facilitate comparison to model estimates.

The developing eye is one of the more sensitive tissues for gestational and neonatal EtOH and methanol (MeOH) toxicity in both rodents and humans (Harris et al., 2000; Katz & Fox, 1991; Pinazo-Durán et al., 1996; Pons et al., 2007; Pons-Vázquez et al., 2008; Strömland, 2004; Strömland & Pinazo-Duran, 2002). Peak maternal BECs as low as 120–150 mg/dL have been shown to alter the structure and function of developing visual systems in rats and mice exposed in utero, via the oral route; with toxicity to dendritic spines in the developing mouse visual cortex and long-term effects on retinal function in rats evaluated using electroretinograms (Cui et al., 2010; Katz & Fox, 1991). In their detailed review, Strömland & Pinazo-Duran (2002) report a range of developmental ocular effects, such as microphthalmia and reduced myelination of the optic nerve. They also include proposed mechanisms of action in rodents and humans, including formation of free radicals, induction of apoptosis, and exposure-induced alterations to genes that encode signaling molecules for cellular growth and differentiation. However, no ocular tissue time-course PK data could be located for EtOH inhalation at any rat life-stage. Rabbit eye tissue (Winok et al., 1983), human vitreous humor (Caplan & Levine, 1990; Jones & Holmgren, 2001; Mackey-Bojack et al., 2000), and rat, mouse, and rabbit lacrimal fluid (via sampling vapor above the lacrimal fluid) concentrations have been reported (Giles et al., 1989, 1988); however, differences in species, route of exposure, and sampling methods complicate interpretation of these data in the present context. In the current report, we hypothesized that the concentration of EtOH in ocular tissue (EEC) would be comparable to BEC, due to the high perfusion rate of the retina (Salter et al., 1998; Strang, 1974), and designed experiments and analytical methods to support investigations of ocular pharmacokinetics. If supported, this hypothesis would suggest that maternal BEC data from ocular toxicity studies could serve as a surrogate for the EEC during fetal development.

Fetotoxicity and developmental delays tend to be more severe when EtOH exposure occurs during critical windows of susceptibility for the brain and other tissues. However, there are questions surrounding the comparability of maternal to fetal BECs and BrECs following inhalation exposure and whether or not the day of gestation influences the magnitude of the tissue concentrations produced by maternal exposure. Epidemiology studies have investigated the impact of EtOH on pregnant women and their fetuses when exposure occurred during the first or subsequent trimesters, though most have dealt with oral exposures. However, inhalation is the primary route of exposure for EtOH in the workplace (industrial production, laboratory, culinary, medical use) and for EtOH-based biofuels, especially during automotive refueling. In the animal literature, few published studies have utilized vapor concentrations sufficient to produce toxicologically-relevant BECs (Nelson et al., 1985), irrespective of the stage of gestation, and no fetal BEC data are available for this route. Additionally, it is difficult to convincingly estimate or predict this information from literature studies where other routes of exposure were utilized. Thus, new data were necessary to support hypotheses surrounding the impact of maternal inhalation exposure on gestational development. With this in mind, we sought to evaluate EtOH concentrations in blood and/or tissues across multiple days of a critical gestational window (days 9–20) using vapor concentrations intended to achieve toxicologically-relevant BECs.

To address the questions posed, experiments were conducted to: (1) investigate the kinetics of EtOH in target tissues; (2) investigate the impact of vapor concentration and type of chamber, specifically nose-only (N-O) or whole-body (W-B), on respiratory uptake; and (3) examine the relationship between maternal and fetal BEC and BrEC, in the context of a PBPK model for pregnancy (Supplementary Figure 1).

Experiment 1 was conducted to provide time-course EtOH concentration data in the blood, brain, liver, and eyes, covering both uptake and clearance of EtOH in the body using N-O exposure in non-pregnant female rats. Non-pregnant females were used to maximize efficiency in the number of pharmacokinetic time points and tissues that could be collected in as few experimental days as possible. Logistically, incorporation of a N-O system improves control over the exposure duration, because rats are loaded onto the chamber after the target concentration is reached and then removed individually for sacrifice without disturbing other rats. Additionally, it is not necessary to wait for the chamber concentration to fall back to a safe exposure limit (for laboratory staff) prior to removal of animals, so a more accurate determination of peak tissue concentration is possible. The N-O method also limits unintended exposures via preening (and subsequent ingestion) or dermal uptake, as well as contamination of ocular tissue through direct contact with vapor. From the modeling perspective, EtOH's rapid clearance from blood complicates accurate characterization of the peak BEC following inhalation exposure. Therefore, the current experimental protocol was designed to facilitate rapid removal from the chamber, sacrifice, and collection of blood within one minute of the desired kinetic time-point.

Experiment 2 was conducted to determine if exposure to high concentrations of EtOH vapor altered respiratory parameters, and if any potential impact differed with respect to the type of chamber used. Literature studies have used both N-O and W-B systems, but no such comparison studies have been reported. Appreciable differences in respiratory parameters such as ventilation rate, due to the type of chamber used, could indicate a need to compensate for this effect when simulating available pharmacokinetic data. Although by a different route, such compensation is routinely performed when adjusting the terms that control infusion rates in models for intravenous injection. Specifically, the infusion-time parameter is changed when administration occurs through a rapid intravenous bolus injection, as opposed to a slow, extended-duration, infusion occurring over several minutes.
or hours. Separate from questions related to chamber types, the EtOH vapor concentration that depressed respiration by 50% (RD50) was 27 314 ppm in mice (Kane et al., 1980), which suggests the potential for measurable respiratory depression at the vapor concentrations used in the current study. Similarly, PBPK models for chemicals that are known to reduce ventilation rates may utilize significantly lower ventilation rates when simulating the exposures scenarios that produced this effect (Dennison et al., 2003). Additionally, sensitivity analyses applied to our PBPK models (Martin et al., 2012) indicated high sensitivity of BECs to respiratory uptake parameters during inhalation exposure, suggesting a model-directed need to evaluate uncertainties surrounding the inhalation route. Therefore, we evaluated eleven standard respiratory parameters, including frequency, tidal volume, and minute volume using both types of chambers.

Experiment 3 was performed with two purposes: (1) to provide a companion PK dataset for a neurodevelopmental toxicology study with pregnant rats (Boyes et al., 2014), and (2) to fill data gaps surrounding the kinetics of inhaled EtOH during gestation. Regarding the first purpose, this PK study replicated the exposure scenario used in a neurodevelopmental toxicology study to document tissue concentrations of EtOH in pregnant rats exposed to the high concentration of EtOH vapor and to verify model-derived estimates in rats exposed to lower concentrations of the vapor. For the second purpose, fetal tissues are often grouped into a single compartment in the PBPK modeling literature, with simulated concentrations from this compartment used to evaluate the potential for fetotoxicity. While it makes sense to address the PK associated with widespread physical deformities in this manner, actual fetal BrECs or BECs would appear to be a more relevant dose-metric for gestational exposures that induce post-partum neurological effects, compared to the single compartment “carcass” concentration. It was not feasible to collect gestational time-course datasets in pregnant and fetal rats across multiple vapor concentrations and gestational days, due to the large number of animals that would be required. Instead, we elected to determine peak, rather than time-course BECs and BrECs in pregnant and fetal rats, and then to compare these data to BECs and BrECs in the toxicity literature and from Experiment 1. Because our neurodevelopmental toxicity study (Beasley et al., 2014; Boyes et al., 2014; Oshiro et al., 2014) required use of W-B chambers to generate large numbers of offspring for behavioral, sensory, and motor activity assessments, and ethical considerations precluded the use of nose-only cones to repeatedly expose pregnant rats, we used a W-B exposure system for this experiment. Regarding target tissues, we assumed that a model capable of accurately simulating both BEC and BrEC in adult non-pregnant and pregnant rats would also accurately simulate the liver and eye concentrations from both life-stages, reducing the need to collect the liver and eyes from both fetuses and adults. This allowed us to focus on rapid collection of fetal blood, brain, and total carcass concentrations from each exposed dam and thereby fill the data gaps surrounding comparability of EtOH concentrations in the fetus, pregnant dam and non-pregnant adult, since BEC and BrEC data were collected in all of the kinetic studies.

These experiments provided novel time-course and peak blood and tissue concentration data for EtOH and respiratory plethysmography (PLY) measurements to support refinement of PBPK models for EtOH. All experiments were conducted at the same target vapor concentrations to link this work to existing toxicity studies and facilitate comparisons across life-stages and methods of exposure. The refined PBPK models improve our ability to reliably predict the kinetics of EtOH across a wide range of exposure scenarios.

Methods

Chemicals

Ethyl alcohol (95% v/v, ACS/USP grade) was used for all exposures (Pharmaco-AAPER, Louisville, KY). Perchloric acid was purchased from JT Baker, Inc. (69–72%, Phillipsburg, NJ), while sodium azide and thiourea were purchased from Sigma Aldrich (St. Louis, MO).

Animals

Adult female non-pregnant (n = 117) and pregnant (n = 12) Long-Evans rats (Charles River Laboratories, Raleigh, NC) were maintained on a 12-h light-dark cycle (lights on at 6:00 a.m.) with a controlled ambient room temperature of 22 ± 2°C (SD) and relative humidity of 50 ± 10% (SD). Non-pregnant females were pair-housed in polycarbonate cages (18780, Maryland Plastics, Inc., Federalburg, MD) with kiln-dried pine shaving bedding (Northeastern Products, Warrensburg, NY). Pregnant rats were received on gestation day (GD) 2 and housed individually with beta chip bedding (Northeastern Products, Warrensburg, NY) and Enviro Dri nesting material (Shepherd Specialty Papers, Watertown, TN), also in polycarbonate cages. Food (PMI 5001 for non-pregnant and PMI 5008 for pregnant, Lab Diet/PMI Nutrition International, Richmond, IN) and water were available *ad libitum* while animals were housed in the US EPA animal facility, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International according to NIH guidelines. The types of food and bedding materials are standard for non-pregnant and pregnant rats in our facility. Animal research protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the US EPA, Office of Research and Development, National Health and Environmental Laboratory. The number of rats, their body weights, EtOH exposure concentrations, and other PK or PLY study-related details are summarized in Table 1. Unless otherwise noted, four animals were used per time point (n = 4), consistent with federal guidelines (40 CFR 795 and OECD 417) for PK studies. The peak vapor concentration (21 000 ppm) was related to the maximum allowable exposure concentration in our facility, which was required to be less than 75% of the lower explosive limit.

Rats exposed N-O (Experiments 1 and 2) were acclimated to the N-O cones for 3 d (10 min, 30 min, 60 min) prior to exposure to mitigate potential confinement stress. To ensure an effective comparison during the plethysmography study, rats assigned to the W-B plethysmography group were placed in small polycarbonate cages of similar dimensions.
Table 1. Kinetic and plethysmography (PLY) studies.

| Study                      | Exposure concentration (ppm) | Body weight (g) | Exposure duration (h/d) | Total rats | Rats/time pt | Tissue collection GD and/or times (h) | Tissues collected |
|----------------------------|-------------------------------|-----------------|-------------------------|------------|--------------|---------------------------------------|------------------|
| Experiment 1:              |                               |                 |                         |            |              |                                       |                  |
| Kinetic-N-O                | 5078.5 ± 217.1                | 244.8 ± 13.2    | Up to 6                 | 19         | 3–4          | 0.5, 2, 6, 6.25, 6.5                  | BL, BR, LI, EY   |
| Non-pregnant               | 10068.5 ± 255.3               | 246.9 ± 14.5    | Up to 6                 | 20         | 4            | 1, 3, 6, 6.25, 6.5                    | BL, BR, LI, EY   |
|                            | 21507 ± 185.3                 | 254.4 ± 9.97    | Up to 6                 | 24         | 4            | 2, 4, 6, 6.5, 7, 7.5                  | BL, BR, LI, EY   |
| Experiment 1:              |                               |                 |                         |            |              |                                       |                  |
| Experiment 2:              |                               |                 |                         |            |              |                                       |                  |
| Non-Pregnant               | 4979 ± 149                    | 263.0 ± 10.3    | 6                       | 4          | 4            |                                       | BL, BR, LI, EY   |
|                            | 10894 ± 625                   | 254.0 ± 13.3    | 6                       | 4          | 4            |                                       | BL, BR, LI, EY   |
|                            | 19851 ± 509                   | 263.8 ± 6.70    | 6                       | 4          | 4            |                                       | BL, BR, LI, EY   |
|                            | 0                             | 250.3 ± 10.5    | 6                       | 4          | 4            |                                       | BL, BR, LI, EY   |
| Experiment 2:              |                               |                 |                         |            |              |                                       |                  |
| Non-Pregnant               | 5508 ± 171                    | 255.3 ± 8.66    | 6                       | 4          | 4            |                                       | BL, BR, LI, EY   |
|                            | 11557 ± 760                   | 250.5 ± 20.8    | 6                       | 4          | 4            |                                       | BL, BR, LI, EY   |
|                            | 20714 ± 721                   | 258.8 ± 20.8    | 6                       | 4          | 4            |                                       | BL, BR, LI, EY   |
|                            | 0                             | 247.3 ± 7.09    | 6                       | 4          | 4            |                                       | BL, BR, LI, EY   |
| Experiment 3: Kinetic-W-B  | 21320 ± 1120                  | 240 to 365      | GD9-20 (6.33h/12d)      | 4          | 4            | 6.33 on GDs 9, 12, 16, 20             | BL, BR           |
| Pregnant w/fetus           | 2.23 ± 0.26                   | GD20 Fetus      | 13.5                    | 44         |              | GD20                                  | BL, BR, FWB      |

BL, Blood; BR, Brain; LI, Liver; EY, Eye; FWB, Fetus Whole body; GD, Gestational Day.

Blood was collected via tail nicks on GD9, 12, and 16, otherwise trunk blood was collected on GD20 and all non-pregnant animals. One rat died prior to the 5079 ppm exposure and was not replaced, leaving 19 rats in the cohort. N-O, Nose-Only; W-B, Whole-Body.

Vapor concentrations were monitored continuously during experiments (mean ± SD), with three vapor concentrations for Experiment 1, four for each chamber type in Experiment 2, and one vapor concentration used for Experiment 3. Body weights (mean ± SD) were recorded the morning of exposure. Exposure durations, number of animals/study and/time point were model inputs. Tissue collection times captured the time-course in adults and peak in pregnant dams/fetuses. Dams 1–4 had 11, 13, 17, and 13 fetuses, respectively, avg. 13.5). Collection times are targets, as the number of seconds between removal and decapitation slightly differed between rats.
to the W-B plethysmographs, for the same duration as rats in the N-O group. Pregnant rats exposed from gestational day 9–20 by the W-B method in Experiment 3 were not acclimated in this manner, because that study was designed to mirror the larger developmental neurotoxicity study which utilized Hazelton H1000 “live-in” W-B exposure chambers, where dams were housed continuously (also gestational day 9–20), and acclimation was not performed (Beasley et al., 2014; Boyes et al., 2014; Oshiro et al., 2014).

**Vapor monitoring and generation**

**Exposure concentration monitoring**

Vapor concentrations were monitored in all experiments using Miran 1A infrared (IR) spectrophotometers (Wilks Foxboro Analytical, South Norwalk, CT), with strip chart recorders. The IRs were tuned to EtOH’s CH\_2-stretch wavelength (10.5 microns, or 952 cm\(^{-1}\) wavenumbers) and calibrated using 100% EtOH (200 proof) at the appropriate vapor concentration (±20%). This wavelength was selected over the stronger CO\_2-stretch wavelength (9.3 microns) because of the high vapor concentrations required. Data are presented as mean and standard deviation (SD) of chamber concentration (ppm). Prior to the start of experiments, a maximum allowable difference window of ±10% of the mean vapor concentration was established to ensure high stability during chamber operation.

**Non-pregnant rat exposure system (Experiment 1)**

To minimize exposure via unintended routes, such as oral ingestion, N-O exposure has been recommended for pharmacokinetic studies (OECD- 417, 2010, CFR-795-231, 2012, CFR-795-232, 2012). Briefly, a 52-port Cannon-style directed flow N-O exposure system was used for kinetic time-course and plethysmography studies (described later). Rat cones were mounted only on the top three rows of the N-O chamber (10–12 ports) to ensure uniform exposures, with the remaining lower ports blocked by plugs. The total flow-rate through the N-O exposure system was maintained at 9.0–9.2L/min during exposures. The dynamic counter-current evaporator that was used to generate exposures was adapted from the design from Miller et al. (1980) and was packed with 4–6 mm Pyrex™ beads to increase evaporative surface area (Supplementary Figure 2a). The evaporator was maintained at 110\(^{\circ}\)C, (10\(^{\circ}\)C above the boiling point of the water component in 95% EtOH) with a heating mantle (GlasCol, Terre Haute, IN) and temperature controller (Omega, Stamford, CT) to ensure complete vaporization. A liquid metering pump (Model# RH0CKC, Fluid Metering Inc., Oyster Bay, NY) was used to deliver the liquid EtOH into the evaporator with pump flow rates adjusted to generate desired vapor concentrations. Vaporized EtOH was mixed with nitrogen (3 L/min) and combined with HEPA-filtered dilution air in a separate mixing vessel before passing through the chamber’s inner plenum and exiting through jets that faced each rat’s nares in the N-O ports. A negative pressure vacuum flow removed exhaled and waste vapor from the breathing zone into the chamber’s outer plenum for disposal.

A chamber distribution and uniformity study was performed by evaluating the vapor concentration at several exposure ports using a gas chromatograph (GC) (SRI, Torrance, CA) and gas-tight syringes. Three measurements were used to assess uniformity within the chamber: Total Port Variability (TPV), Within Port Variability (WPV), and Between Port Variability (BPV). TPV is the total measured variability between the sampled ports. WPV represents fluctuation of the mean concentration when the chamber is stable. BPV represents the actual variability between all ports and was determined using the formula BPV = TPV – WPV (Higuchi et al., 1990). Though the difference between ports (BPV) would ideally be zero, a value of 0.05, or 5%, would be considered excellent with this system.

**Plethysmograph exposure system (Experiment 2)**

Four W-B plethysmographs for unrestrained rats and four N-O plethysmographs (Models PLY3023 and PLY4213, respectively, Buxco Electronics, Inc., Wilmington, NC) were used to collect data from non-pregnant female rats. The vapor generator from Experiment 1 supplied EtOH to all plethysmographs concurrently (n = 8 per day). Combined air flow was ~12 L/min, with 3.2 L/min total for the four N-O plethysmographs on the N-O chamber and 2.0–2.1 L/min each (~8 L/min total) for the four W-B plethysmographs. Respiratory measurements were collected using Buxco Biosystem XA version 2.05 software, including: frequency (F, breaths/min), tidal volume (Vt, mL/breath), minute volume (Ve, mL/min), peak inspiratory and expiratory flow (PIF, PEF, mL/s), inspiratory and expiratory time (TI, TE, s), enhanced pause (Paus, unitless), and Penh (unitless). A proprietary algorithm within the software automatically pre-screens measurements for acceptability, and rejects values falling outside of a pre-programmed range. Components of the plethysmography system and its operation are described elsewhere (Gordon et al., 2013; Hamelmann et al., 1997; Shannahan et al., 2010) and are unchanged from previous studies, with the exception of the N-O plethysmographs, which we had not previously utilized. The N-O plethysmographs were used following the manufacturer’s instructions, with the exception that the pneumotachometers, which convert air flow in the plethysmograph into quantifiable signals representing changes in air pressure, were moved from the cone’s rear sidewall to the back of the rear plunger (similar to N-O plethysmographs for mice), to prevent interference with the screen by fur or dander.

For calibration purposes, each chamber or tube was injected with a 3mL bolus of air and the response recorded in the system software. A result of <0.2% error between calibrations was considered acceptable. Frequency and volume measurements were confirmed using a small animal ventilator (Inspira ASV, Harvard Apparatus, Inc., Holliston, MA).

For W-B exposure, rats were transferred to the plethysmographs and allowed to move freely, curl up, and preen during exposure. Each W-B plethysmograph included a thin cardboard sheet on the bottom of the unit to absorb urine and feces for sanitary reasons (discarded after exposure). For the N-O plethysmographs, rats were fitted with a latex collar, placed in the cone, and mounted onto the N-O chamber for
Pregnant rat exposure system (Experiment 3)

Exposures were performed using custom made, stainless steel W-B exposure chambers (39.2 L) described previously (Kenyon et al., 2008; Oshiro et al., 2011). Methodological details for EtOH vapor generation were similar to those described in Experiment 1. Briefly, liquid EtOH was dispersed by four fluid metering pumps (Model# RHOCKCLF, Fluid Metering Inc., Syosset, NY) into heated airflow through each of the four chambers was ~20 L/min. The rise-time of EtOH vapor concentrations (t90, time to 90% of target) was ~20 min (0.33 h). Each chamber was sampled repeatedly in a circular 1–4 cycle via the IR and monitored for stability (mean ± SD). To determine the mean ± SD of vapor concentrations, all data points for each day were combined and the results analyzed across both the inter-day periods and the overall study. Due to the single unit steel construction, and glass front door of the chamber, it was not practical to conduct a distribution study similar to the BPV assessment in Experiments 1 and 2. However, due to the small volume of the chambers and high flow rates, the presence of areas of significantly poor distribution and uniformity are unlikely.

Tissue kinetic studies (N-O-non-pregnant & W-B-pregnant)

N-O-non-pregnant kinetics (Experiment 1)

Non-pregnant females were exposed to target concentrations of 5000, 10,000, or 21 000 ppm for a maximum of 6 h. Kinetic data were collected both during and following exposures (including 0.5 h into exposure and up to 1.5 h after exposure) to capture the loading, peak, and clearance kinetics of inhaled EtOH across a wide range of blood and tissue concentrations. Experimental details are provided in Table 1. No more than 12 rats were exposed on any given day and exposures were performed using a balanced ABCCCA design (5000, 10,000, 21,000, 21,000, 10,000, 5000 ppm).

To collect tissues, rats were removed from the chamber at designated time points and either immediately sacrificed, or returned to their individual home cages for sacrifice at post-exposure (clearance) time points. The home cages were located in a secondary containment area with separate clean air supply and ad libitum access to food and water. Rats were sacrificed via decapitation without anesthesia. Trunk blood was immediately collected into heparinized vials and vortexed to ensure mixing of heparin and blood. Liver, brain, and eyes were subsequently collected and homogenized or treated (described below), placed on ice, and then transferred to a −20°C freezer where they remained frozen until analysis (Ferrari et al., 2006; Kristoffersen et al., 2006). Blood and tissue collection times were recorded.

Immediately following excision from the carcass, liver and eyes were treated with an ice-cold inhibition cocktail to limit ex vivo metabolic activity during storage and analysis. The cocktail contained an internal standard (1-propanol) and perchloric acid, sodium azide, and thiourea to inhibit metabolism by specific enzymatic and non-enzymatic systems (alcohol dehydrogenase (ADH), microsomal EtOH oxidizing system, catalase, and oxyhemoglobin) (Steenaart et al., 1985). Liver tissue was rapidly minced in the cocktail using iris scissors. Eyes were not immediately minced and could not be homogenized due to the small mass. Brain samples were immediately treated with ice-cold phosphate buffer solution and homogenized thoroughly using a stand homogenizer with metal blades (Polytron, Kinematica, Inc., Bohemia, NY) prior to storage, as per established methods for chemicals such as toluene (Oshiro et al., 2011). Blood and brain samples were treated with the inhibition cocktail upon removal from the freezer, prior to analysis. Recovery studies indicated that BEC and BrEC were higher and more consistent across samples when the cocktail was added during the thawing process (data not shown).

PLY-plethysmography and plethysmograph kinetics (Experiment 2)

Respiratory parameters were recorded in non-pregnant female rats (n = 32, four rats per concentration for each method) inhaling EtOH vapor (0, 5000, 10,000, or 21,000 ppm) for 6 h using either N-O plethysmograph tubes or W-B plethysmograph chambers. Prior to conducting the full study, pilot studies were performed on a small group of rats (not shown) to ensure optimum system operation of both types of chambers and that rats tolerated the plethysmographs well. Blood and brain samples were collected from the 21,000 ppm W-B scenario, to ensure agreement between PBPK model simulations of peak BEC and BrEC and data collected under this scenario. As blood and tissues were collected from rats exposed in N-O cones during Experiment 1, at essentially the same concentration and duration, additional samples from N-O exposed rats were not collected.

To evaluate concerns that EtOH was lost from the brain tissue during homogenization at the time of sacrifice, homogenization was compared with rapid mincing with scissors during the PLY-W-B pilot studies (21 000 ppm, 6 h). To reduce rat-to-rat variability resulting from the small sample size in this pilot study (n = 2/group), half-brains from each of two rats in each group were combined with half-brains from the other rats in the same group, yielding the equivalent of two “whole” brain samples per group. Two vials of combined half-brains were then homogenized with a stand mixer in phosphate buffered saline, and the two other pairs of half-brains were minced on ice with iris scissors, and then combined without homogenization in two other vials.

W-B- pregnant kinetics (Experiment 3)

Pregnant dams (n = 4) were exposed to EtOH at 21 000 ppm for 6.33 h/d from GD9 through GD20 (12 d total) to evaluate...
the PBPK model for pregnancy. The duration of 6.33 h was selected to account for chamber vapor rise times, ensuring that each rat received 6 full hours of exposure at the target concentration, reproducing a companion toxic effects study (Beasley et al., 2014; Boyes et al., 2014; Oshiro et al., 2014).

Blood was collected at the end of exposure using tail nicks and capillary tubes on GD9, 12, and 16. Dams were decapitated on GD20, with blood and maternal brain collected as previously described. Fetuses were removed from the dam and amniotic sacs and placentas discarded. Though the model contains a placental compartment, we elected to focus on rapid collection and processing of the fetal blood, brain, and carcass, in lieu of the placentas from each fetus. Following removal, each fetus was then immediately decapitated with scissors and sealed in a vial for storage (−20 °C) until analysis.

Our original PBPK model of the maternal–fetal unit described the pregnant female with a single fetus compartment. The present experiment provided an opportunity to evaluate a more detailed model, which included an expanded fetal compartment with defined blood, brain, liver, and “other tissues” compartments. To do this, two subsets of fetal tissues were collected. For the first subset, fetal blood and brain samples were collected using capillary tubes to collect fetal trunk blood (~50–100 μL per fetus), and fetal brain was collected as per the adult method. To collect enough tissue for the analytical method, three brain samples from the same litter were combined per vial. In the second subset of fetuses from each dam, individual whole fetuses were homogenized to determine the fetal carcass EtOH concentration (fCEC). Ocular and liver tissues were not collected. For model development, fetal brain and liver tissue partition coefficients were assumed to be the same as those from adults. Therefore, the relationships between achieved fetal blood and tissue concentrations should also be equivalent to those from adults.

Statistical analysis

Eleven respiratory parameters were monitored continuously using the N-O and W-B plethysmographs, with the data exported from the operating software in 10-min averages. The resulting data were non-normally distributed. Therefore, a non-parametric Friedman one-way repeated measures analysis of variance on ranks (Sigma Plot 12.3, Systat Software, Inc., San Jose, CA) was performed to identify differences between the datasets collected at the four exposure concentrations. When statistical differences were detected, a non-parametric Tukey test was performed for all pairwise comparisons of ranked means between the concentration groups. The threshold for statistical significance was \( p < 0.05 \) for all measurements. In addition to the statistical analysis of the raw data for each parameter, the area-under-the-curve (AUC) was determined from the time-course data for each parameter using SigmaPlot (Sigma Plot 12.3, Systat Software, Inc.). Unless otherwise stated, data are reported as mean, standard deviation, and relative change (±%) in AUC.

Tissue analysis

Tissues were analyzed using a headspace-GC with flame ionization detector (HS-GC-FID) (Agilent 6890 GC-FID with 7694 auto-sampler and HP Chemstation Software (Agilent Technologies, Inc., Revision Number B.03.02, Santa Clara, CA). Method details are listed in Supplementary Table 1.

Prior to analysis, samples were removed from −20 °C storage and allowed to equilibrate on ice before transfer to headspace vials. Aliquots of blood or tissue were added to 20 mL headspace vials containing an n-propanol standard, capped, and then immediately placed on the auto-sampler. Samples were incubated for 0.5 h each at 70 °C to allow for phase equilibration. All exposed samples were analyzed with laboratory control samples, matrix blanks, and laboratory control duplicates. For quantification of EtOH, acetaldehyde (AALD), and MeOH, matrix-matched calibration curves were generated with linear regressions of \( \sim 0.99 \) using the n-propanol internal standard. Independent calibration verification standards were used and method validation studies were performed. Laboratory reporting limits (lower limits of quantitation) were 0.1, 0.02, and 0.1 mg/dL for EtOH, AALD, and MeOH, respectively. Control limits were determined to be ±20% for spiked samples. The relative percent difference for duplicate samples was also determined to be 20%. Pathological changes were not evaluated. AALD was quantified in these inhalation PK studies to provide data on the production of this toxic metabolite in adult and fetal animals. Collection of these data would also identify any deficiencies in the performance of the inhibition cocktail. MeOH was quantified to provide information on the potential impact of inhalation exposure to EtOH on the metabolism of endogenous alcohols.

PBPK modeling

Original model literature and justification for refinements

Initial model development was reported by Martin et al. (2012) and coded in acslX (v2.5.0.6, The Aegis Technologies Group, Inc., Huntsville, AL). In brief, the Pastino et al. (1997, 2000) models for multiple routes of exposure in the adult rat were modified to provide capability for simulation of BEC and tissue concentrations across multiple rat life-stages, including adult, pregnant, and neonatal periods (Yoon et al., 2000) models for multiple routes of exposure in the adult rat and tissue concentrations across multiple rat life-stages, including adult, pregnant, and neonatal periods (Yoon et al., 2009a,b). Additionally, the Pastino et al. (1997) model description for respiration was replaced with a description of upper respiratory tract (URT) absorption first reported for isopropanol and acetone (Clewell III et al., 2001) that accounts for the “wash-in wash-out” behavior of water soluble vapors in the URT through inclusion of mucus partitioning and a first-order uptake and clearance constant (kUrtC). Iterative testing using our earlier models indicated that a kUrtC value of 12 L/h/kg\(^{0.75}\) provided reasonable approximations to BEC data available from Pastino et al. (1997), Scarino et al. (2009) and Nelson et al. (1985), at concentrations from 200 up to 20,000 ppm. However, in comparison to data from Ferko & Bobjyock (1977) and others (not shown), kUrtC values from 8 to 12 L/h/kg\(^{0.75}\) were also capable of reasonably fitting these data sets; suggesting a need for clarification of this parameter. Regarding this uncertainty around kUrtC, there was evidence for concentration dependence in URT scrubbing in the data and model simulations from Clewell et al. (2001). Specifically, their model was capable of reproducing the inhalation exposure...
data, using a range of kUrtC values for isopropanol. A central value for kUrtC was eventually used by Clewell et al. (2001) for simulations across all datasets, which led to high quality approximations of the data at all but the highest exposure concentration.

For these EtOH models, sensitivity analyses were performed in an attempt to identify the parameters that most impacted BECs during inhalation. The results indicated that BECs were highly sensitive to adjustments of kUrtC and ventilation rate (QPC, L/h/kg0.75). Therefore, the data collected in the current report will be useful in addressing this uncertainty and will inform descriptions of uptake via inhalation when higher vapor concentrations are used.

Model refinements

Adult rat

A compartment for the eyes was added and described using a single flow-limited compartment (Figure 1). Parameters for this rapidly-perfused tissue compartment were collected from the literature and are listed in Table 2. The adult rat blood, liver, and brain descriptions remain unchanged from the original model from Martin et al. (2012). Modifications to account for respiratory effects are described later (in section “PLY-Plethysmography Results”).

Pregnant rat

The model for pregnant rats was largely unchanged from the previous version, with the exceptions that the single lumped fetal compartment in the model from Martin et al. (2012) was tested against a version containing a more detailed description of the fetus, as per Yoon et al. (2009a,b), with specific compartments for fetal brain and liver (Figure 2). The description of maternal respiratory uptake was also modified, in response to plethysmography data (described in section “PLY-Plethysmography Results”). Partition coefficients were assumed to be equivalent at both life-stages. For the fetus, most organs have formed or are in the process of forming by GD20. However, differences in partitioning between immature tissues and blood could result in more complex kinetic behavior than would be accounted for by a single well mixed compartment. Therefore, the refined dam and fetal model structure was also compared to maternal and fetal BEC time-course data following a GD20 oral gavage (2000 mg/kg) of Wistar dams (Hayashi et al., 1991). This step was conducted to gauge the impact of model refinements on the model’s predictive capabilities for an alternate exposure route. The kinetics of EtOH in the eye were evaluated using the adult model and adult tissues. As ocular tissues were not collected from dams and pregnancy status is not expected to change the partitioning of EtOH in the eye, a defined compartment for this tissue was not included in the pregnant model.

Sensitivity analysis

It was important to identify model parameters which were sensitive to three specific conditions: (1) route of exposure (inhalation versus oral); (2) gestational state (non-pregnant, pregnant GD1, or pregnant GD20); and (3) internal dose-metric used (peak BEC versus AUC (mg/dL × h) in blood). To evaluate route of exposure, a 1000 ppm 6 h vapor exposure was simulated and parameter sensitivities determined for the inhalation route. In order to provide a comparable peak BEC by the oral route, for comparison with the inhalation route, simulations were performed iteratively until the single oral (bolus) dose that produced the same peak BEC was achieved. This dose was then used to facilitate the inhalation versus oral exposure comparison. The same approach was replicated with the dams. To determine the influence of gestational state, the pregnant model was initiated on either of the two GDs and the resulting sensitivities compared to one another. To investigate the impact of the internal dose-metric, the AUC of a 1000 ppm 6 h inhalation exposure was determined and iterative simulations were performed until the oral dose associated with this AUC was identified. This approach produces comparable AUCs but different peak BECs, as the kinetics of the two routes differ. For all analyses, a 1% proportional change in each parameter value was used, with the sensitivity coefficients normalized to the response variable and parameter. Sensitivity coefficients of 0–0.19 were treated as “not sensitive,” 0.2–0.49 as having “low
sensitivity,' 0.5–0.75 ‘‘moderate sensitivity,’’ and 0.75–1 or more as ‘‘highly sensitive.’’

Results
Exposure concentration monitoring (chamber performance)

The standard deviations in the N-O chamber during Experiments 1 and 2 were less than 10% of the mean vapor concentration (Table 1). In Experiment 1, the TPV ranged from 0.071 to 0.092, WPV ranged from 0.092 to 0.093, and BPV ranged from 0.023 to 0.00. Negative BPVs are an artifact of the calculation and indicate effectively negligible variability.

During Experiment 2, rats in W-B plethysmographs generally curled up and slept with their heads tucked under their torsos at 0 or 5000 ppm vapor. This rarely occurred with the 10 000 ppm and 21 000 ppm studies, where rats tended to sleep on their sides in an elongated position. Rats in the 21 000 ppm group had an unsteady gait and appeared intoxicated, but at no time showed any signs of pain or trauma during or after exposure. Similar signs of intoxication were not observed at 0 or 5000 ppm, and only occasionally with 10 000 ppm EtOH vapor. Regarding the distribution study, TPVs ranged 0.09–0.026, WPVs held at 0.08, and BPVs ranged 0.071 to 0.009.

In Experiment 3, 21 of the 451 individual chamber concentration values recorded during the GD9 – 20 period (Table 1) were above, and 13 were below the target ±10% difference window. When these values are included in the daily average, only two values were above ±10% of the difference window. Both of these outliers occurred in one chamber, in which the value was 10.4% above the mean on GD15 and 12.1% on GD16. In addition, the rat in this

Table 2. PBPK Model parameters.

| Parameter | Adult rat | Pregnant rat/Fetus | Fetal submodel |
|-----------|-----------|--------------------|----------------|
| QPC, Breathing Rate (L/h/kg\(^{0.75}\)) | 24.75 | 24.75 | NA |
| QCC, Cardiac Output (L/h/kg\(^{0.75}\)) | 14.0 | 14.6 | QCI\(_f\) × Vfet |
| Blood Flows (% cardiac output) | | | |
| QBrC, Brain | 2.0 | 3.0 | 10.55 |
| QLC, Liver | 25.0 | 18.3 | 6.1 |
| QPC, Fat | 7.0 | 7.0 | – |
| QR, Rapidly Perfused | 76.0-QBr-QL | 76.0-QBr-QL | – |
| QS, Slowly Perfused | 24.0-QF | 24.0-QF-QMam | – |
| QMamC, Mammary | – | 0.2 | – |
| QE, Eyes | 0.01629 | – | – |
| QAlv, Alveolar | | | – |
| QOth, Other Tissues | – | – | QCI\(_f\)×(QL\(_f\)+QBrn\(_f\)) |
| Tissue volumes (% body weight) | | | |
| VBrnC, Brain | 0.6 | 0.6 | Eqp\(_f\) |
| VLivC, Liver | 4.0 | 3.4 | Eqp\(_f\) |
| VFatC, Fat | 7.0 | 10.0 | – |
| VSI, Stomach Lumen | 2.0 | 2.0 | – |
| VRap, Rapidly Perfused | 9.0-VBrn-VLiv | 9.0 × BWInit-VLiv-VBrn | – |
| VSlw, Slowly Perfused | 82.0-VFat | 82 × BWInit-VFat-Vmam | – |
| VmamC, Mammary | – | 1.0 | – |
| VBrn | 0.00104 | – | – |
| VAlv | 0.7 | 0.7 | – |
| VmucC, Mucus (L/kg) | 0.01 | 0.01 | – |
| VOther Tissues | – | – | Vfet-(VBrn\(_f\)+VLiv\(_f\)+VBld\(_f\)) |
| VBld\(_f\) | – | – | 6.76 |
| Metabolism constants | | | |
| V\(_{max}\)_LC, Hepatic V\(_{max}\) (mg/h/kg\(^{0.75}\)), Fitted | 272 | 272 | – |
| KML, Hepatic Km (mg/L) | 23 | 23 | – |
| V\(_{max}\)_GC, Gastric V\(_{max}\) (mg/h/kg\(^{0.75}\)), Fitted | 300 | 300 | – |
| KMG, Gastric Km (mg/L) | 18 400 | 18 400 | – |
| KAC, Gastric Uptake (kg\(^{0.25}\)/h) | – | 1.8 | – |
| Partition coefficients | | | |
| PL, Liver:Blood | 0.81 | 0.81 | 0.81 |
| PB, Fat:Blood | 0.11 | 0.11 | – |
| PS, Slowly Perfused:Blood | 0.80 | 0.80 | – |
| PR, Richly Perfused:Blood | 0.95 | 0.95 | – |
| PBr, Brain:Blood | 0.87 | 0.87 | 0.87 |
| PB, Blood:Air and Mucous:Air | 2140 | 2140 | 2140 |
| Pmam, Mammary:Blood | 0.80 | 0.80 | 0.80 |
| Ppla, Placenta:Blood | – | 0.80 | 0.80 |
| PEyes, Eyes | | | PR |
| POther, Other tissues | – | – | 0.80 |
| Diffusion constants | | | |
| PAFC, Permeability-Area Product (L/h/BW Fetus\(^{0.75}\)) | – | 0.99 | – |

\(^{4}\) [ILSI] International Life Sciences Institute, 1994, \(^{b}\)Pastino et al., 2000, \(^{c}\)Kaneko et al., 1994, \(^{d}\)Clewell et al., 2001, \(^{e}\)Salter et al., 1998, \(^{f}\)Determined from rats in this study, \(^{g}\)Yoon et al., 2009a.
chamber received only 4 h of exposure on GD16, due to a malfunctioning vapor generator. Due to the short duration, small number, and low potential impact of these transient excursions on the resulting kinetic data, the results remained acceptable for our purposes. The single BEC data point collected from the dam exposed for 4 h on GD16 was analyzed and considered to be a rough indicator of post-exposure clearance.

Kinetic studies

N-O-non-pregnant kinetics (Experiment 1)

Blood samples were collected within an average of approximately 20 s of the designated sacrifice time. Liver samples were excised and placed in the inhibition cocktail within 1 min. Brain samples were excised and homogenized in buffer within ~2 min. Eyes were removed and placed in the inhibition cocktail by ~3 min following sacrifice. The full tissue dataset is provided in Table 3.

W-B-non-pregnant kinetics (Experiment 2)

All data are listed in Table 3 and Supplementary Table 2. Fetuses collected on GD20 have marginal metabolic capabilities, do not respire (negating loss by exhalation), and were decapitated within a few seconds of removal from exsanguinated dams. As a result, we expect that these data accurately reflect the peak EtOH concentrations achieved in the fetus.

PLY-plethysmography results (Experiment 2)

For brevity, analysis of only the most relevant data for the model (frequency, tidal volume, and minute volume, Table 4) is described. The full set of plethysmography data are provided in Supplementary Table 3(a) and (b). Breathing frequencies (F) in the control groups were similar in both types of plethysmographs, with overlapping standard deviations. Statistically significant differences in frequencies were detected for control and EtOH concentration groups exposed using the N-O method, $\chi^2(3) = 33.40, p < 0.001$. The Tukey test indicated that frequencies at 21 000 and 5000 ppm, but not 10 000 ppm, were significantly different from

“Results: PLY-Non-Pregnant Kinetics (Experiment 2)” below, which may have led to lower than expected BrECs.

W-B-pregnant kinetics (Experiment 3)

All data are listed in Table 3 and Supplementary Table 2. Fetuses collected on GD20 have marginal metabolic capabilities, do not respire (negating loss by exhalation), and were decapitated within a few seconds of removal from exsanguinated dams. As a result, we expect that these data accurately reflect the peak EtOH concentrations achieved in the fetus.
Table 3. Summary of time-course adult and repeated exposure dams/fetus kinetic study data.

| Adult rats | Acetaldehyde | Methanol | Ethanol |
|------------|--------------|----------|---------|
| Exposure   | Mean ± SD (mg/dL) | Mean ± SD (mg/dL) | Mean ± SD (mg/dL) |
| Tissue     | Time (h) | BL | Br | LI | EY | BEC | BrEC | LEC | EEC |
|-------------|---------|----------------|-----------------|-----------------|----------------|----------------|----------------|----------------|----------------|
| 5000        | 0.5     | 0.62 ± 0.41  | 0.27 ± 0.01  | BLQ           | 0.21 ± 0.08  | 0.21 ± 0.08  | 0.21 ± 0.08  | 0.21 ± 0.08  | 0.21 ± 0.08  |
|             | 2       | 0.73 ± 0.28  | 0.33 ± 0.06  | 0.23 ± 0.01  | 0.23 ± 0.01  | 0.23 ± 0.01  | 0.23 ± 0.01  | 0.23 ± 0.01  | 0.23 ± 0.01  |
|             | 6.5     | 0.24 ± 0.04  | 0.28 ± 0.06  | 0.09 ± 0.04  | 0.09 ± 0.04  | 0.09 ± 0.04  | 0.09 ± 0.04  | 0.09 ± 0.04  | 0.09 ± 0.04  |
| 10000       | 3       | 0.27 ± 0.3  | 0.30 ± 0.03  | 0.28 ± 0.02  | 0.28 ± 0.02  | 0.28 ± 0.02  | 0.28 ± 0.02  | 0.28 ± 0.02  | 0.28 ± 0.02  |
|             | 6.5     | 0.74 ± 0.25  | 0.30 ± 0.01  | 0.09 ± 0.03  | 0.09 ± 0.03  | 0.09 ± 0.03  | 0.09 ± 0.03  | 0.09 ± 0.03  | 0.09 ± 0.03  |
|             | 21000   | 1.25 ± 0.79  | 0.38 ± 0.06  | 0.05 ± 0.03  | 0.05 ± 0.03  | 0.05 ± 0.03  | 0.05 ± 0.03  | 0.05 ± 0.03  | 0.05 ± 0.03  |

| Pregnant rats | Acetaldehyde | Mean ± SD (mg/dL) | Methanol | Mean ± SD (mg/dL) | Ethanol | Mean ± SD (mg/dL) |
|---------------|--------------|-------------------|----------|-------------------|---------|------------------|
| Exposure      | Time (h)    | BL | Br | BL | Br | BL | Br | BL | Br | BL | Br | BL | Br |
| 21000         | GD9         | 0.23 ± 0.06  | BLQ | NC | 4.62 ± 0.47 | 142.7 ± 34.96 | 62.5 ± 8.76 | 14.5 ± 0.38 | 50.1 ± 7.56 | 197 ± 8.24 |
|               | GD12        | 1.12 ± 0.35  | BLQ | NC | 25.4 ± 0.17 | 35.2 ± 8.76  | 9.3 ± 0.38  | 19.6 ± 0.38  | 28.1 ± 8.76  | 49.3 ± 8.76  |
|               | GD16        | 0.10 ± 0.02  | 0.37 ± 0.05 | 0.09 ± 0.03  | 0.09 ± 0.03  | 0.09 ± 0.03  | 0.09 ± 0.03  | 0.09 ± 0.03  | 0.09 ± 0.03  |
|               | GD20        | 0.10 ± 0.02  | 0.37 ± 0.05 | 0.09 ± 0.03  | 0.09 ± 0.03  | 0.09 ± 0.03  | 0.09 ± 0.03  | 0.09 ± 0.03  | 0.09 ± 0.03  |
| Fetal rats    | GD9         | 0.04 ± 0.01  | 0.07 ± 0.01 | 0.07 ± 0.01  | 0.07 ± 0.01  | 0.07 ± 0.01  | 0.07 ± 0.01  | 0.07 ± 0.01  | 0.07 ± 0.01  |
|               | GD12        | 0.16 ± 0.05  | 0.16 ± 0.05  | 0.16 ± 0.05  | 0.16 ± 0.05  | 0.16 ± 0.05  | 0.16 ± 0.05  | 0.16 ± 0.05  | 0.16 ± 0.05  |
|               | GD16        | 0.16 ± 0.05  | 0.16 ± 0.05  | 0.16 ± 0.05  | 0.16 ± 0.05  | 0.16 ± 0.05  | 0.16 ± 0.05  | 0.16 ± 0.05  | 0.16 ± 0.05  |
|               | GD20        | 0.16 ± 0.05  | 0.16 ± 0.05  | 0.16 ± 0.05  | 0.16 ± 0.05  | 0.16 ± 0.05  | 0.16 ± 0.05  | 0.16 ± 0.05  | 0.16 ± 0.05  |

BLQ, Below Limit of Quantitation; NC, Not Collected; Tissues: BL, blood; LI, liver; EY, eyes; BEC, fetal blood EtOH; BrEC, fetal brain EtOH; LEC, fetal carcass EtOH; AALD specific: fBL, fetal blood; fBr, fetal brain; fWB, fetal whole body.
control \((p < 0.05)\). Specifically, frequencies were affected in a non-dose-related manner, being reduced at 21 000 ppm and increased at 5000 ppm. Significant differences were also detected between the groups using the W-B method, \(\chi^2(3) = 35.348, p < 0.001\). Frequencies at 21 000 and 5000 ppm were significantly lower than control \((p < 0.05)\). Frequencies were also reduced by exposure to 10 000 ppm, but the effect was not significant.

Tidal volumes in the N-O groups were approximately twice the values of the W-B groups (Table 4). Differences between the N-O-exposed groups were significant, \(\chi^2(3) = 61.83, p < 0.001\). Statistically significant reduction of tidal volume was observed at all three EtOH vapor concentrations \((p < 0.05)\). There were also significant differences between W-B-exposed groups, \(\chi^2(3) = 52.67, p < 0.001\). Data at 5000 ppm were significantly higher than control, and 21 000 ppm significantly lower \((p < 0.05)\).

There were significant differences in the minute volumes recorded under both the N-O. \(\chi^2(3) = 68.55, p < 0.001\) and the W-B. \(\chi^2(3) = 61.38, p < 0.001\), scenarios (Table 4). For the N-O method, minute volumes were reduced by all three vapor concentrations, however only the impact of 21 000 ppm \((p < 0.05)\), was significantly different from control. For the W-B method, exposure to EtOH at 21 000 ppm produced a significant reduction in minute volume, while 10 000 ppm \((p < 0.05)\) produced a small but significant increase. The minute volume data from the N-O plethysmographs were also significantly different \((p < 0.05)\) from the W-B plethysmograph data; a factor most likely related to the difference in recorded tidal volume (see Discussion section). However, relative to their respective controls, a consistent ~20% reduction in minute volume was observed for both the N-O and W-B datasets at 21 000 ppm.

These findings, and the high standard deviations around the mean values, indicated that simply replacing the original model’s ventilation rate with the mean ventilation rates at 5000, 10 000, or 21 000 ppm may be less representative of the impact of EtOH on respiration than the relative effect on the area under the minute volume curve (AUC, mL/min \(\times\) min, 0–6 h). The AUC encompasses the total amount of air inhaled during the 6-h exposure period. The AUC was determined for each parameter collected during Experiment 2 and the change in AUC (unit \(\times\) h, 0–6 h) relative to control (Strachan et al., 2002) was calculated. The model ventilation rate was multiplied by the ratio of exposed to control AUCs, resulting in an adjusted QPC. The adjusted QPC was then incorporated into equation 1 at 21 000 ppm.

The impact of exposure on minute volume reached statistical significance at 21 000 ppm, and the magnitude (%) of the effect was generally consistent across both W-B and N-O systems. However, the results at 5000 or 10 000 ppm did not meet these criteria; therefore, QPC was not adjusted at the two lower vapor concentrations.

To incorporate the effect of EtOH on minute volume into the model, an equation was added,

\[
k_{\text{urtC}} = 1.3412 \times (\text{QPC}) - 19.7889 \tag{1}
\]

where the wash-in/wash-out constant, \(k_{\text{urtC}} (\text{L/h/kg}^{0.75})\), is a function of the ventilation rate, QPC (L/h/kg\(^{0.75}\)). This equation includes the two most sensitive parameters
influencing uptake in the model and was developed by simulating inhalation exposure datasets and iteratively changing both QPC and $k_{urtC}$ in order to achieve the same peak BEC. The QPC and $k_{urtC}$ values were then plotted together and a linear regression performed. The regression was then used as the initial equation for $k_{urtC}$, and was later statistically optimized (Matlab Software, Mathworks, Inc, Natick, MA).

Revised PBPK model performance

Adult rat-PBPK model evaluation (Experiment 1)

Simulations of BECs, BrECs, and EECs are shown for each exposure concentration, while LECs are only shown for 21 000 ppm (Figures 3–5). Liver samples were collected at 5000 and 10 000 ppm, however the resulting data were below the limit of quantitation (not shown). Several BECs and BrECs in the 5000 and 10 000 ppm groups were also near or below the reporting limit, resulting in plots with sparse data points. In all cases, simulated blood or tissue concentrations that were within a factor of two of the in vivo data were considered acceptable (IPCS, 2010).

At 5000 ppm (Figure 3a–c) and 10 000 ppm (Figure 4a–c), simulation lines generally fell within, or just outside, the range of the BEC data at most time points. Those that were not fit by the simulation, such as the 6h BEC at 5000 ppm, were overpredicted by <2-fold. BECs collected at 21 000 ppm were well approximated by simulations (Figure 5a–d). BrECs at 21 000 ppm were also well approximated, but overpredicted at 5000 and 10 000 ppm; always by <2-fold. EECs were quantitatively higher than BECs at 5000 and 10 000 ppm, but closely tracked BECs at 21 000 ppm. EEC simulations consistently bisected the EEC data. The simulated LEC overpredicted several time-points at 21 000 ppm, but fell within the range of the data at the end of exposure and 1 h post-exposure. Refer to Table 3 for a comparison of all blood and tissue data across the three vapor concentrations.

PLY-non-pregnant kinetics (Experiment 2)

After incorporating the study-specific bodyweights (323.0 ± 9.20 g), and the exposure scenario (21073 ± 363 ppm, 6 h), the simulated peak BECs for this pilot study were in agreement with the observed data, while BrBECs were
underpredicted. The observed and simulated peak BECs in these rats, were 167.25 ± 34.65 mg/dL and ~150 mg/dL, respectively. The observed and simulated peak BrECs were 169.42 ± 24.36 mg/dL and ~130 mg/dL, respectively. The underprediction of BrEC (~23%) may be related to our manipulations of the brain tissue. Separating out the homogenized and minced samples from the overall mean BrEC of 169.42 ± 24.36 mg/dL, the homogenized sample BrECs were 184.33 ± 29.43 mg/dL and the minced were 154.52 ± 5.04 mg/dL.

**Pregnant rat-PBPK model evaluation (Experiment 3)**

For Experiment 3, the model for pregnant rats accurately reproduced peak BECs on all four blood collection days, as well as maternal BrEC (Figure 6a–c) on GD20. There were no clear and consistent patterns in the BEC data across the gestational period that would suggest a change in BECs over time. For the dam that received only 4 h of exposure on GD12, but was sacrificed at the end of the normal 6 h exposure period, the BEC was 9 mg/dL. As the trend in the post-exposure clearance after 4 h of exposure is comparable to that produced after 6 h, this BEC was accurately reflected by the adjusted (4 h) simulation (Figure 6c). When the fetus was described as a single compartment, and simulations of the EtOH concentration in this compartment were compared to the fBEC, fBrEC and fCEC data, the simulation underpredicted these data (Figure 7a–c). After inclusion of specific compartments for fetal tissues, the model predictions of fBEC, fBrEC, and fCEC reproduced the observed data points on GD20 (Figure 8a–c). Blood and brain concentrations were consistent between dams and fetuses (Figure 8a and b).

In comparison to an oral gavage dataset at a comparable peak maternal BEC of ~200 mg/dL (Hayashi et al., 1991), the refined model approximated the peak maternal BEC and fBEC data, though clearance data were underpredicted by the simulation lines for BEC and fBEC (Figure 8d, gray lines). A further iterative reduction in \( V_{\text{max,LC}} \) of 36% to 150 mg/h/ kg\(^{0.75}\) produced a simulated BEC and fBEC that more closely tracked the in vivo clearance data, though with a modest increase (~10%) in the simulated peak (Figure 8d, black lines).

**Acetaldehyde and methanol (Experiments 1 and 3)**

Concentrations of AALD in tissues (Table 3) were orders of magnitude lower than concentrations of EtOH, as expected. The highest AALD concentrations in blood were from dams.
exposed to 21 000 ppm (0.23–1.12 mg/dL). MeOH was also detected in blood, with the highest concentrations (2.5–4.3 mg/dL) achieved in dams at 21 000 ppm (Table 3).

Sensitivity analysis

Adult rat model

The results of the sensitivity analyses performed for the adult rat model are reported in Supplementary Figure 3(a) and the abbreviations of the sensitive parameters are defined in Table 2. For route of exposure considerations, a 1000 ppm 6 h exposure produces a peak BEC of 0.58 mg/dL. The oral dose required to produce the same peak BEC in the adult model is 55 mg/kg. Route dependencies were evident in the analysis, with the BEC produced via the inhalation route being most sensitive to QPC, QLivC, QCC, kUrtC, VmaxLC and KmL. For the oral route, BEC was at least moderately sensitive to VmaxLC, the gastric uptake rate (KAC), KmL, and QLivC. For the AUC metric of concentration in blood (mg/dL/C2), the comparable oral dose for a 1000 ppm/6 h vapor exposure, is 248.4 mg/kg. As with peak BEC, route dependencies were observed when using the AUC metric, though were of different magnitudes than for peak BEC. AUC was very sensitive to VmaxLC and KAC, sensitive to KmL, and moderately sensitive to BW, KmG, VSLwC, QCC, and VmaxGC.

Pregnant rat model

The results of the various sensitivity analyses performed for the pregnant rat model are reported in Supplementary Figure 3(b) and the abbreviations of the sensitive parameters are defined in Table 2. The model was particularly sensitive to route of exposure and gestational day. For inhalation on GD20, the model was very sensitive to QPC, QLivC, and QCC, and less sensitive to kUrtC, VmaxLC, and VSlwC. Similar coefficients (±0.03) were recorded for the same exposure on GD1, with the exception that the direction of the sensitivity for VSLwC was positive on GD1 and became negative by GD20, reflecting changes in pregnancy-related tissues that are slowly perfused. The oral exposure scenarios that produced the same peak BEC were 74.5 mg/kg on GD1 and 86.5 mg/kg on GD20. BEC was very sensitive to VmaxLC, KAC, and KmL, and moderately sensitive to QLivC and QSLwC. The same oral exposure parameters from simulation on GD1 remained sensitive, but with different magnitudes and directions for KmL and VmaxLC, depending on the day. For the AUC condition, doses of 272.8 mg/kg on GD1 and

Figure 5. Experiment 1: Blood, brain, liver, and eyes at 21 000 ppm. (a) BEC, (b) BrEC, (c) EEC, and (d) LEC. Lines represent simulations, symbols represent data points.
284.6 mg/kg on GD20 were used. The resulting coefficients differed somewhat by day, with the directions of sensitivity of AUC to $V_{\text{max}, \text{LC}}$ and $V_{\text{Slw}, \text{C}}$ on GD1 opposite to the directions on GD20. The change in direction for $V_{\text{Slw}, \text{C}}$ is likely attributable to growth in gestation-related tissues as pregnancy progressed.

**Discussion**

This report combines a collection of novel pharmacokinetic data with the use of these data to evaluate and refine a series of life-stage PBPK models for non-pregnant adult and pregnant rats, as well as developing fetuses during gestation. In each inhalation pharmacokinetic study, BEC and BrEC were collected to enable quantitative comparisons between life-stages. Liver, eyes, and fetal tissues were collected to ensure that model predictions accurately portrayed the *in vivo* behavior of EtOH in these target tissues. Additionally, AALD and MeOH were quantified in most of these tissues to enable comparison with toxicity data (see Discussion section *Acetaldehyde and Methanol*). Plethysmography studies were performed to investigate the impact of exposure on respiratory parameters and to improve the predictive capabilities of the PBPK models for the inhalation route.

The PBPK models remain largely unchanged from our earlier work (Martin et al., 2012); however, two areas of uncertainty were considered and addressed during this course of this study: the fetal and respiratory descriptions. The description of the fetus from the original model for pregnant rats was further parameterized to include blood, brain, and liver tissues. Second, because BECs were especially sensitive to parameters that influence respiratory uptake, the description of these parameters was refined using plethysmography data. After including these changes, the refined PBPK models were used to produce simulations of a variety of exposure scenarios, with the comparison of these simulations to *in vivo* data used to evaluate the PBPK models for EtOH.

**Pharmacokinetic data**

The distributions of measured values of BECs and BrECs were consistently similar in the adult, pregnant, and fetal rats, as shown by the overlapping standard deviations, indicating similar distribution of EtOH across tissue compartments during these life-stages. The mean peak BECs of non-pregnant rats in Experiment 1 moderately exceeded those of pregnant dams in Experiment 3 by ~18% on GD20 and by ~33% on GD16 (largest difference between mean BECs),
under similar exposure scenarios. Similarly, peak BrECs from non-pregnant rats exceeded those of the pregnant dams by an equivalent amount (~22%) on GD20. In contrast, mean BECs and BrECs of dams and fetuses were essentially equivalent (Table 3). Overall, these modest differences in the mean, peak, BECs and BrECs between pregnant and non-pregnant rats support the utility of studying EtOH kinetics in non-pregnant rats as an indicator of internal doses during pregnancy.

To evaluate the ability of the models to predict EtOH concentrations at other sites of reported toxicity and/or metabolism, liver and eye tissues were collected in Experiment 1, and fetuses were collected in Experiment 3. Regarding these tissues, LECs have been previously reported following oral dosing by Eriksson and Sippel (1977). However, this is the first report of EECs in rats. In Experiment 1, BEC and EEC data were generally similar for adult rats, and were well approximated by the PBPK model (≤2× of the in vivo data). While the model would ideally include development of an ocular compartment for the fetus and then make similar BEC-EEC comparisons across life-stages, the physiological parameters and growth equations necessary to support such a compartment are unavailable in the literature. Instead a transitive assumption is suggested, as follows: BECs in adult rats approximate EECs, and it is reasonable to assume a similar relationship between the maternal BECs and EECs in dams. Thus, because maternal BECs and fetal BECs were shown to be equivalent in Experiment 3, maternal BECs should approximate both fetal BECs and EECs. This means that maternal BECs collected during studies reporting fetal ocular toxicity are reasonable indicators of EtOH concentrations in the developing eye.

**Acetaldehyde and methanol**

Dams that were exposed to 21 000 ppm EtOH had the highest concentrations of AALD in their blood (0.23–1.12 mg/dL). These concentrations are similar to data obtained from Wistar rat dams (~0.18–0.23 mg/dL) that consumed a liquid diet containing 5% (w/v) EtOH for 15 d of gestation (14.6–18.4 g/kg BW/d) (Guerri & Sanchis, 1986). In a toxicity study that administered this dose (5% EtOH diet) throughout gestation, exposure to EtOH damaged the optic nerve (Pinazo-Duran et al., 1993).

The concentrations of MeOH in maternal blood peaked at 2.5–4.3 mg/dL (21 000 ppm EtOH for 6.33 h/d for 12 d), but were not considered to be toxicologically relevant (Table 3). Nelson et al. (1985), exposed pregnant rats to 5000–20 000 ppm of MeOH vapor for 7 h/d for 20 d resulting in concentrations in blood that ranged from ~100 to
865 mg/dL, respectively. Significant skeletal and visceral malformations resulting from exposure to MeOH vapor were present only at concentrations of 865 mg/dL in blood. Therefore, the MeOH detected in blood following exposure to EtOH vapor was likely the result of endogenous buildup from normal physiological processes (Jones et al., 2009).

Like EtOH, MeOH is metabolized by ADH and is subject to competitive metabolic inhibition when EtOH concentrations saturate the ADH pathway (Jones et al., 2009). When EtOH and MeOH were present in equimolar concentrations, a 90% inhibition of MeOH oxidation has been reported by Makar et al. (1968).

Plethysmography studies

Plethysmography studies quantified changes in ventilation during exposure (Experiment 2). For the W-B plethysmographs, data collected from the control (0 ppm) group were consistent with the literature for this type of chamber. The mean values for respiratory frequency, tidal volume, and minute volume were 213 breaths/min, 1.32 mL/breath, and 247 mL/min, respectively, in the current study, which compare favorably with values of 178.3 breaths/min, 1.35 mL/breath, and 242.5 mL/min, respectively (Higuchi et al., 2004). Exposure to EtOH decreased respiratory frequencies 4–15% compared to controls. EtOH also increased tidal volumes 2–12% at 5000 and 10,000 ppm, and decreased them by 8% at 21,000 ppm. EtOH increased minute volumes 5% in the 5000 and 10,000 ppm groups, and decreased them by 19% at 21,000 ppm.

In Experiment 2, the N-O plethysmograph cones yielded data that were generally either consistent with literature for control rats or higher than previous reports. The mean values for respiratory frequency, tidal volume, and minute volume in controls in the current study were 164 breaths/min, 2.78 mL/breath, and 450 mL/min, respectively, compared to literature values of 144–170 breaths/min, 1.6–2.2 mL/breath, and 171–355 mL/min, respectively, in male and female rats at bodyweights of 159–301 g (Chen et al., 1989; de Madron & Mauderly, 1988; Mauderly, 1986; Sabourin et al., 1992). Exposure to EtOH increased respiratory frequencies 5–10% at 5000 and 10,000 ppm and decreased frequencies by a similar amount at 21,000 ppm. EtOH increased tidal volumes by 10–16% across 5000–21,000 ppm. EtOH decreased minute volumes.
volumes ~10–22% across the three vapor concentrations, with the largest decrease of 22% occurring in rats exposed to 21 000 ppm; which is generally consistent with the effect of 21 000 ppm using the W-B method. For comparison, in a large compendium of physiological parameters for model development (Brown et al., 1997) minute volumes in unanesthetized rats range from 118 to 514 mL/min, assuming a 250 g rat.

The effect of EtOH on respiratory parameters may be related to direct effects in respiratory tissue, such as the sensory response demonstrated in studies to determine RD50s (stimulation of trigeminal nerve) (Kane et al., 1980; Kuwabara et al., 2007), high BECs, or a combination of both. Increasing BECs have been linked to concurrent decreases in respiratory parameters (Linowiecki et al., 1992; Ren et al., 2012). Ren et al. (2012) determined that adult male Sprague–Dawley rats (300–400 g) that were dosed intraperitoneally with 2 g/kg EtOH and placed into W-B plethysmographs had statistically significantly lower tidal volumes (81 ± 6% of control) and minute volumes (77 ± 8% of control), while respiratory frequency (98 ± 9% of control) was not affected. The dose of 2 g/kg was associated with a peak BEC of 160 mg/dL in that study. In the current study, exposure to 21 000 ppm vapor produced a higher peak BEC, but a somewhat lower magnitude of depression of tidal and minute volumes compared to the Ren et al. (2012) study. The difference may be attributable to the route of exposure and its impact on the pharmacokinetics of EtOH. In Experiment 1, BECs increased slowly during exposure to 21 000 ppm, peaked at 6 h, and cleared slowly, while intraperitoneal injections produce a rapid rise and fall in BECs. This rapid change in BEC may be in-part responsible for the more severe response in the Ren et al. (2012) study. In the current study, minute volumes in all rats declined over the first hour of exposure. Data from rats exposed to 5000 and 10 000 ppm stabilized after this period at the reduced levels, similar to how BECs in these dose groups eventually reached and maintained a steady state concentration. However, minute volumes in rats exposed to 21 000 ppm continued to decline slowly over the 6 h exposure duration, similar to the slow increase in BEC over time. Since exposure to 21 000 ppm is near the RD50 (see Introduction) for EtOH vapor (27 314 ppm) and the effects observed during the plethysmography study reflect the trend in the BEC data, it is likely that the overall effect is a combination of both a direct effect on respiratory tissue and the resulting BECs.

We initially planned to incorporate the average minute volumes to estimate deposition in the PBPK model; however, it was determined that this value was sensitive to transient spikes and movement artifacts, as evidenced by the large standard deviations. Instead, the AUCs from each EtOH-exposed group were compared to their respective control groups. This analysis indicated that AUC accurately accounted for the effect of EtOH on respiration while mitigating the impact of the spikes in minute volume. To incorporate the effect of EtOH on minute volume, we determined the percentage reduction present in each group of rats and generated an equation that reflected the change. The equation had the added benefits of controlling kUrtC and the ability to retain accepted QPC values from the literature that accurately described the literature-derived pharmacokinetic data in our previous report (Martin et al., 2012). Overall, incorporation of this correction factor produced model simulations that were within a factor of two or better compared to the datasets collected at 21 000 ppm, while maintaining the capability to predict EtOH kinetics under normal respiration at lower vapor concentrations.

**Adult rat model**

The range of vapor concentrations in Experiment 1 bridges the gap between the datasets from Pastino et al. (1997) at 200 and 600 ppm, Scarino et al. (2009) at 1000 and 3000 ppm, and the higher 21 000 ppm exposure concentration. Simulations replicated all data within a factor of two or better, demonstrating that the clearance profiles of blood, as well as tissues, change between 10 000 and 21 000 ppm, as metabolism nears and then becomes saturated. The simulated peak BEC at 21 000 ppm was on the lower side of the data range at 6 h. It is unlikely that a simulation capable of fitting the increasing 2 and 4 h time points would also be capable of perfectly fitting the mean at 6 h and still maintain the post-exposure clearance profiles across the various blood and tissue data sets.

In the tissues, BrEC data collected at 5000 and 10 000 ppm were often near or below the limit of quantitation, especially during very early (0.5 h) and late time-points (6.5 h), though data that were successfully quantified were generally simulated within a factor of ~2. EECs were consistently above the limit of quantitation at the same time points. Vitreous humor reportedly serves as a reservoir for EtOH in the eyes of post-mortem humans killed in accidents, and is used to determine EtOH concentrations, in the absence of blood, at the time of death (Caplan & Levine, 1990). This reservoir function may explain why EECs remained above the limit of quantitation but not BrECs. Inter-sample variability was similar between EEC and BEC data, with simulations bisecting the range of the EEC data at most time points across the three concentrations tested. The peak EEC (214 mg/dL) and BEC (222 mg/dL) at 21 000 ppm, were in excess of the peak BEC of 118 mg/dL which has been associated with alterations in retinal function in pups whose dams consumed a liquid diet containing 35% EtOH-derived calories during gestation (Katz & Fox, 1991). In the liver, the slight overprediction of LECs at 21 000 ppm may be attributable to uncertainties surrounding the liver blood flow (QL), as evidenced by the results of the sensitivity analyses, or to other parameters. Alternatively, the metabolism inhibition cocktail may have been only partially effective, or its action not rapid enough to prevent a significant loss of EtOH. Due to difficulties in homogenizing liver samples and the time-burden associated with ensuring complete removal of liver tissue from the homogenizer blades between each sample, we chose to thoroughly mince this tissue in the ice-cold cocktail. As minced samples may expose less tissue surface area to the cocktail than homogenized samples, isolated pockets of tissue may have remained metabolically active for an extended period.
Pregnant rat model

Two versions of the pregnancy model were evaluated. When the fetal unit was treated as a single compartment, maternal BEC and BrEC were well simulated, while fetal BECs, BrECs and CECs were underpredicted. We hypothesized that this underprediction was the result of dilution of circulating EtOH in the total fetal compartment volume. Additionally, EtOH in fetal tissues is subject to partitioning and distribution in vivo, which may not be fully accounted for using the simplified model description. Inclusion of the alternative version, with compartments for target tissues, provided more representative simulations of the fetal BEC, BrEC and CEC data. Both descriptions adequately described the maternal data. Therefore, when only maternal data are available, such as maternal BECs from toxicity studies, and fetal parameters are unavailable (number of gestating fetuses, their final birthweight, etc.), the single-compartment fetal description actually offers a less complex solution with reasonable and useful estimates. When fetal data are available, a more highly parameterized fetal compartment improves the accuracy of the predictions. Additionally, linking effects or processes in specific fetal tissue compartments to concentrations of EtOH within those compartments is only possible when those compartments are included. Examples would be tracking within those compartments is only possible when those specific fetal tissue compartments to concentrations of EtOH the predictions. Additionally, linking effects or processes in parameterized fetal compartment improves the accuracy of useful estimates. When fetal data are available, a more highly parameterized fetal compartment improves the accuracy of the predictions. Additionally, linking effects or processes in specific fetal tissue compartments to concentrations of EtOH within those compartments is only possible when those compartments are included. Examples would be tracking concentrations of EtOH in the fetal brain during critical points in development or changes in tissue concentrations as organs mature.

When the refined fetal sub-model was used to simulate an oral gavage dataset, peak BEC and fBEC were well approximated, but the rate of clearance was overpredicted. Reducing the hepatic $V_{\text{max}}$ resulted in an improved fit to the later clearance time points, both in the dam and fetus with only small effect (10%) on the simulated peak BEC (Figure 8d). This parameter change is supported by a report that the specific activities of hepatic P450 2E1 (19% of non-pregnant activity) and ADH1 (95% of non-pregnant activity) were reduced in pregnant rats dosed intragastrically with EtOH (Badger et al., 2005). Reduced metabolic clearance is also consistent with lower Cp4-450 activity that has been reported to occur in humans as gestation progresses (He et al., 2005; Syme et al., 2004). Alternatively, post-exposure interactions between EtOH and AALD, such as reverse metabolism of AALD to EtOH, have been previously considered for oral exposures (Umulis et al., 2005). Conversion of AALD back to EtOH results in a slower than expected decline in EtOH concentrations. The reduction in $V_{\text{max}}$ necessary to reproduce this dataset may, at least in part, also be reflecting the effect of this post-exposure interaction between AALD and EtOH.

Overall, controlled inhalation exposures were performed to refine life-stage PBPK models for EtOH in the rat. This is the first report of time-course kinetic data in brain, liver and ocular tissues following inhalation exposures and represents the first example of plethysmography measurements at these vapor concentrations. To our knowledge, this is also the first report of fetal EtOH data obtained following repeated gestational inhalation exposures in rats, as well as the utilization of these data in a PBPK modeling context.

Conclusions

The PBPK models reported in this study accurately describe the kinetics of EtOH across a variety of exposure scenarios and tissues. The models have direct applicability for simulations of blood and tissue concentrations produced during developmental neurotoxicology studies where dams were exposed and the occurrence of a range of behavioral, cognitive and other adverse effects assessed in the offspring. This suite of PBPK models may also be useful for route-to-route extrapolations and determinations of internal dose metrics for studies of EtOH toxicity, addiction, or pharmacokinetics in the literature. Additionally, these life-stage models provide a basis for developing more complex models of mixtures of EtOH and gasoline to investigate potential metabolic interactions between EtOH and the various hydrocarbons.

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Declaration of interest

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Supplementary material available online.

**Supplementary Figures** 1–3

**Supplementary Tables** 1–3